Chronic Lymphocytic Leukemia: the B cell receptor and beyond

Alice Muggen

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Chronic Lymphocytic Leukemia: the B cell receptor and beyond

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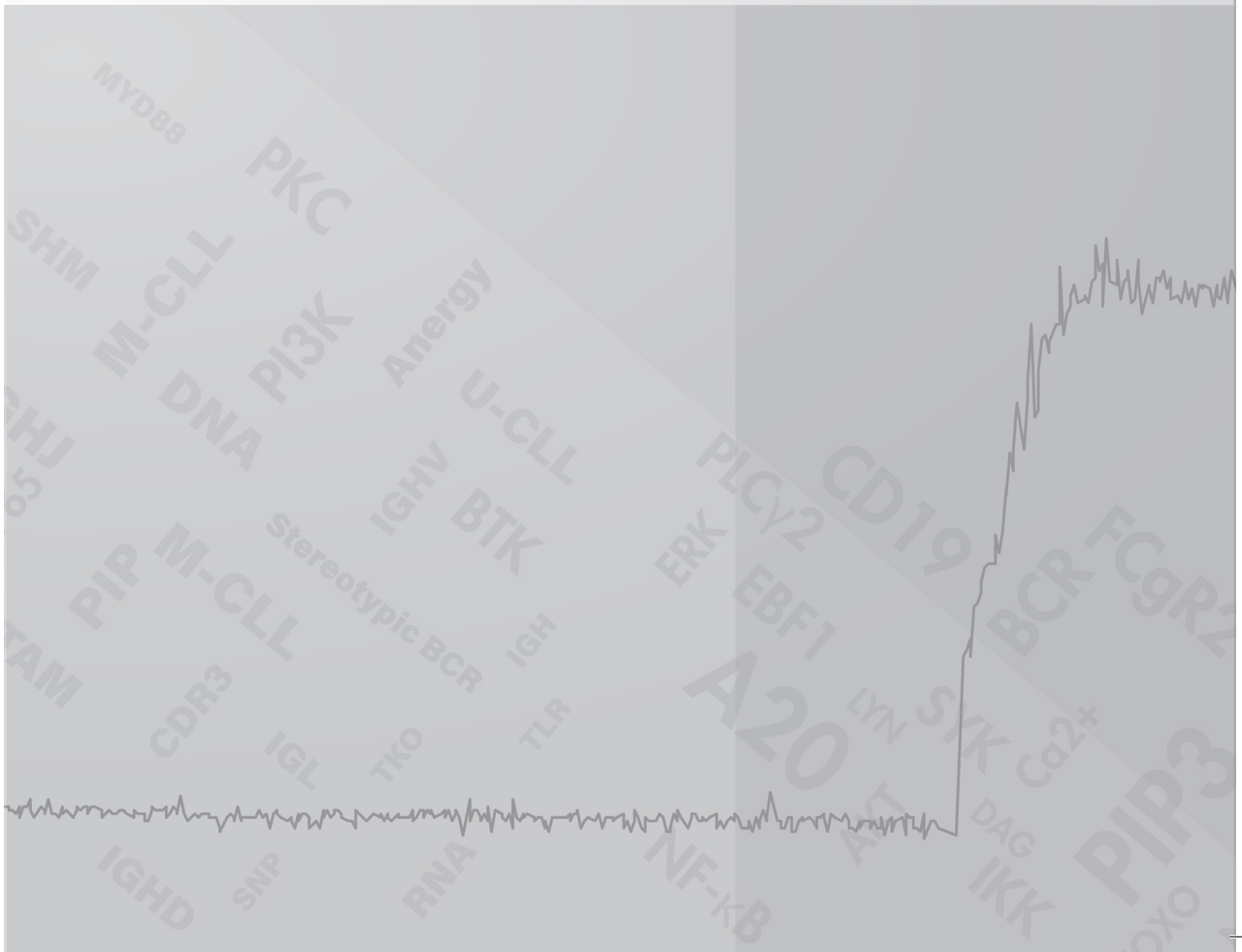

GENERAL INTRODUCTION

B cells in health and disease

All living organisms, including humans, are challenged by pathogens, such as viruses, bacteria, fungi, and parasites during their life. Therefore, a good functioning immune system is required to defend the organism against illness, or even death, caused by infections. The immune system consists of an innate arm, which forms a first line of defense, and an adaptive arm that can mount a pathogen-specific response. B and T lymphocytes both belong to the adaptive immune cells and express antigen receptors, which are specific for pathogen-derived antigens. These specific antigen receptors are called B cell receptor (BCR) and T cell receptor (TCR) molecules depending on the cell of origin. An enormous diversity of these antigen receptors is required to be able to recognize the immense number of antigens that might be encountered during life. To achieve this, each B and T cell expresses a unique BCR or TCR respectively, which contains a variable domain consisting of three framework regions (FR) and three complementarity determining regions (CDR), the latter being involved in actual antigen recognition. The variable domain is formed in a stochastic manner through V(D)J recombination processes in the antigen receptor genes (see below for more detail).

B cells are key players in humoral immunity. Binding of an antigen to the BCR induces the activation of a signaling cascade formed by several kinase proteins which can phosphorylate each other as well as other signaling molecules including linkers and lipases to induce proliferation and differentiation processes. Upon BCR activation and a second signal B cells proliferate and differentiate into memory cells or into plasma cells, which secrete their BCR as immunoglobulins (Ig).

The development of B cells requires checkpoints to prevent the recognition of self-antigens and thereby autoimmune disease development, and to keep B cell proliferation and activation in check to prevent leukemia and lymphoma formation. Nonetheless, significant numbers of B cells exhibit BCRs, which are to some extent autoreactive. Hence, additional control mechanisms regulate B cell activation by harmful antigen specificities. Via controlling BCR signaling strength the genesis of autoimmune diseases or B cell malignancies can be prevented. Unfortunately, this control mechanism in BCR signaling as induced by self-antigens or by chronic infections cannot always prevent the development of leukemias or lymphomas.

Human B cell differentiation

Early B cell development in the bone marrow

To be able to recognize an immense variety of antigens, an unlimited variety of BCR/Ig is required. The first step to establish this variation already occurs early in B cell differentiation. B cells develop in the bone marrow and the initial differentiation step after the stem cell

phase concerns the transition from a common lymphoid progenitor into the first progenitor B cell stage, the pro-B cell [1] (**Figure 1**). This specification process involves the concerted action of several transcription factors including PAX-5, which serves a the commitment factor of the B cell lineage [2]. In this pro-B cell stage the formation of the BCR starts at the immunoglobulin heavy chain locus (IGH). The IGH locus contains 38-46 functional variable genes (IGHV), 23 diversity genes (IGHD), and 6 joining (IGHJ) genes. In the pro-B cell stage recombination normally involves one of the IGHD genes and IGHJ genes through the induction of double strand DNA breaks by the lymphocyte-specific recombination activating genes 1 and 2 (RAG1/RAG2); these breaks are subsequently repaired by general DNA repair proteins. This is followed by recombination of a IGHV gene to the previously formed D-J junction. After transcription, the newly formed V-D-J exon will be spliced to the μ-constant region (Cμ) exons [3] (**Figure 2**). In the pre-B cell stage, co-expression of this Ig heavy chain occurs together with the surrogate light chain, consisting of the VpreB and λ5 subunits, and together they form the pre-BCR. This pre-BCR is required to test the fitness of the Ig heavy chain to be expressed on the cell surface (reviewed in [4]). Subsequently, recombination of the V to J genes of the Ig light chains will be performed. This will first occur for the Ig kappa (IGK) light chain alleles, which contain in human 34-38 IGKV genes and 5 IGKJ genes, and when this does not

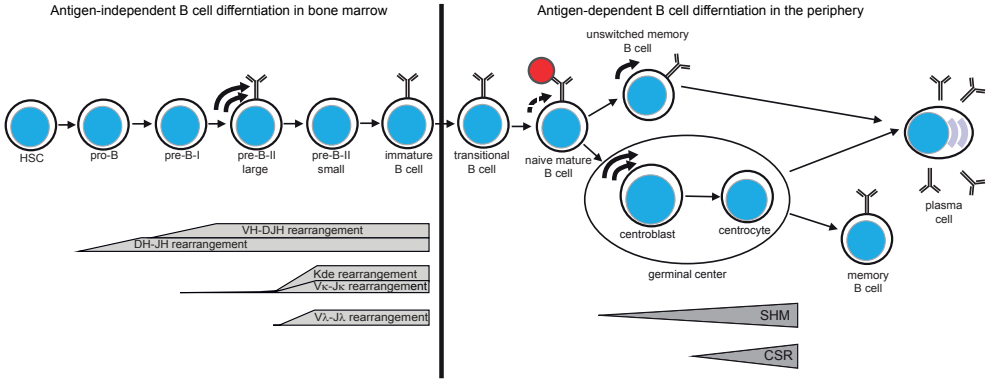

Figure 1. B cell differentiation.

Schematic overview of the phases of B cell differentiation from hematopoietic stem cells to memory B cells and plasma cells. The antigen-independent early B cell development stages in the bone marrow are defined by ordered IG gene rearrangements. In the periphery, antigen-dependent B cell differentiation takes place. Upon antigen recognition naïve mature B cells migrate to lymphoid follicles and initiate a T cell-dependent GC reaction, where the cells undergo extensive proliferation, somatic hypermutation (SHM) and class switch recombination (CSR). Alternatively, the B cells can undergo a T cell-independent differentiation in the marginal zone leading to largely unswitched memory B cells. (Adapted from the thesis of H. IJspeert)

succeed in a productive Ig light chain, this process will be continued on the Ig lambda (IGL) alleles, which have 29-33 IGLV and 5 IGLJ genes [5, 6].

This V(D)J recombination process induces a great variety in BCR molecules, but there are additional ways to create further diversity in the junction between the V, D, and J genes. This can be mediated by exonucleases, which can remove nucleotides. Moreover, RAG1/RAG2 together with DNA repair proteins can create palindromic nucleotides (P-nucleotides) through asymmetric hairpin opening, and terminal deoxynucleotidyl transferase (TdT) can

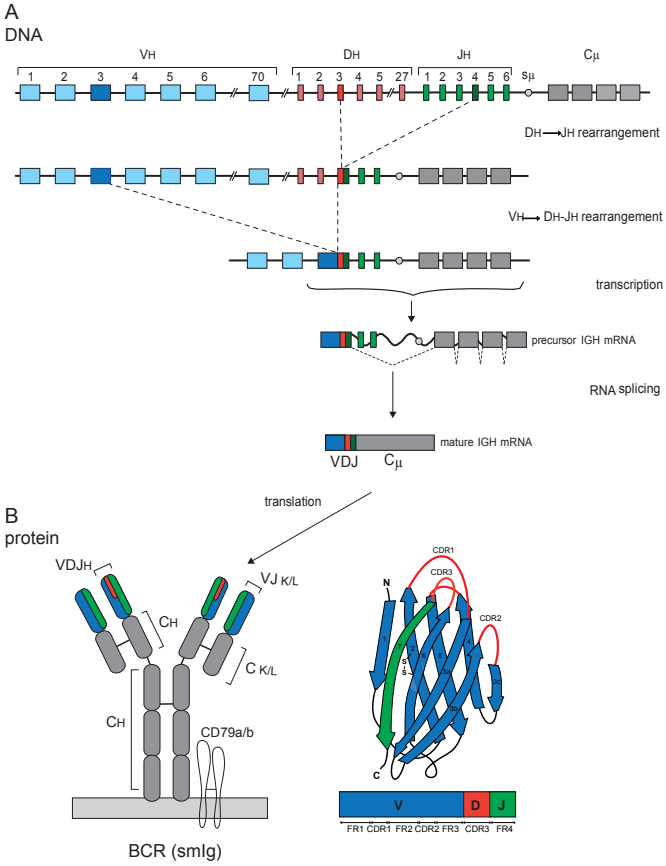

Figure 2. B cell receptor formation.

A. Rearrangement of the IGH locus starts with random recombination of one IGHD gene to one IGHJ gene, followed by rearrangement of this D-J joint to IGHV. Subsequently, the VDJ-exon is transcribed into immature mRNA, and forms after splicing with the Cμ exons the Igμ mature mRNA, which is translated into Ig μ heavy chain protein. After rearrangement of the IGK / IGL locus into a functional light chain protein, these are expressed as a functional BCR (surface IgM) on the plasma membrane together with CD79a/b (B, left panel). **B,** right panel. The IGHV domain is composed of four FR regions that are separated by three CDR regions, which are essential for antigen binding by forming loops. (Adapted from the thesis of M. Rother)

randomly add nucleotides (N-nucleotides). To complete formation of the junction, the coding ends of the V-, D-, and J-genes are ligated [7]. This junction defines the essential part for antigen recognition, the CDR3 [8].

The combination of the Ig heavy and Ig light chain will form the BCR, which is subsequently checked for autoreactivity. Upon recognition of self-antigen, the autoreactive B cell will either go into apoptosis, or become anergic, by limiting the response of BCR stimulation via the downstream signaling cascade. Alternatively, receptor editing, through replacement of the Ig light chain due to ongoing recombination events at the Ig light chain loci, can take place, which can rescue a B cell that was initially self-tolerant [9].

When these processes lead to a functional BCR, the B cell will leave the bone marrow and enter the circulation as a transitional B cell (**Figure 1**). At this point in development the B cells will start to express IgD BCRs, next to the IgM configuration, as a result of alternative splicing of the BCR mRNA [10]. These B cells are functionally immature, since they do not respond to BCR stimulation. Transitional B cells are characterized by the expression of the immune modulatory molecule CD5 and by CD24 and CD38, and constitute 5-10% of total B cells in the peripheral blood of adults [11]. Maturation of the transitional B cells into naive mature B cells is initiated by downregulation of CD24 and CD38 and finally CD5, which makes the cells fully competent to respond to antigenic stimulation [12].

Antigen-dependent B-cell differentiation

Naive mature B cells require binding to their cognate antigen to induce BCR signaling, and subsequently need a second signal to differentiate further. This additional signal is provided by activated T cells via CD40 ligand (CD40L), which interacts with CD40 on the B cells. T cell-dependent B cell responses induce the formation of germinal centers (GC). B cells within the GC will undergo extensive proliferation, accompanied by affinity maturation induced by somatic hypermutation (SHM) (**Figure 1**).

This SHM process is initiated by the enzyme activation-induced cytidine deaminase (AID), which randomly and bi-directionally introduces DNA alterations on single strand DNA of transcribed IGHV and IGK/IGL V genes [13], by starting with the deamination of cytidine (C) into uracil (U). This generates a mismatch between the newly formed U nucleotide and the pre-existing guanine (G) nucleotide on the corresponding position in the complementary DNA strand. When cells proliferate this U nucleotide can be recognized as thymine (T) by high-fidelity polymerase, which results into a C to T transition mutation. As an alternative, the U nucleotide can also be removed by uracil-N-glycosylase (UNG), which results into an abasic site that can be targeted by the base excision repair machinery and can induce both transition and transversion mutations in all of the four nucleotides. Another possibility is that the U-G mismatch will be recognized by the mismatch repair machinery which also can induce both transition and transversion mutations into A-T bases (reviewed in [14]) (**Figure 3**).

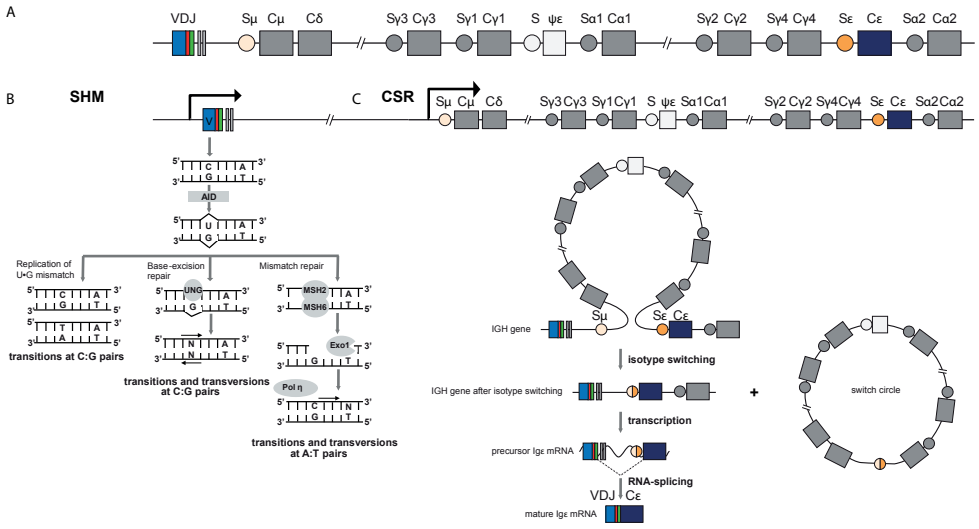

Figure 3. Somatic hyper mutation and class switch recombination.

A. The Ig heavy chain is composed of the variable region, which is essential for antigen recognition, and one of a series of nine constant region genes, which determine the functional properties of an antibody. **B.** Somatic hypermutation (SHM) starts with transcription of the variable region, when AID deaminates the C residues into U in single stranded DNA. The resulting mismatch can be repaired in three different ways. Via replication of the U-G mismatch, removal of U by UNG and subsequent repair via base-excision repair (BER), or when the U mismatch is recognized and repaired by the mismatch repair (MMR) machinery.

C. For class switch recombination (CSR), AID targets Ig switch regions and thereby causes multiple single strand DNA breaks in close proximity to each other, which can lead to double strands DNA breaks. When two of these Ig switch regions are in close proximity, they can be recombined, and the intervening DNA is looped out and excised.

SHM mostly occurs in the GC but can also take place in the marginal zone [15, 16]. Although, SHM can occur throughout the V genes of both heavy and light chains, it is especially apparent in the CDR1 and CDR2 regions [17]. The functionality of the now altered BCR is selected by its affinity for the cognate antigen [14].

AID mediates an additional GC process, namely class-switch recombination (CSR), the process whereby the constant region of the heavy chain is replaced to allow the B cell to switch to another Ig isotype. AID targets switch regions that contain multiple AID hotspot motifs and that are located upstream of the different constant regions. The CSR process also starts with the deamination of C into U. When AID induces two a-basic sites in close proximity this can induce a double strand DNA break. If this occurs in the μ-switch region and a switch region upstream of another constant gene, this can trigger class switching to IgG, IgA, and IgE (encoded by Cγ, Cα, or Cε respectively) (reviewed in [14]) (**Figure 3**). CSR does not alter antigen recognition itself, but changes the function of the BCR since Ig-isotypes

have different characteristics [18]. IgM is secreted as a pentamer and its main function is in complement activation [19]. IgG is most abundantly found in serum, can act locally in tissue, and is mainly involved in neutralization of pathogens, even intracellularly [20]. IgA functions in mucosal tissues, especially in the gastro-intestinal tract and is coating bacteria that are present [21]. Finally, IgE is involved in parasitic infections and allergic responses [22]. The SHM and CSR processes will trigger the formation of high affinity memory B cells and Ig-producing plasma cells [23]. The growth factors BAFF and APRIL support these processes via interaction with TACI on B cells [24].

Alternatively, naive B cells can be activated by antigens in a T cell-independent manner. In this scenario an additional signal occurs via an innate receptor such as a toll-like receptor (TLR), or via strong activation of multiple BCRs by antigen with a repetitive composition such as carbohydrate and lipid structures. This T cell-independent activation can occur in the marginal zone of the spleen - mostly directed against blood-borne more innate antigens - or in mucosal tissues [25]. BAFF and APRIL support these B cells to proliferate, partly undergo CSR to IgG or IgA, and differentiate into plasma blasts and finally into plasma cells (reviewed in [26]).

Malignant transformation of B cells

The molecular processes that occur during B cell development pose a potential risk of genomic instability, which might contribute to subsequent leukemia or lymphoma development (reviewed in [27]). The formation of the BCR during early B cell development requires the introduction of double strand DNA breaks in the genome by RAG1 and RAG2, which might also induce deletions or chromosome translocations involving non-IG genes including PAX5 and IKAROS, and thereby contribute to malignant transformation of precursor B cells into B cell precursor acute lymphoblastic leukemia (BCP-ALL) [28]. In the onset of BCP-ALL, also translocations (such as *BCR-ABL1*) are important, as well as mutations in genes that encode factors involved in (pre-)BCR signaling, or that encode transcription factors that are essential in B cell development [27, 29, 30]. BCP-ALL is relatively predominant in children and young adults.

In mature B cells, SHM and CSR processes can induce double strand breaks and genetic modifications, e.g. leading to IG translocations, involving the MYC and BCL genes, or leading to the induction of mutations in SHM hotspot-like regions, which might contribute to transformation and result in the mature types of leukemias and lymphomas, making AID key regulator in these processes [31, 32, 33]. These mature lymphoid malignancies are classified based on histological and cytological characteristics, localization, immunophenotype and genetic abnormalities. Many of these features reflect the differentiation stage of the B cell from which the leukemia or lymphoma originated [27]. Follicular lymphoma, the most common type of B cell lymphoma is derived from GC B cells [34]. Also, diffuse large B cell lymphoma seems to be derived from activated B cells in the GC, in which auto-antigenic recognition plays a

role [35]. On the far end of the B cell differentiation spectrum are plasma cells that can give rise to multiple myeloma, a plasma cell malignancy, which suppresses normal immune cell development in the bone marrow [36].

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in elderly individuals in the Western world. CLL is a multifactorial disease, in which both cell-intrinsic genetic alterations as well as cell-extrinsic stimulation via the microenvironment and the BCR play key roles.

BCR-induced stimulation in CLL

Chronic antigenic stimulation is thought to be relevant in the onset of CLL , particularly in CLL that carry a BCR with SHM (mutated CLL, M-CLL), highlighting their origin from post-GC memory B cells [37]. In contrast, CLL without SHM (also termed unmutated CLL, U-CLL) are derived from cells that potentially have been activated but that have not gone through the GC reaction [37]. M-CLL and U-CLL show different prognosis, with M-CLL being the more indolent form of disease [38]. Nowadays, signaling molecules functioning downstream of the BCR are important targets for therapy, further corroborating the importance of BCR activation in CLL.

Microenvironmental interactions in CLL

B cells are strongly dependent upon interactions with the microenvironment for their survival and further maturation. CLL cells also have particular interactions with other cells in the bone marrow and in lymphoid organs in certain niches, the so-called proliferation centers. The microenvironment in these proliferation centers consists of cells which are normally present in these organs, such as mesenchymal stromal cells in bone marrow and nurse-like cells (NLC) in lymphoid organs. However, these cells co-evolved with the CLL cells through cellular interactions in order to establish a beneficial microenvironment. This CLL microenvironment shows similarities with that of other tumor environments, including blood vessel formation to provide more nutrients, local production of growth factors, and protection against the elimination of the tumor cells by immune cells (reviewed in [39, 40]).

NLC are a type of macrophages that are derived from blood monocytes and are able to activate the BCR by presenting antigen to the CLL cells. They provide homing signals through secretion of the chemokines CXCL12 and CXCL13 [41]. CLL cells express the chemokine receptors CXCR4 and CXCR5 and can sense chemokine gradients. NLC also express BAFF and APRIL, which induce proliferation and survival of CLL cells via the BAFF receptor and the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) [42].

T cells also play an important role in the activation and survival of CLL cells via CD40-CD40-L interaction [43]. In CLL patients the T cell subset distribution is shifted, and a more

exhausted phenotype is apparent. This change in phenotype coincides with impaired T cell effector function (tumor cell killing) and reduced synapse formation capacity [40, 44, 45].

Genetic alterations in CLL

Several genetic alterations play a role in the onset and clonal evolution of CLL. It is suggested that *passenger* mutations in normal counterpart cells accumulate and could act as founders in leukemogenesis [46]. The next steps of clonal evolution are induced by the so-called *driver* mutations, which are generally clonal aberrations that are found across patients. Examples of such driver aberrations are 13qdel (deletion of the short arm of chromosome 13), trisomy 12, and mutations in myeloid differentiation primary response gene 88 (*MYD88*), encoding a key signaling molecule downstream of Toll-like receptors, which all seem specific drivers in many B cell malignancies, including CLL [46, 47, 48]. Subclonal mutations direct the next phase in disease progression, and are often induced by therapy. These affect clonal evolution and induce an increase in proliferation and a decrease in apoptosis over other CLL cells [46, 49]. Among these mutations are loss-of-function mutations in tumor protein 53 (*TP53*) and ataxia telangietasia mutated (*ATM*) genes as well as gain-of-function mutions in *RAS* , *NOTCH1*, and splicing factor 3B subunit 1 (*SF3B1*) genes [46, 47]. Spliceosome component SF3B1 is often found to be mutated in CLL and is associated with poor outcome. *SF3B1* mutation was found to induce comprehensive changes in splicing and gene expression across several pathways including DNA damage response, Notch signaling, and telomere maintenance [50]. *NFKBIE* mutations are associated with a more advanced stage of CLL. The *NFKBIE* gene encodes IκBε, which regulates NF-κB in healthy B cells. A particular *NFKBIE* mutation has been reported to lead to a truncated protein with an abrogated function, and thereby loss of its inhibitory effect on NF-κB [51]. Another recently identified driver mutation is found in the early growth response 2 (*EGR2*) gene, which functions as a transcription factor involved in haematopoeisis and downstream of ERK in BCR signaling. The EGR2 missense mutations lead to altered expression of EGR2 target genes in CLL and are associated with a poor prognosis [52]. In addition to mutations in coding genes, also non-coding mutations were identified to play a role in CLL [53]. Overall, innovations in next generation sequencing in the last decade are shedding more and more light on the genes involved in onset and evolution of CLL.

Scope of this thesis

Next to the above described microenvironmental interactions and genetic alterations, B cell receptor-driven signaling is believed to essentially contribute to the immunopathogenesis of human CLL. The overall aim of this thesis is to evaluate functional abnormalities in the BCR signaling process in human CLL cells and to define their impact on CLL pathogenesis.

In **Chapter 1** an overview is presented on human B cell development and on malignant transformation of B cells, with a special focus on CLL and the role of the microenvironment

and genetic alterations. In **Chapter 2** the role of signaling pathways in B cells and their important roles in human CLL are reviewed. Furthermore, an overview is given on novel therapies which target these signaling pathways in human CLL. It has been reported that CLL B cells have increased autonomous Ca^{2+} signaling, in contrast to other B cell malignancies or healthy B cells. As it remained unknown whether the increase in Ca^{2+} signaling was related to the mutation status of the BCR, we aimed to explore in **Chapter 3** whether differences exist in the basal Ca^{2+} signaling between U-CLL and M-CLL. In **chapter 4**, we wanted to further characterize the differences in BCR responsiveness detected in human CLL and study the underlying molecular mechanisms. Relatives of CLL patients are known to have an increased risk in developing this disease. In **Chapter 5** the importance of the BCR as well as genetic predisposition are investigated in the context of familial CLL cases. In **chapter 6**, we aimed to evaluate the effect of aging on B cell subset distribution and BCR repertoire in healthy individuals, and compared this with the BCR repertoire and stereotypic BCR features in human CLL. Finally, the implications of the studies described in this thesis are critically discussed in the general discussion (**Chapter 7**).

References

1. Kondo, M., Weissman, I.L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-672 (1997).
2. Mikkola, I., Heavey, B., Horcher, M. & Busslinger, M. Reversion of B cell commitment upon loss of Pax5 expression. *Science* **297**, 110-113 (2002).
3. Alt, F.W., Yancopoulos, G.D., Blackwell, T.K., Wood, C., Thomas, E., Boss, M. *et al.* Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO J* **3**, 1209-1219 (1984).
4. Hendriks, R.W. & Middendorp, S. The pre-BCR checkpoint as a cell-autonomous proliferation switch. *Trends Immunol* **25**, 249-256 (2004).
5. Ehlich, A., Schaal, S., Gu, H., Kitamura, D., Muller, W. & Rajewsky, K. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell* **72**, 695-704 (1993).
6. van der Burg, M., Tumkaya, T., Boerma, M., de Bruin-Versteeg, S., Langerak, A.W. & van Dongen, J.J. Ordered recombination of immunoglobulin light chain genes occurs at the IGK locus but seems less strict at the IGL locus. *Blood* **97**, 1001-1008 (2001).
7. Bassing, C.H., Swat, W. & Alt, F.W. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* **109 Suppl**, S45-55 (2002).
8. Wardemann, H., Yurasov, S., Schaefer, A., Young, J.W., Meffre, E. & Nussenzweig, M.C. Predominant autoantibody production by early human B cell precursors. *Science* **301**, 1374-1377 (2003).
9. Lang, J., Ota, T., Kelly, M., Strauch, P., Freed, B.M., Torres, R.M. *et al.* Receptor editing and genetic variability in human autoreactive B cells. *J Exp Med* **213**, 93-108 (2016).
10. Yuan, D., Witte, P.L., Tan, J., Hawley, J. & Dang, T. Regulation of IgM and IgD heavy chain gene expression: effect of abrogation of intergenic transcriptional termination. *J Immunol* **157**, 2073-2081 (1996).

11. Sims, G.P., Ettinger, R., Shirota, Y., Yarboro, C.H., Illei, G.G. & Lipsky, P.E. Identification and characterization of circulating human transitional B cells. *Blood* **105**, 4390-4398 (2005).
12. van Zelm, M.C., Szczepanski, T., van der Burg, M. & van Dongen, J.J. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med* **204**, 645-655 (2007).
13. Senavirathne, G., Bertram, J.G., Jaszczur, M., Chaurasiya, K.R., Pham, P., Mak, C.H. *et al.* Activation-induced deoxycytidine deaminase (AID) co-transcriptional scanning at single-molecule resolution. *Nat Commun* **6**, 10209 (2015).
14. Chaudhuri, J., Basu, U., Zarrin, A., Yan, C., Franco, S., Perlot, T. *et al.* Evolution of the immunoglobulin heavy chain class switch recombination mechanism. *Adv Immunol* **94**, 157-214 (2007).
15. Martin, A., Bardwell, P.D., Woo, C.J., Fan, M., Shulman, M.J. & Scharff, M.D. Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* **415**, 802-806 (2002).
16. Colombo, M., Cutrona, G., Reverberi, D., Bruno, S., Ghiotto, F., Tenca, C. *et al.* Expression of immunoglobulin receptors with distinctive features indicating antigen selection by marginal zone B cells from human spleen. *Mol Med* **19**, 294-302 (2013).
17. Messmer, B.T., Albesiano, E., Messmer, D. & Chiorazzi, N. The pattern and distribution of immunoglobulin VH gene mutations in chronic lymphocytic leukemia B cells are consistent with the canonical somatic hypermutation process. *Blood* **103**, 3490-3495 (2004).
18. Stavnezer, J. Antibody class switching. *Adv Immunol* **61**, 79-146 (1996).
19. Lu, J.H., Thiel, S., Wiedemann, H., Timpl, R. & Reid, K.B. Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. *J Immunol* **144**, 2287-2294 (1990).
20. Mallery, D.L., McEwan, W.A., Bidgood, S.R., Towers, G.J., Johnson, C.M. & James, L.C. Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc Natl Acad Sci U S A* **107**, 19985-19990 (2010).
21. Berkowska, M.A., Schickel, J.N., Grosserichter-Wagener, C., de Ridder, D., Ng, Y.S., van Dongen, J.J. *et al.* Circulating Human CD27-IgA+ Memory B Cells Recognize Bacteria with Polyreactive Igs. *J Immunol* **195**, 1417-1426 (2015).
22. Sutton, B.J. & Gould, H.J. The human IgE network. *Nature* **366**, 421-428 (1993).
23. Weller, S., Faili, A., Garcia, C., Braun, M.C., Le Deist, F.F., de Saint Basile, G.G. *et al.* CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A* **98**, 1166-1170 (2001).
24. Yu, G., Boone, T., Delaney, J., Hawkins, N., Kelley, M., Ramakrishnan, M. *et al.* APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. *Nat Immunol* **1**, 252-256 (2000).
25. Weill, J.C., Weller, S. & Reynaud, C.A. Human marginal zone B cells. *Annu Rev Immunol* **27**, 267-285 (2009).
26. Cerutti, A., Cols, M. & Puga, I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* **13**, 118-132 (2013).
27. Rickert, R.C. New insights into pre-BCR and BCR signalling with relevance to B cell malignancies. *Nat Rev Immunol* **13**, 578-591 (2013).
28. Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D. *et al.* Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* **446**, 758-764 (2007).

29. Shojaee, S., Chan, L.N., Buchner, M., Cazzaniga, V., Cosgun, K.N., Geng, H. *et al.* PTEN opposes negative selection and enables oncogenic transformation of pre-B cells. *Nat Med* **22**, 379-387 (2016).
30. Swaminathan, S., Klemm, L., Park, E., Papaemmanuil, E., Ford, A., Kweon, S.M. *et al.* Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia. *Nat Immunol* **16**, 766-774 (2015).
31. Otto, C., Scholtysik, R., Schmitz, R., Kreuz, M., Becher, C., Hummel, M. *et al.* Novel IGH and MYC Translocation Partners in Diffuse Large B-Cell Lymphomas. *Genes Chromosomes Cancer* **55**, 932-943 (2016).
32. Ghazzaui, N., Saintamand, A., Issaoui, H., Vincent-Fabert, C. & Denizot, Y. The IgH 3' regulatory region and c-myc-induced B-cell lymphomagenesis. *Oncotarget* **8**, 7059-7067 (2017).
33. Casellas, R., Basu, U., Yewdell, W.T., Chaudhuri, J., Robbiani, D.F. & Di Noia, J.M. Mutations, kataegis and translocations in B cells: understanding AID promiscuous activity. *Nat Rev Immunol* **16**, 164-176 (2016).
34. Bende, R.J., Smit, L.A., Bossenbroek, J.G., Aarts, W.M., Spaargaren, M., de Leval, L. *et al.* Primary follicular lymphoma of the small intestine: alpha4beta7 expression and immunoglobulin configuration suggest an origin from local antigen-experienced B cells. *Am J Pathol* **162**, 105-113 (2003).
35. Young, R.M., Wu, T., Schmitz, R., Dawood, M., Xiao, W., Phelan, J.D. *et al.* Survival of human lymphoma cells requires B-cell receptor engagement by self-antigens. *Proc Natl Acad Sci U S A* **112**, 13447-13454 (2015).
36. Podar, K. & Jager, D. Targeting the immune niche within the bone marrow microenvironment: The rise of immunotherapy in Multiple Myeloma. *Curr Cancer Drug Targets* (2017).
37. Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Durig, J. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* **209**, 2183-2198 (2012).
38. Degan, M., Bomben, R., Bo, M.D., Zucchetto, A., Nanni, P., Rupolo, M. *et al.* Analysis of IgV gene mutations in B cell chronic lymphocytic leukaemia according to antigen-driven selection identifies subgroups with different prognosis and usage of the canonical somatic hypermutation machinery. *Br J Haematol* **126**, 29-42 (2004).
39. Burger, J.A., Ghia, P., Rosenwald, A. & Caligaris-Cappio, F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* **114**, 3367-3375 (2009).
40. Choi, M.Y., Kashyap, M.K. & Kumar, D. The chronic lymphocytic leukemia microenvironment: Beyond the B-cell receptor. *Best Pract Res Clin Haematol* **29**, 40-53 (2016).
41. Burger, J.A., Quiroga, M.P., Hartmann, E., Burkle, A., Wierda, W.G., Keating, M.J. *et al.* High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. *Blood* **113**, 3050-3058 (2009).
42. Nishio, M., Endo, T., Tsukada, N., Ohata, J., Kitada, S., Reed, J.C. *et al.* Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood* **106**, 1012-1020 (2005).
43. Granziero, L., Ghia, P., Circosta, P., Gottardi, D., Strola, G., Geuna, M. *et al.* Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. *Blood* **97**, 2777-2783 (2001).
44. Ramsay, A.G., Johnson, A.J., Lee, A.M., Gorgun, G., Le Dieu, R., Blum, W. *et al.* Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J Clin Invest* **118**, 2427-2437 (2008).

45. Riches, J.C., Davies, J.K., McClanahan, F., Fatah, R., Iqbal, S., Agrawal, S. *et al.* T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood* **121**, 1612-1621 (2013).
46. Landau, D.A., Carter, S.L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M.S. *et al.* Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* **152**, 714-726 (2013).
47. Puente, X.S., Pinyol, M., Quesada, V., Conde, L., Ordonez, G.R., Villamor, N. *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* **475**, 101-105 (2011).
48. Abruzzo, L.V., Herling, C.D., Calin, G.A., Oakes, C., Barron, L.L., Banks, H.E. *et al.* Trisomy 12 chronic lymphocytic leukemia expresses a unique set of activated and targetable pathways. *Haematologica* **103**, 2069-2078 (2018).
49. Zhao, Z., Goldin, L., Liu, S., Wu, L., Zhou, W., Lou, H. *et al.* Evolution of multiple cell clones over a 29-year period of a CLL patient. *Nat Commun* **7**, 13765 (2016).
50. Wang, L., Brooks, A.N., Fan, J., Wan, Y., Gambe, R., Li, S. *et al.* Transcriptomic Characterization of SF3B1 Mutation Reveals Its Pleiotropic Effects in Chronic Lymphocytic Leukemia. *Cancer Cell* **30**, 750-763 (2016).
51. Mansouri, L., Sutton, L.A., Ljungstrom, V., Bondza, S., Arngarden, L., Bhoi, S. *et al.* Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. *J Exp Med* **212**, 833-843 (2015).
52. Young, E., Noerenberg, D., Mansouri, L., Ljungstrom, V., Frick, M., Sutton, L.A. *et al.* EGR2 mutations define a new clinically aggressive subgroup of chronic lymphocytic leukemia. *Leukemia* (2017).
53. Puente, X.S., Bea, S., Valdes-Mas, R., Villamor, N., Gutierrez-Abril, J., Martin-Subero, J.I. *et al.* Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature* (2015).

Chapter 2

Targeting signaling pathways in chronic lymphocytic leukemia

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ABSTRACT

Various signal transduction pathways have been implicated in the pathogenesis of chronic lymphocytic leukemia (CLL), which is characterized by the progressive accumulation of monoclonal $CD5^+$ B cells in the blood. B cell receptor (BCR) signaling appears to have a crucial role in disease onset and is thought to be induced by self- or non-self-antigen recognition leading to chronic stimulation. Several of the kinases functioning downstream of the BCR are aberrantly expressed or constitutively activated in CLL. Yet, these kinases have additional roles, particularly in chemokine receptor signaling, which is essential for homing and survival of CLL cells in lymphoid organs, or in toll-like receptor signaling. Recently, small molecule inhibitors of kinases in the BCR signaling pathway have shown impressive anti-tumor activity in clinical trials. Remarkably, the observed durable responses in CLL patients were accompanied by transient increases in blood lymphocyte numbers, indicating the importance of these kinases in chemokine receptor signaling. In this review, we therefore highlight the role of BCR signaling and the important other associated signal transduction cascades for CLL cells and give an overview of novel agents that target these specific pathways and were shown to be successful for CLL treatment in clinical trials.

GENERAL INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most frequently occurring type of leukemia in adults in the Western world. CLL is characterized by accumulation of a monoclonal population of small B cells with a typical immunophenotype ($CD19^{+}$, $CD20^{dim}$, $CD5^{+}$, $CD23^{+}$, $CD27^{+}$, $CD43^{+}$, surface Ig^{dim}) in the blood. Over 95% of cases are diagnosed above 50 years of age. To date, the overall 5-year relative survival for CLL patients is 79.2% [1]. Nevertheless, there is a large difference in survival between individual CLL patients, varying from several months to a normal life expectancy [1]. This heterogeneity in survival reflects the biological heterogeneity known for CLL, which is considered a multifactorial disease.

In CLL several genetic aberrations are found that have prognostic value. For instance, there are multiple chromosomal aberrations that together occur in approximately 80% of CLL cases. These mainly concern deletions of chromosomal regions 17p, 11q, or 13q, and trisomy of chromosome 12. These chromosomal deletions are associated with the loss of the *TP53* and *ATM* genes, and the *miR15a* and *miR16-1* microRNA genes, respectively; so far the relevant gene involved in trisomy 12 is unknown. Deletion of 17p and deletion of 11q are associated with a poor disease outcome, while deletion of 13q as a single event is associated with a milder form of disease (reviewed in [2]). Over the last few years several novel mutations with prognostic value have been identified by next generation sequencing approaches. Mutations found in the *NOTCH1* [3] and *SF3B1* genes [4] are associated with progressive disease, while mutations in *MYD88* [3] are rare and appear to be associated with an indolent form of disease. Although some of the identified mutations appear to act as early driver mutations playing a role in disease onset, mutations are never present in 100% of the clones [5]. Other mutations may act later in evolution of the CLL clone and seem to be more important for disease progression. From all genetic studies it is however clear that neither the B cell receptor (BCR) itself, nor its signaling pathway is directly targeted by mutations.

Instigated by the idea that CLL is generated from different stages of B cell maturation and activation, several cellular origins of CLL have been suggested. Marginal zone B cells [6], post-germinal center (GC) memory B cells [7], $CD5^{+}$ B cells [8, 9], and IL-10 expressing regulatory B cells [10] have all been mentioned as the normal B cell counterpart from which CLL cells would derive. On the basis of somatic hypermutation status (SHM) of the immunoglobulin heavy chain variable (IGHV) genes of the BCR, it is clear that CLL cells could derive from pre- or post-GC B cells. CLL patients can thus be grouped into mutated CLL (M-CLL) and unmutated CLL (U-CLL). This division is also clinically relevant because U-CLL tend to have an unfavorable prognosis, with a more aggressive course of the disease and shorter time to first treatment, while M-CLL is associated with a more indolent disease form with a relatively favorable prognosis [11, 12].

Approximately one-third of all CLL cases can be grouped on the basis of their restricted IGHV, IGHD, and IGHJ gene usage, and similarities in length and amino acid sequence of the complementarity determining region 3 (CDR3) [13]. These so-called stereotypic BCRs are found in multiple CLL patients and the analyses of large cohorts of CLL patients enabled their clustering into at least 19 major subsets which contain ~12% of all CLL cases; another 18% of CLL cases belong to minor subsets, as was found in a study which included 7424 CLL patients [13]. The finding of BCR stereotypy is indicative of a contribution of similar antigens, thus implicating antigenic stimulation and thereby BCR specificity in CLL pathogenesis [13]. Recently, it has been shown that BCR stereotypy in CLL not only has biological impact, but also bears clinical significance. Distinct BCR stereotypic subsets appeared to associate with differences in time to first treatment, thus showing added prognostic value over U-CLL/M-CLL status and cytogenetic aberrations [14, 15].

Taken together, these findings demonstrate that important prognostic information resides in the BCR molecules and indicate an important role of the BCR and downstream signaling pathways. Recent clinical trials have provided evidence that the therapeutic effect of various kinase inhibitors of the BCR signaling pathway, particularly including the Bruton's tyrosine kinase (BTK) inhibitor, ibrutinib, and the phosphatidyl-inositol-3-kinase δ-isoform (PI3Kδ) inhibitor, idelalisib, is also dependent on the role of these kinases in chemokine receptor signaling. Therefore, in this review, we aim to discuss the role of BCR signaling and important associated other signal transduction cascades for CLL cells. We will give an overview of novel agents that target these specific pathways and were shown to be successful in clinical trials.

ROLE OF ANTIGENIC STIMULATION IN CLL PATHOGENESIS

In addition to the stereotypic BCR repertoire in CLL, other lines of evidence support an important role for antigen-driven BCR signaling in CLL pathogenesis. In particular, CLL B cells show low surface IgM expression and often an anergic response to BCR ligation, suggesting chronic BCR stimulation and signaling. Nevertheless, the degree of anergy is not equal for every CLL, as we will point out below. Anergy is a cellular condition that keeps a B cell capable of binding to its antigen, without inducing proper BCR activation, and so full activation is hampered. This is a strategy of the immune system for silencing auto-reactive B cells, which can in this way persist in the periphery [16]. The finding that BCR unresponsiveness can be restored upon culture *in vitro* supports that the BCR on CLL cells is engaged by antigen *in vivo* [17]. Based on CLL expression profiling studies that show upregulation of gene signatures consistent with BCR and nuclear factor-κB (NF-κB) activation, it has been proposed that the lymph node micro-environment is essential for the expansion of CLL cells in so-called proliferation centers [18].

For several CLL-derived BCRs a binding antigen has been identified. In general, most U-CLLs were found to express poly-reactive, low affinity BCRs, which bind self- or non-self-antigens, such as DNA, insulin, LPS, apoptotic cells, vimentin, myosin heavy chain 2A (MYH2A), and phosphoryl choline-containing antigens including oxidized low-density lipoprotein (LDL) 19, 20, 21, 22. BCRs from stereotypic subset #1 bind to oxidized LDL 20, 22, 23. Stereotypic subset #2 recognizes protein L, a cell-wall protein of the commensal gut bacterium Peptostreptococcus magnus 24 and in addition binds to cofilin-1 22. Several stereotypic CLL subsets, including #3, #6, and #7, are known to use the IGHV1-69 gene, which is very common in CLL and is mostly associated with U-CLL. CLL-derived BCRs containing IGHV1-69 and IGHV3-21 were found to react with the cytomegalovirus protein pUL32 25. Recently, two reports demonstrated that two distinct stereotypic M-CLL subsets have specificity for the Fc-tail of IgG, and thereby have so-called rheumatoid factor activity 26, 27. Stimulation of these specific stereotypic CLL cells with IgG indeed resulted in their proliferation 26. In addition, Hoogeboom et al. 28 identified a stereotypic M-CLL subset with IGHV3-7 gene usage with an extremely short CDR3 of 5-6 amino acids. This stereotypic receptor is highly specific for a potent antigen called β-(1,6)-glucan, which can be found in yeast and filamentous fungi. Antigenic stimulation of CLL cells expressing this particular stereotypic BCR induces proliferation 28. Collectively, these data provide support for an important role for antigen-driven BCR signaling in CLL pathogenesis.

In contrast to these studies, Dühren-von Minden *et al*. [29] observed that CLL-derived BCRs can be stimulated independently of external antigens, because of the presence of an internal epitope in framework 2 (FR2) of the IGHV domain that is recognized by their CDR3. This recognition induces an increased level of antigen-independent, basal or 'autonomous' signaling of the BCR, as demonstrated by increased cytoplasmic Ca^{2+} levels compared with BCRs from non-malignant B cells. Remarkably, this cell-autonomous BCR signaling was not observed in other B cell malignancies, including myeloma, mantle cell lymphoma, follicular lymphoma, and marginal zone lymphomas. These observations were predominantly made in an innovative *in vitro* assay using triple knockout (TKO) cells, which are BCR-free, conditionally signaling-competent mouse B-lineage cells. Upon transduction of human CLL-derived BCRs (or CLL-derived CDR3s) increased Ca^{2+} signaling was observed, while other B cell lymphoma-derived BCRs needed anti-BCR stimulation to induce Ca^{2+} signaling [29]. When the conserved FR2 epitope was mutated, leading to an amino acid change, the autonomous signaling was lost. Conversely, when the CDR3 region of the CLL-derived BCR was introduced in an originally non-autonomous signaling BCR, this BCR became autonomously active. In addition, another internal epitope in FR3 of both IGHV and Ig light chain V genes was identified [30], which may play a role in autonomous signaling in CLL as well.

These findings fueled the controversy whether antigen-dependent or cell-autonomous stimulation would be important in CLL pathogenesis, but at the same time they further underlined the relevance of the BCR in this disease. Importantly, the two contrasting

observations may not be mutually exclusive, because it is conceivable that some level of autonomous signaling may be enhanced by ligand-dependent, antigen-driven BCR signaling. In support of this hypothesis, we recently found that the degree of cell-autonomous signaling differed between CLL subgroups, when we analyzed basal Ca^{2+} signaling in a series of primary stereotypic or heterogeneous U-CLL and M-CLL [31]. We observed that basal signaling was not uniformly enhanced in CLL B cells, but particularly increased in M-CLL and thus associated with CLL IGHV mutational status. From these findings we concluded that this might reflect the distinct cellular origin of M-CLL and possibly a different anergic state induced by repetitive or continuous antigen binding *in vivo* [31]. Perhaps CLL clones originate as antigen-dependent clones that become more autonomous and gain increased basal Ca^{2+} signaling upon mutation of critical amino acids in their CDR3 region.

IMPORTANT SIGNAL TRANSDUCTION PATHWAYS IN MATURE B CELLS

Based on the mutations and signal transduction aberrations identified in CLL cells, as well as the successful treatment of CLL by small molecule inhibitors, it is becoming clear that three main signal transduction routes, BCR signaling, chemokine receptor signaling, and toll-like receptor (TLR) signaling, are crucial for survival of malignant CLL cells in patients. Here, we first describe these signaling pathways in non-malignant mature B cells.

B cell receptor (BCR) signaling

The BCR serves as an antigen receptor and is essential for B cell fate. Surface Ig is the main component of the BCR signaling complex which senses the environment for molecules that bind with significant avidity. The BCR complex at the cell surface further consists of the non-covalently associated Igα (CD79a) and Igβ (CD79b) molecules, which together form a heterodimer via a disulphide bridge, and the BCR co-receptor CD19 (**Figure 1**). After antigen binding to the BCR the SRC-family protein tyrosine kinase LYN phosphorylates the first immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residue of the cytoplasmic tails of both Igα and Igβ [32]. This first phosphorylated ITAM tyrosine residue serves as a docking site for another protein tyrosine kinase, SYK, which can phosphorylate both ITAM tyrosines [32, 33]. LYN additionally phosphorylates tyrosine residues in the cytoplasmic tail of CD19, which facilitates binding and activation of PI3K and the guanine nucleotide exchange factor VAV [33, 34, 35]. PI3K can also be recruited by the cytoplasmic B cell adapter for PI3K (BCAP) [36]. Class IA PI3K isoforms are composed of a heterodimer between a p110 catalytic subunit and a p85 regulatory subunit. The three different catalytic subunits, p110α, p110β and p110δ, are encoded by separate genes and have distinct roles. PI3Kδ (p110δ, encoded by *PIK3CD*) is

predominantly expressed in leukocytes, including B cells [37]. It is known that PI3Kδ plays a key role in B cell function, since inhibiting this specific isoform leads to reduced activation downstream in the BCR signaling cascade and disturbed GC-formation [38]. PI3K phosphorylates PIP2 to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), which then recruits BTK to the plasma membrane [39]. Next, BTK can be fully activated through phosphorylation by SYK and LYN at the Y551 position, which is followed by BTK autophosphorylation at position Y223 [40, 41, 42]. This activation of BTK can be modulated by SH2-domain containing

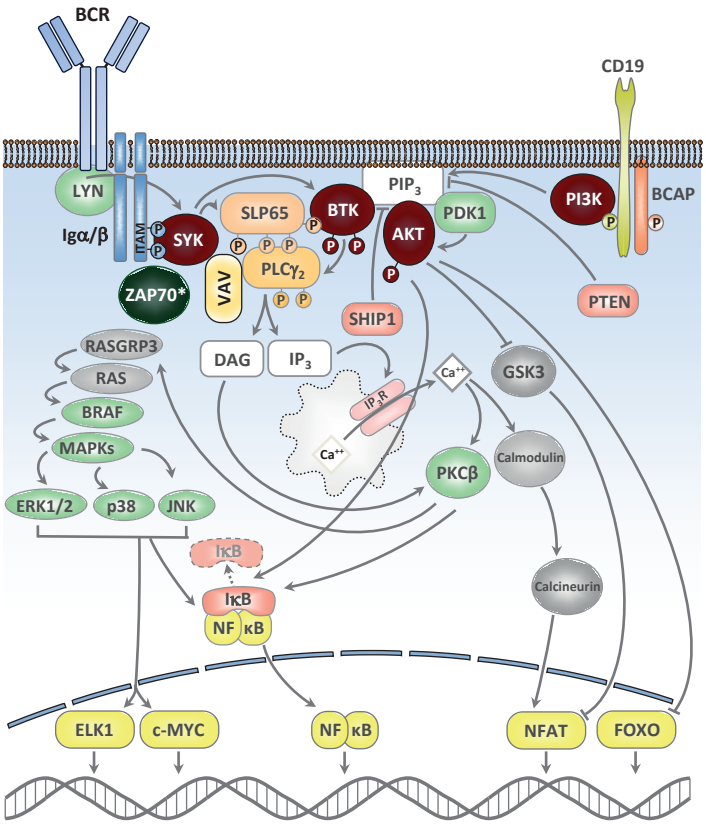


Figure 1. CLL treatment targets in the B cell receptor signaling pathway.

Upon antigen binding by the B cell receptor (BCR), signaling is initiated by LYN-mediated phosphorylation of ITAMs in the cytoplasmic tail of Igα/β and CD19, resulting in recruitment of SYK and PI3K, respectively. PI3K generates PIP_3 to enable membrane recruitment of BTK. Phosphorylation of SLP65 by SYK induces a docking platform for BTK and $PLC\gamma_2$, and formation of the BCR micro-signalosome, resulting into phosphorylation of $PLC\gamma_2$ by BTK. This induces an influx of Ca^{2+}, finally resulting in subsequent activation of ELK1/c-Myc, NF-κB, NFAT and FOXO transcription factors. In CLL, several kinases are upregulated and ZAP70 might be aberrantly expressed (see text for details). The kinases that are targeted by inhibitors described in this review are indicated in red.

inositol-5'-phosphatase-1 (SHIP1) and phosphatase and tensin homolog (PTEN), which both inhibit association of BTK to the membrane by dephosphorylating PIP3. Recruitment of SH2 domain-containing leukocyte protein of 65 kDa (SLP65, or B cell linker, BLNK) is essential for further downstream signaling, as it serves as a scaffold for the signaling molecules SYK and BTK, and thereby creates a docking site for phospholipase Cγ2 (PLCγ2) [43, 44]. PLCγ2 is activated by phosphorylation through BTK at positions Y753 and Y759, which is important for its lipase activity. The SLP65-mediated recruitment of BTK and PLCγ2 into the complex, finalizes the formation of a so-called micro-signalosome, which consists of PI3K, VAV, SLP65 and PLCγ2 [45, 46] (see **Figure 1**). Inferred from *in vitro* findings in the A20 B cell line, BTK can – independently of its kinase activity - play a role in the recruitment of PIP5K. This generates a positive feedback loop via PIP2, which acts as a substrate for both PI3K and PLCγ2 [47]. In this way, BTK stimulates PIP3 production that is essential for its own activation. After the activation of PLCγ2 by BTK, it cleaves PIP2 to generate the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), which leads to the branching of the further downstream signaling pathways [48], although these also partially overlap. IP3 is involved in the regulation of intracellular Ca^{2+} levels after binding to its receptors on the endoplasmic reticulum membrane. It thereby activates, via calmodulin and calcineurin, the transcription factor nuclear factor of activated T cells (NFAT) [49]. DAG mediates the activation of protein kinase Cβ (PKCβ), which is required for the activation of several mitogen-activated protein kinase (MAPK) family members like the proto-oncogene B-RAF (BRAF), mitogen activated protein kinase kinase (MEK), extracellular signal-regulated kinases 1 and -2 (ERK1 and -ERK2), and other downstream MAPK targets like Jun N-terminal kinase (JNK), P38, and NF-κB pathway components [50] (see **Figure 1**).

Another major branching point, besides that of PLCγ2, functions further upstream in the BCR signaling cascade and is induced by the activation of serine/threonine kinase AKT by PI3K. The downstream effector of PI3K, Ser/Thr kinase phosphoinositide-dependent kinase 1 (PDK1) is required for the phosphorylation of AKT at the cell membrane. Fully activated AKT proceeds to the cytoplasm to facilitate pro-survival signaling 51. Important AKT targets are NFAT, forkhead transcription factors (FOXOs) and NF-κB 52. Collectively, these partially parallel BCR signaling routes determine the outcome of BCR stimulation, which may be very diverse, including proliferation, survival, cellular differentiation, apoptosis, or anergy.

Chemokine receptor signaling

Chemokine receptors are G-protein-coupled receptors, of which 19 have been described so far. Given that there are 50 known chemokines, this indicates a redundancy in the function of chemokines [53]. Chemokine receptor pathways in B cells are essential for their trafficking, homing and homeostasis. The chemokine receptors CXCR4 and CXCR5 are highly expressed on the surface of B cells [54]. Binding of one of their agonists to the extracellular domains of the

chemokine receptor induces conformational changes in the seven-transmembrane spanning domain. This facilitates interactions with the intracellular heterotrimeric G-proteins and allows transmission of the signal (see **Figure 2**) [55]. Heterotrimeric G-proteins are composed of α, β, and γ (Gα, Gβ, and Gγ) subunits. Upon dissociation of the Gα subunit, the Gβγ subunits can activate several downstream targets of the chemokine receptor. Both Gα and Gβγ subunits can activate PI3K independently, which in turn results in the activation of AKT and MAPK [56]. The PI3K-mediated increase in PIP3 leads to a local recruitment of proteins containing PIP3-pleckstrin homology (PH) domains, including BTK, and is essential for membrane anchorage [57]. Next to PI3K activation, both Gα and Gβγ subunits can directly bind to BTK via the PH and Tec homology (TH) domain [58]. The tyrosine kinases LYN and SYK play a role in chemokine receptor signaling as well, as was observed in B cells that lack LYN or SYK [59]. These kinases were found to activate BTK after CXCR4 ligation[59], which was confirmed by the finding that CXCL12-induced ligation of CXCR4 in the presence of a SYK inhibitor reduces BTK Y551 phosphorylation [60]. Through activation of SLP65 and PLCγ2, cleavage of PIP2 into DAG and IP3 occurs, which will finally result in the activation ERK1/2, and several transcription factors, including NF-κB and NFAT [56]. Importantly, in the absence of BTK, B cells show impaired integrin-mediated adhesion and migration in response to CXCL13 [59]. Moreover, also *in vivo* homing of B cells to lymphoid organs was impaired, as was shown with adoptive transfer of BTK-deficient B cells into mice [59]. The role described for BTK in B cell homing, is also essential for retention of CLL cells in lymphoid organs, as was also clearly visible in the BTK inhibitor trials in which lymphocytosis was observed after start of treatment [61]. This will be discussed in more detail later in this review.

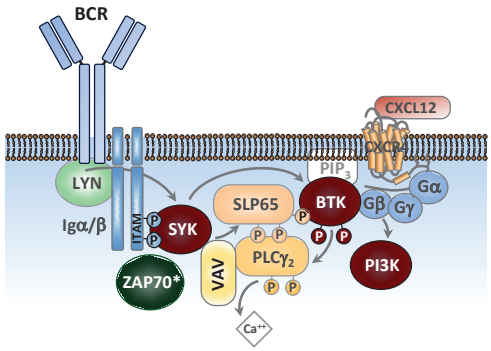


Figure 2. Roles for BTK and SYK in chemokine receptor signaling.

A schematic overview of the chemokine receptor signaling in B cells and CLL cells, with a dual role for several of the signaling molecules within the BCR signaling pathway. CXCL12 induces CXCR4 activation after binding. Heterotrimeric G protein subunits interact with BTK, which via interaction with SLP65, VAV, and PLCγ2, into Ca^{2+} release and further downstream activation of transcription factors (see text for details). The kinases BTK, SYK, and PI3K which are targeted by inhibitors described in this review, are indicated in red.

Toll-like receptor signaling

TLRs are pattern recognition receptors which are characterized by extracellular leucine-rich repeats and intracellular Toll/interleukin-1 receptor domains. TLRs typically recognize structurally conserved molecules derived from pathogens, *e.g.* lipopolysaccharides, flagellin, double-stranded RNA, or bacterial DNA. TLRs play an essential role in both innate and adaptive immunity. TLR1, TLR4 (at low levels, increased levels are found during inflammatory conditions), TLR6, TLR7, TLR9 and TLR10 are all expressed in human B cells, although the expression levels vary according to differentiation status [62]. Upon ligand interaction, TLRs homodimerize or heterodimerize, which leads to activation of downstream signaling pathways and finally results in cytokine release and co-stimulatory molecule upregulation [63]. Upon activation most TLRs recruit the adaptor protein myeloid differentiation primary response 88 (MYD88), with the exception of TLR3 that is MYD88-independent [63]. MYD88 then activates the recruited interleukin-1 receptor-associated kinase (IRAK) family members by its own or in combination with an additional adaptor molecule toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP). IRAK1, IRAK2, and IRAK4 interact with the E3 ubiquitin ligase tumor necrosis factor receptor-associated factor 6 (TRAF6) [63]. Interestingly, it was shown in macrophages that SYK is present in both TRAF3- and TRAF6-containing complexes, in which SYK has opposing regulatory roles [64]. In addition, BTK interacts with four different proteins downstream of TLR (as outlined in **Figure 3**), i.e. directly downstream of the TLR by the interaction with the TIR domain of the TLR itself, with MYD88 and TIRAP, and also within the complex of the IRAK family members with IRAK1, thus underlining its importance in regulation of the TLR signaling pathway. Activation of this final complex results in IκB and MAPK activation and shuttling of the transcription factors NF-κB and AP-1 to the nucleus [65, 66].

Taken together, it is clear that SYK and BTK occupy a crucial position in both BCR and TLR signaling in B lymphocytes and therefore interconnect these two signaling pathways. However, it is currently unknown how B cells integrate adaptive BCR and innate TLR activation.

SIGNALING CASCADES IN CLL

BCR signaling in CLL

CLL B cells show aberrant BCR signaling, whereby differences are found between U-CLL and M-CLL. Because in CLL B cells chronic engagement of the BCR by self- or non-self-antigens is thought to occur *in vivo* (described above), surface expression of IgM is generally down-modulated. Moreover, CLL B cells often show an anergic response and several of the kinases downstream of the BCR show aberrant expression levels or constitutive activation.

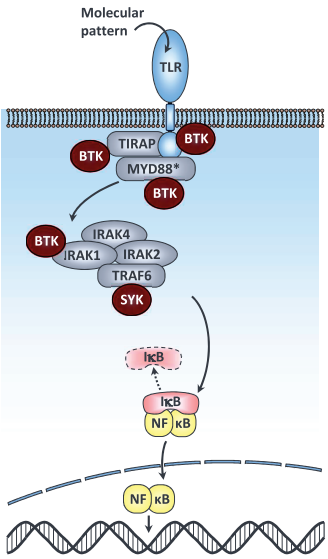


Figure 3. Roles for BTK and SYK in toll-like receptor signaling .

A schematic representation of the TLR signaling pathway. Upon TLR engagement, BTK interacting molecules TIRAP and MYD88 are recruited. Via interaction with a complex formed by IRAKs and TRAF6, which is stabilized by SYK, downstream activation of the transcription factor NF-κB is induced. For CLL, MYD88 mutations leading to aberrant activation of the pathway have been reported (see text for details). The kinases BTK and SYK, which are targeted by inhibitors described in this review, are indicated in red.

The tyrosine kinases LYN and SYK are overexpressed and constitutively phosphorylated [67, 68]. Both expression and phosphorylation of SYK correlate with disease prognosis, being higher in U-CLL than in M-CLL [68, 69]. In addition, there is clear evidence for a role of constitutively activated PI3K, particularly the PI3Kδ isoform, in survival of CLL B cells [70]. BTK expression levels are 2-3 fold increased in CLL [71], whilst BTK is constitutively phosphorylated in a substantial proportion of CLL cases [71], although its expression or activation cannot be correlated with prognosis [71]. A crucial role for BTK in CLL leukemogenesis was confirmed in CLL mouse models, showing that either CLL-like disease did not develop in the absence of BTK [72], or that BTK inhibition significantly delayed the development of CLL [73].

Aberrant expression of the SYK-related tyrosine kinase ZAP70, which is normally expressed in T cells, where it signals downstream of the T cell receptor (reviewed in [74]), is associated with U-CLL and an aggressive form of the disease [75]. Surface expression of the cyclic ADP ribose hydrolase glycoprotein CD38 also differs between CLL samples, and its expression regularly coincides with ZAP70 expression [76, 77, 78, 79]. $CD38^+$ CLL cells have an increased BCR signaling capacity, as is revealed by increased Ca^{2+} influx upon anti-IgM stimulation, compared with

CLL cells that do not express CD38 [80]. Furthermore, CD38 is not expressed homogeneously in all CLL cells of $CD38^+$ cases, whereby $CD38^+$ cells are thought to represent a proliferating compartment within a CLL clone [79, 80, 81]. PLCγ2 phosphorylation levels are increased in CLL, compared with healthy donor B cells [82] and this signal transducer is clearly activated in CLL B cells following BCR stimulation [83]. ZAP70 expression in CLL is known to enhance PLCγ2 phosphorylation upon BCR stimulation [84]. Recently, evidence was provided that primary CLL cells generally show a higher basal Ca^{2+} signaling level than B cells derived from peripheral blood of healthy controls [29, 85]. As discussed above, division of CLL samples on the basis of their IGHV gene SHM status revealed a significantly higher basal Ca^{2+} signaling level in the M-CLL subgroup compared with U-CLL. The degree of basal Ca^{2+} signaling did not correlate with other BCR characteristics, including Ig expression level, the length/ composition/charge of HCDR3, or FR2/FR3 sequences. It therefore appears that differences in basal Ca^{2+} signaling are directed by the SHM status of the CLL subgroup, reflecting e.g. a distinct cellular origin or a different anergic state induced by repetitive or continuous antigen binding *in vivo* [85]. However, it remains possible that the observed differences in basal Ca^{2+} signaling between U-CLL and M-CLL are not directly related to their IGHV gene SHM status, but instead reflects differences in proximal BCR signaling pathways between the two CLL subgroups.

The U-CLL and M-CLL subgroups also show differences in alterations in the activation of molecules that are positioned more downstream in the BCR signaling cascade, including ERK, NF-κB and NFAT [69, 86]. Constitutive phosphorylation of ERK was found to be associated with NFAT activation and translocation to the nucleus and as a consequence a higher anergic state [86]. Interestingly, it was recently reported that even though phosphorylated ERK (p-ERK) levels are quickly upregulated upon BCR ligation in M-CLL, there is also rapid loss of the signal, while in U-CLL ERK activation is higher and more prolonged. This finding of enhanced intensity and duration of p-ERK activation in U-CLL supports the idea that U-CLL cells may be better equipped to transmit BCR-derived signals and thus be more responsive to BCR activation compared with CLL cells from M-CLL cases, which appear to be more anergic [17]. Moreover, pronounced ERK activation in M-CLL required simultaneous BCR and TLR stimulation, while for U-CLL monovalent stimulation was sufficient to induce pronounced p-ERK induction [87].

Mutations in BRAF in CLL are considered as one of the early driver mutations that play a role in disease onset [88], even though they have only been found in ~3% of CLL patients [88, 89]. The most commonly found BRAF mutation in other cancers (V600E) is rarely found in CLL. BRAF mutations in CLL concern recently described mutations that target amino acids in the P-loop of this kinase. An example is the BRAF-G469R mutation, which leads to constitutive activation of ERK, although weaker than activation induced by the V600E mutation [88]. In a recent report, a melanoma patient with a BRAF-V600E mutation who was treated with the specific BRAF inhibitor, vemurafinib, was found to develop CLL within 3 months of therapy induction. An increase in p-ERK was seen in the CLL cells of the patient. This seems paradoxical

because BRAF inhibition is expected to result in reduced ERK activation. However, upon constitutive SYK activation, as is often seen in CLL, the BRAF inhibition was overcome via RAS, resulting in increased ERK activation and disease onset [90]. RAS mutations, both in NRAS and KRAS, are found in ~3 % and ~2% of CLL patients, respectively [5]; however, their functional role in altering BCR signaling in CLL is so far unknown. Recently, inactivating mutations of the NF-κB inhibitor epsilon (NFKBIE) were identified in 7-10% of CLL patients and were more often found in U-CLL and associated with poor outcome of disease [88, 91]. Since NFKBIE binds to NF-κB components, thus trapping the complex in the cytoplasm and preventing it from activating genes in the nucleus, the identified inactivating mutations might lead to release of NF-κB towards the nucleus [88, 91]. Finally, NF-κB activation was found to be higher in U-CLL than in M-CLL [69] and is associated with increased survival of CLL cells [92].

Taken together, these findings demonstrate that downstream BCR signaling is dissimilar between U-CLL and M-CLL, which could at least partly explain the more aggressive behavior of U-CLL and the milder form in M-CLL associated with a higher anergic state. Moreover, the expression or activation status of BCR signaling molecules and CD38 have prognostic impact, because they affect – in conjunction with genetic abnormalities in CLL and the BCR mutation status – the initiation, accumulation or expansion of CLL clones. It is to be expected that knowledge obtained from treatment of CLL patients with kinase inhibitors will provide crucial information how the BCR signaling contributes to clinical course and outcome of CLL.

Chemokine receptor signaling in CLL

The interaction with a supportive microenvironment in bone marrow and lymph nodes is essential for homing, survival and proliferation of CLL cells. Stromal cells, macrophages and nurse-like cells in these tissues provide signals via secretion of high levels of chemokines such as CXCL12 and CXCL13 [93]. Interaction with their respective corresponding chemokine receptors CXCR4 and CXCR5 on CLL cells is required for trafficking and homing. CLL cells actually have increased expression levels of these chemokine receptors [92, 93, 94, 95, 96]. Activation of CXCR4 and CXCR5 results in ERK1/2 and STAT3 activation, which are both important for cell survival [97, 98]. CLL cells themselves secrete high levels of the BCR signaling-dependent chemokines CCL3 and CCL4, which are important for interaction with T cells [99]. As already described above for normal B cells (see also **Figure 2**), several kinases important for BCR signaling are important for chemokine receptor signaling in CLL cells as well. Especially, inhibition of BTK, SYK and PI3K kinases has shown this importance since lymphocytosis is induced *in vivo*, as will be further discussed below.

TLR signaling in CLL

Recently, an activating mutation in the TLR signaling molecule MYD88, causing a L265P transition, was discovered to be present in ~3% of CLL cases, especially in M-CLL. It appeared

that this mutation induces activation of STAT3, IκBα, and NF-κB, via phosphorylation. In addition, an inactivating mutation in MYD88, E52DEL, was identified in two of 363 CLL patients [3], however the biological relevance of this mutation in CLL pathogenesis needs to be established. In another patient cohort which only included U-CLL and stereotypic subset #2 patients only 1% of patients showed MYD88 mutations [100]. A newly published study showed that 4% of CLL cases have activating MYD88 mutations, and additionally identified mutations in TLR2 and IRAK1 which were activating or leading to truncations, respectively. The mutations found in the TLR pathway in this cohort were exclusively identified in M-CLL [101].

The L265P MYD88 mutation is identified to act as a driver mutation in CLL pathogenesis and its clonal evolution [5]. This particular mutation is mostly found in M-CLL patients younger than ~50 years of age and with low CD38 or ZAP70 expression, and thus in patients with a favorable outcome of disease [102]. The L265P MYD88 mutation is also detected in other B cell malignancies, including almost 80% of patients with Waldenström's macroglobulinemia [103] and a substantial fraction of activated B cell – diffuse large B cell lymphoma [104], which indicates the importance of this particular mutation in pathogenesis of several types of B cell malignancies next to CLL. Interestingly, these B cell malignancies parallel CLL in that BTK inhibition showed antitumor activity in clinical trials (see Hendriks *et al.* for review [105]). On the basis of co-immunoprecipitation studies in Waldenström's macroglobulinemia it has been proposed that the L265P MYD88 mutation preferentially binds and activates BTK, leading to enhanced survival through NF-κB activation. It is likely that this mechanism, which would interconnect BCR and TLR downstream pathways - is also active in L265P MYD88-expressing CLL cells.

TARGETING SIGNALING PATHWAYS IN CLL MOUSE MODELS

Mouse models of human cancers provide a useful tool to elucidate the mechanisms that account for the natural history of the disease and evaluate the effect of different therapeutic approaches prior to human clinical trials. To date, various CLL-like mouse models have been generated using transgenesis and gene targeting approaches (see Simonetti *et al.* for an excellent recent review [106]). Of these various CLL mouse models only the Eμ-TCL1 and IgH. TEμ models showed complete disease penetrance (100%), with no obvious monoclonal B cell lymphocytosis stage preceding CLL development. Here we will discuss the characteristics and usefulness of these two CLL models for the study of human disease.

The Eμ-TCL1 mouse model was generated based on overexpression of TCL1 under the control of a VH promoter and the IGH intronic enhancer (Eμ) in the B cell lineage [107]. The immunophenotypic profiling of Eμ-TCL1 mice revealed that these animals spontaneously

develop hyperplasia of the $CD5^+IgM^+$ B cells, initially in the peritoneal cavity and then in lymph nodes, spleen, bone marrow and peripheral blood. Sequence analysis of BCR rearrangements from Eμ-TCL1 mice demonstrated a skewed murine VH repertoire with particular involvement of IGHV11, IGHV12 and IGHV4 gene families. Furthermore, analysis of IGHV gene SHM status demonstrated that TCL1-derived leukemias are identical and minimally divergent from germ-line sequences, similar to human U-CLL. The HCDR3s in Eμ-TCL1 mice are often long and contain multiple neutral tyrosine and serine residues. They also contain many (~55%) positively and negatively charged residues at or adjacent to the VH–D and D–JH junctions, deriving from simple and complex rearrangement events including insertion of non-templated nucleotides [108].

Since the Eμ-TCL1 mouse model stands very close to human CLL in terms of leukemia phenotype, antigen-receptor and disease course, it has been extensively used to study pathogenic mechanisms leading to CLL [106]. Recently, it has been applied as a pre-clinical tool to investigate the efficacy and potential side effects of novel therapeutic agents. The Eμ-TCL1 leukemias have been tested for the effects of the BTK inhibitor, ibrutinib, or the SYK inhibitor, fostamatinib, (described below), following adoptive transfer in immunodeficient severe combined immunodeficiency or syngeneic mice respectively [109, 110]. Both inhibitors delayed CLL disease progression in these adoptive transfer Eμ-TCL1 leukemia mouse models. Mimicking clinical observations in patients [61, 111], treatment of mice transplanted with Eμ-TCL1-derived leukemias with either ibrutinib or fostamatinib resulted in a transient increase in CLL numbers in the peripheral blood concurrently with a decreased tumor load in the spleen [109, 110].

A second model, the IgH.TEμ CLL mouse model was generated based on sporadic expression of the SV40 T oncogene in mature B cells [112]. SV40T is a potent oncogene able to transform many cell types and has been implicated in the etiology of various cancers, including B cell malignancies [113]. Sporadic expression was achieved by insertion of a SV40 T antigen gene in opposite transcriptional orientation in the heavy chain locus between the IGHD and IGHJ regions, in the presence (IgH.TEμ) or absence (IgH.T) of an extra copy of the Eμ enhancer [112]. Leukemic cells present in these mice displayed many characteristics also found in human CLL, in particular in U-CLL patients. At 6-9 months of age, most *IgH.TE*μ mice showed accumulation of a monoclonal $CD5^+IgM^+$ IgD^{low} B cell population. IGHV sequence analysis revealed preferential usage of unmutated VH11.2 and non-stochastic usage of D and J genes, strikingly similar to those observed in Eμ-TCL1 mice. Interestingly, unlike Eμ-TCL1 mice, two $VHJ558^+$ leukemias from IgH.TEμ CLL mice manifested extensive SHM, thereby providing an animal model for both U-CLL and M-CLL and demonstrating that pathways activated by the SV40 T antigen play important roles in CLL pathogenesis. The CDR3s of IgH. TEμ mice were enriched for serine/tyrosine residues and contained multiple charged amino acids that might confer CDR3 flexibility and favor poly-reactivity.

Furthermore, the IgH.TEμ CLL mouse model came up as the first mouse model to demonstrate the importance of BTK in CLL development. CLL formation was absent in BTK-deficient IgH.ETμ mice [72]. Conversely, transgenic overexpression of human BTK specifically in B cells under the control of the CD19 promoter (CD19-hBTK transgene) accelerated and increased CLL formation in IgH.TEμ mice. Increased CLL susceptibility of BTK-overexpressing B cells in IgH.TEμ; CD19-hBTK tumors was associated with frequent occurrence of CLL clones that expressed non-stereotypical BCRs. These BCR characteristics comprise of increased Igλ usage and the presence of long HCDR3 regions, frequently containing tyrosine stretches thereby substantiating contribution of BTK-mediated BCR signaling to CLL development [72].

In conclusion, due to complete disease penetrance and phenotypic relatedness of disease to CLL in humans, both Eμ-TCL1 mice and IgH.TEμ mice provide useful pre-clinical models for understanding the pathogenesis of CLL. In this regard, the Eμ-TCL1 mouse model has been extensively used in elucidating the functional role of specific molecules in the onset and progression of CLL in vivo in crosses of Eμ-TCL1 mice with several transgenic and knockout mouse models [106].

TARGETING OF SIGNALING PATHWAYS IN HUMAN CLL BY SMALL MOLECULE INHIBITORS

Components of the biologically relevant signaling pathways in CLL, as earlier described in this review, have become important innovative targets for therapy strategies over the last decade. To date, several of these small molecule kinase inhibitors are under investigation – either as a monotherapy or in combination strategies - in CLL patients and in patients with other types of B cell malignancies (outlined in **Table 1**). Impressive clinical results are obtained for several of these inhibitors which target signaling pathways, and these will be further outlined in this review.

BTK inhibitor, ibrutinib

Considering the significant expression levels and the important role of BTK in several signaling pathways implicated in CLL pathogenesis, BTK seemed an important target for CLL therapeutic intervention strategies. Ibrutinib (PCI-32765, Pharmacyclics), is an orally available potent inhibitor that irreversibly and covalently binds to the cysteine at position 481 in the BTK kinase domain and thereby blocks its kinase activity. Although ibrutinib does not prevent Y551 phosphorylation of BTK by SRC-like kinases and SYK, it prevents BTK autophosphorylation at position Y223 [114].

Ibrutinib was originally described in 2007 as one compound of a series of irreversible BTK inhibitors and its *in vivo* efficacy was first evaluated in a mouse model of rheumatoid

arthritis [114]. It was subsequently reported that CD40- or BCR-activated CLL cells cultured in the presence of ibrutinib showed abrogated survival pathways, including ERK and PI3K signaling and NF-κB shuttling to the nucleus. Ibrutinib treatment increased apoptosis and resulted in a reduction in survival signals induced by the microenvironment, and in decreased interaction with stromal cells [71]. In the Eμ-TCL1 mouse model of CLL, ibrutinib reduced disease progression and caused a transient early lymphocytosis (described above). Ibrutinib inhibits cell migration in response to tissue homing cytokines CXCL12 and CXCL13, consistent with a crucial role for BTK in chemokine-controlled B cell migration and homing [59], and it reduces DNA synthesis [109]. Ibrutinib was also found to reduce secretion of the BCR-dependent chemokines CCL3 and CCL4 by CLL cells, both *in vitro* and *in vivo* [109]. A more detailed explanation of the effects of ibrutinib on chemokine-controlled adhesion and migration of CLL cells was provided by De Rooij *et al*., who showed that the drug inhibits integrin α4β1-mediated adhesion to fibronectin and VCAM1 [60]. Furthermore, ibrutinib prevented CXCL12-, CXCL13-, and CCL19-induced signaling, adhesion, and migration of primary CLL cells. These findings indicate that ibrutinib disables retention and homing of CLL cells in their growth- and survival-inducing microenvironment in lymph nodes and bone marrow, which might well explain the CLL regression observed in clinical trials [60]. For MCL it was additionally shown that treatment with ibrutinib reduced the expression of the chemokine receptor CXCR4 on MCL cells, whereas lowered plasma chemokine levels were seen [115]. It is thus likely that expression of CXCR4 will also be reduced in CLL upon treatment with ibrutinib. To date, little is known regarding the interplay between chemokine receptor signaling and BCR signaling during B cell activation. Therefore, it is not clear whether ibrutinib mainly acts directly on chemokine receptor signaling, or whether ibrutinib downregulates chemokine responsiveness more indirectly through its effect on BCR signaling (See **Figure 2**). In this context, it has been shown that BCR signaling strength can model CXCL13-mediated B cell migration, whereby strong BCR signals halt B cell migration and weak signaling allow B cells to migrate in response to CXCL13 [116]. Conversely, CXCL13/CXCR5 signaling may have costimulatory function in BCR-triggered B cell activation. Because integrin-mediated adhesion plays a critical role in B cell activation, it is conceivable that ibrutinib may additionally act through effects on integrin activity (since BCR signaling also controls integrin $\alpha 4\beta 1$ (VLA-4)-mediated adhesion of B cells, through SYK, BTK and PI3K) [117].

In a first phase 1 clinical trial with various different relapsed/refractory B cell malignancies, including 16 CLL patients, it was observed that ibrutinib was safe and well-tolerated with mild-to-moderate toxicity. It showed a clinical response in these patients with an overall response rate (ORR) of 79% without any signs of progressive disease [118]. Therefore, an additional phase 1b/2 study was started in 85 refractory and relapsed CLL patients, mostly having high-risk disease. Two different doses of ibrutinib were tested in this study, but no significant differences were found between the two groups in ORR, which was ~71%.

The observed responses were found to be independent of various adverse prognostic characteristics, including advanced-stage disease, the number of previous therapies, and presence of 17p/*TP53* deletion. Notably, the ORR was dependent on IGHV mutation status: ~77% and ~33% in U-CLL and M-CLL patients, respectively. Because an additional ~42% of M-CLL patients showed a partial response with lymphocytosis, inclusion of these patients would bring the ORR of MCLL close to that of UCLL [61]. The observed progression free survival (PFS) rate was ~75% and the overall survival (OS) was ~83%, at 26 months after start of therapy, implying that durable remissions were seen in patients with relapsed/refractory CLL upon ibrutinib treatment [61]. It was found in this phase 1b study that proliferation of CLL cells was directly inhibited by ibrutinib. Moreover, activities of BTK and PLCγ2 as well as downstream signaling molecules, AKT and ERK, were all coordinately downregulated over time. This strongly suggests that blocking proliferation via inhibition of BTK-mediated signaling may contribute to clinical responses in ibrutinib-treated CLL patients [119]. Importantly, in CLL patients, ibrutinib induced a transient increase in blood lymphocyte levels, which coincided with a reduction in lymph node or spleen size. This asymptomatic lymphocytosis is explained by an inhibitory effect of ibrutinib on BTK function in chemokine receptor signaling, which causes re-distribution of CLL cells from the tissue compartments into the peripheral blood [120, 121]. The ibrutinib-induced lymphocytosis resolved in most patients within eight months and developed at similar frequencies (~80%) in patients with U-CLL and M-CLL [61]. Nevertheless, in U-CLL patients, lymphocyte counts normalized more rapidly and more frequently. A fraction of CLL patients showed prolonged lymphocytosis, lasting >1 yr, but PFS was not reduced compared with CLL patients with limited or transient lymphocytosis. Thus it appears ibrutinib-induced prolonged lymphocytosis does not indicate a suboptimal response or early relapse [61, 73].

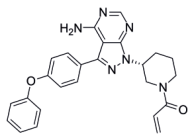
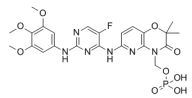
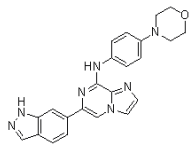
Recently, the results of ~3 year follow-up of ibrutinib treatment were published [122]. Patients who did not receive any other treatment on forehand, showed an ORR of ~84% (~23% and ~61% complete and partial response, respectively). Relapsed/refractory CLL patients showed an even higher ORR of ~90% (~7% complete and ~83% partial response). Three years after start of therapy both median PFS and median OS were not reached. It appears that prolonged ibrutinib treatment is well tolerated and induces increased and improved responses over time [122]. This shows the potential of ibrutinib to improve quality of life of CLL patients for extended time, providing a significant advance in CLL treatment options. Moreover, the authors reported that toxicity associated with ibrutinib, including cytopenia, fatigue, diarrhea and infections, diminished with continued treatment. It is important to note, however, that in this study only a low number of high-risk patients with cytogenetic abnormalities (which may incidentally develop ibrutinib resistance, see below) were included [122]. Recently, atrial fibrillation was reported as a potentially therapy-limiting adverse effect occurring in ~3.5-~6.5% of ibrutinib-treated patients [122, 123]. Although

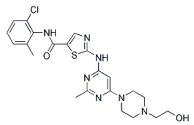
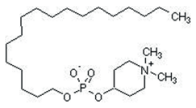
BTK and its family member TEC kinase were found to be expressed in human heart tissue, further experiments are required to establish functional effects of ibrutinib on myocytes [123]. Importantly, infectious complications with ibrutinib treatment are quite limited and might partially be related to the potential of ibrutinib to inhibit its TEC-family member interleukin-associated T-cell kinase (ITK). ITK inhibition promotes T-helper-1 cell differentiation and was shown to diminish infection morbidity [124]. Thus, it cannot be excluded that the usage of very specific BTK inhibitors that have limited specificity for related kinases might in this context even be disadvantageous.

Relapses of ibrutinib monotherapy are very recently described and occur in approximately ~5% of CLL patients, but the mechanisms of resistance are largely unidentified. One patient who first had a partial response after eleven months upon treatment with ibrutinib, showed a relapse at 21 months after treatment initiation. RNA sequencing revealed a thymidine-to-adenine mutation at nucleotide 1634 of the BTK complementary DNA, leading to a C481S cysteine-to-serine mutation in BTK at the binding site of ibrutinib. Structural modeling suggested that the C481S mutation would change irreversible binding of ibrutinib to reversible binding and thereby allow BTK activation. The C481S mutation was detected in samples collected when progressive disease was first noted (~88% of reads) and before dose escalation (~92% of reads), but not in those collected before ibrutinib administration or while the patient was having a response. These findings strongly suggesting that the mutation was acquired during therapy [125]. This was recently confirmed by highly sensitive molecular methods, showing that among CLLs not previously exposed to ibrutinib, the C481S variant is absent or undetectable [126]. The BTK mutation leading to a C481S substitution was found in five additional relapsed CLL patients, using a whole exome sequencing approach. Furthermore, in ibrutinib-resistant patients three distinct mutations in PLCγ2 were identified. The R665W and L845F substitution mutations in PLCγ2 are both potential gain-of-function mutations that lead to autonomous BCR activity, whilst the mutation leading to S707Y substitution was previously described as an activating mutation. In this way, these PLCγ2 mutations are believed to overcome BTK inhibition [125]. These mutations have been found in a minority of patients and therefore the vast majority of relapses upon ibrutinib treatment cannot be explained by these mutations alone. It is remarkable that resistance was particularly observed in patients with increased genomic instability, including those with del(17p13.1), del(11q22.3), or a complex karyotype [125]. Therefore, patients with increased genomic instability may benefit most from combination therapies designed to limit development of ibrutinib resistance.

Collectively, these findings demonstrate that ibrutinib is a potent therapeutic agent for treating CLL as a monotherapy. However, relapses occur associated with mutations in BTK and PLCγ2. To date, it is unknown how frequently these mutations occur, and which other mechanisms of resistance may explain the cases not carrying a mutation in these two genes. Furthermore, it is not clear which combination therapy might best circumvent these events.

Table 1. Small molecule kinase inhibitors used in clinical trials for CLL

Compound name	Target (IC50)	Mode of action	Selectivity	Clinical trials reported in CLL (References)
Ibrutinib (PCI 32765) 	BTK 0.5 nM	• Irreversible covalent binding to C481 • Inhibits BTK kinase activity, and thus prevents Y223 auto-phosphorylation • Does not prevent BTK Y551 phosphorylation	• High cross-reactivity with BLK and BMX (IC50 = 0.5 nM), which contain a cysteine that aligns with C481 in BTK • Cross-reactivity with ITK (IC50 = 11 nM), which affects T cell-mediated immunity in infectious disease models • Low cross-reactivity with TEC (IC50 = 78 nM), LYN (IC50 = 200 nM) and SYK (IC50 >10,000 nM)	• CLL/SLL [111] OR (71%); CR (13%); nPR, (3%) and PR (55%) • Relapsed CLL/SLL [59] • ORR (71%); PR (15-20%); blood lymphocytosis (78%) accompanied with reduction in lymph node and spleen size; Estimated 26-month PFS (75%) and OS (83%)
Fostamatinib (R788) 	SYK 41 nM	• Prodrug of the active metabolite R406 • R406 binds to the ATP-binding pocket of SYK • Inhibits SYK kinase activity as an ATP-competitive inhibitor	• Cross reactivity with other targets including the Flt-3, Ret, c-Kit, Lck, and JAK-1/3 tyrosine kinases, the adenosine A3 receptor, as well as several additional kinases and non-kinase targets, often at concentration ≤ 41 nM	• CLL/SLL [122] ORR (55%; 95% CI: 23%-83%); median PFS 6.4 months (95% CI: 2.2-7.1 months)
Entospletinib (GS-9973) 	SYK 7.7 nM	• Orally bio-available, selective SYK inhibitor	• 13-fold to >1000-fold cellular selectivity for SYK	• relapsed/refractory CLL 136 ORR (61%; 95% CI: 44.5%-75.8%) all PR; Median PFS 13.8 months (95% CI: 7.7 months, not reached); median DOR Not reached yet (95% CI: 6.5 months, not reached)

Compound name	Target (IC50)	Mode of action	Selectivity	Clinical trials reported in CLL (References)
Idelalisib 	PI3Kδ 2.5 nM	• Potent, highly selective small-molecule inhibitor of PI3Kδ isoform	• Selective for p110δ (IC50 = 2.5 nM) • 40-400-fold greater selectivity for p110δ than p110α (IC50 = 820 nM), p110β (IC50 = 565 nM) or p110γ (IC50 = 89 nM)	• relapsed/refractory CLL[130] • ORR (72%); PR with treatment-induced lymphocytosis (33%); median TTR (1.0 month); median DOR (16.2 months); median PFS (15.8 months)
Dasatinib 	Multikinase 5.0 nM	• Binds to the ATP-binding site of the active and inactive conformation of the ABL kinase domain	• Reversible multi-kinase inhibitor, originally developed as a SRC-kinase and ABL inhibitor; • high cross-reactivity with the TEC family kinases TEC (IC50 = 297 nM), c-Kit (IC50 = 79 nM) and the SRC- family kinases LYN and SRC	• relapsed CLL [137] PR (20%; 90% CI: 6–44%); nodal responses (60%); 50% reduction of all nodal and extranodal masses and blood absolute lymphocyte counts in 4/9 patients.
Perifosine 	AKT 4.7 µM	• Targets PH domain of Akt • causes decrease in Akt Ser473 and Thr308 phosphorylation	• Perifosine inhibits either AKT1 or AKT3 phosphorylation	• relapsed and/or refractory CLL/SLL [139] Death (15 out of 16 patients); Median EFS (3.9 months), median OS (9.7 months) Early discontinuation due to insufficient response (56%) or disease progression/ transformation (19%).

Abbreviations: CI, confidence interval; CLL, Chronic Lymphocytic Leukemia; CR, Complete Response; DOR, Duration of Response; EFS, Event free survival; nPR, nodular Partial Response; OR, Objective Response; ORR, Overall Response Rate; OS, Overall Survival; PFS, Progression Free Survival; PR, Partial Response; SLL, Small Lymphocytic Lymphoma; TTR, time to response.

The first study in which ibrutinib was given in combination with rituximab (anti-CD20 antibody) therapy showed that combination of these therapeutics is safe [127]. It was additionally shown that the combination of ibrutinib and rituximab induced responses in a large proportion of patients with high-risk CLL. Responses were durable, and ~78% of patients were free of disease progression and OS at 18 months was ~84% [127]. One of the most notable findings in this study was the short duration of ibrutinib-associated redistribution of lymphocytosis. Fewer patients had persistent lymphocytosis than with single-agent treatment, likely caused by the addition of rituximab [127]. Collectively, these data show promise for the use of ibrutinib in combination with other treatment strategies, as is currently being further investigated. Likewise, a recent Phase 1b study showed that ibrutinib enhanced the efficacy of immunochemotherapy (with rituximab and bendamustine or with fludarabine, cyclophosphamide, and rituximab), without additive toxicities [128]. Because the response rates to ibrutinib in combination with bendamustine and rituximab were higher than to ibrutinib alone, it now becomes important to assess overall safety and efficacy profiles of combination therapies in clinical trials, particularly given the additional toxicity of chemotherapy. For instance, the combination of rituximab and bendamustine with the addition of ibrutinib or a placebo is currently investigated in relapsed/refractory CLL patients in phase 3 trials [128, 129].

SYK inhibitors (fostamatinib, entospletinib, P505-15, and PRT318)

SYK plays an important role in BCR-mediated survival and is constitutively activated in CLL [68, 130]. Kinase activity of SYK leads to the assembly of the BCR micro-signalosome by connecting SLP65, BTK and PLCγ2 (see **Figure 1**). Moreover, inhibition of SYK signaling prevented CLL cells from interacting with the microenvironment and promotes apoptotic signals [68, 131]. Therefore, targeting SYK represents a promising therapeutic approach for CLL treatment.

It has been demonstrated that the SYK inhibitor, fostamatinib (R788, Astra Zeneca/Rigel Pharmaceuticals), decreases phosphorylation of the proximal BCR signaling regulators SLP65 and PLCγ2, as well as key players in more downstream pathways such as ERK, AKT, and NF-κB. Fostamatinib - a pro-drug which is converted to an active drug, R406 with activity against SYK [132] – is the first oral SYK inhibitor in development as a novel therapeutic approach for lymphoid malignancies. Fostamatinib effectively inhibits BCR signaling in vivo, resulting in reduced proliferation and survival of CLL cells and significantly prolonged survival of Eμ-TCL1 mice [110]. In fact, fostamatinib was initially developed for treatment of autoimmune disease; in a phase 2 study in rheumatoid arthritis patients SYK inhibition by fostamatinib reduced disease activity, although substantial adverse effects were observed [133]. In the first phase 1/2 clinical trial investigating fostamatinib in relapsed B-cell NHL and CLL, clinical efficacy was noted in a variety of B cell malignancies with the highest response rate observed in CLL/SLL patients [134]. Sixty-eight patients with various recurrent B cell malignancies were

treated with fostamatinib in a phase 2 study. The highest response rate (~55%) was observed in patients with SLL/CLL, and their median PFS was 6.4 months. Common toxicities observed in this study included diarrhea, fatigue and cytopenia [134], which might be related to off-target activity against related kinases, including Fms-like tyrosine kinase (FLT3), Janus-like kinases (JAK) or lymphocyte-specific protein tyrosine kinase (Lck). All treated CLL patients had increased peripheral blood lymphocyte counts in the first period after treatment initiation, which showed that the effects of SYK inhibition were not limited to BCR signaling. Indeed, it was found that SYK inhibition also affected expression of CCL3 and CCL4 in nurse-like cell co-cultures and its sensitivity to chemotaxis by CXCR4 and CXCR5 68, 99.

Entospletinib (Gilead Sciences, Inc.) is another SYK inhibitor that is highly selective and orally efficacious and under clinical evaluation for CLL patients [135]. In a phase 2, open-label, single-arm safety and efficacy study entospletinib was evaluated in a cohort of 41 patients with relapsed/refractory CLL. The estimated PFS rate was ~70% at 24 weeks after start of therapy, and the ORR was ~61%, which only included partial responses. Most of the evaluable patients (~95%) experienced a reduction in tumor size, including all patients with a 17p deletion/*TP53* gene mutation, or with other genetic abnormalities linked to poor prognosis. Additionally, ~62% of the evaluable patients showed a tumor reduction of at least ~50% [136]. Additionally, an *in vitro* study in CLL samples showed that entospletinib reduced AKT phosphorylation, and that this agent in combination with the PI3Kδ inhibitor, idelalisib (discussed below) showed synergistic inhibition of CLL cell viability and disrupted BCR and chemokine receptor signaling, compared to its use as single agent [137]. To obtain more insight into the clinical effects of entospletinib, evaluation will be required in a larger series of CLL patients as is currently being performed.

Recently, preclinical characteristics of two novel small molecule SYK inhibitors, P505-15 and PRT318 (both Portola Pharmaceuticals), were evaluated. In contrast to fostamatinib, which is relatively non-specific, these novel inhibitors are highly selective, as e.g. P505-15 (also known as PRT062607) has an anti-SYK activity that is >80-fold higher than its affinity for other kinases. Both P505-15 and PRT318 effectively decreased CLL viability after BCR stimulation and in nurse-like cell co-cultures. Parallel to previous findings for fostamatinib also secretion of CCL3 and CCL4 and migration towards the CXCL12 and CXCL13 chemokines was inhibited [132]. Oral administration of P505-15 produced dose-dependent anti-inflammatory activity in rodent models of rheumatoid arthritis [138], whereas in a xenograft model in mice this compound prevented BCR-mediated splenomegaly [139]. Treatment of primary CLL cells with P505-15 *in vitro* resulted in a concentration-dependent decrease in AKT phosphorylation. Although P505-15 manifested modest single agent activity against CLL survival, the compound was synergistic with fludarabine at nanomolar concentrations, which is important given the dose-limiting toxicity associated with fludarabine treatment [139].

Taken together, the reported results of the first clinical studies of the SYK inhibitors, fostamatinib and entospletinib, in CLL indicate that the observed ORRs are slightly lower (~55% and ~61% respectively) than those found for ibrutinib and idelalisib (see below), which were ~70%. However in-depth comparisons of the various clinical trials of individual inhibitors is complicated by differences in patient inclusions, e.g. proportions of patients with high-risk patients with cytogenetic abnormalities, treatment history or age, and variation in follow-up time. So, it remains possible that the ORRs with SYK inhibitors in CLL are not that different from those with BTK or PI3Kδ inhibitors. The preclinical studies on novel SYK inhibitors, P505-15 and PRT318, provide a strong basis for clinical development of these novel compounds in CLL, next to the traditional SYK inhibitors, fostamatinib and entospletinib. Particularly, since an optimal treatment strategy for an individual patient is not only determined by the observed ORRs for a particular inhibitor, but also depends on how well the therapy is tolerated, the observed quality of life improvement and whether the patients develops resistance.

PI3K inhibitor idelalisib (CAL-101, GS-1101)

Expression of PI3Kδ is restricted to cells of hematopoietic origin, whereby in B cells it plays a key role in proliferation and survival. As mentioned above, the PI3K pathway is constitutively activated in CLL and this is dependent on PI3Kδ [70]. The highly-selective oral PI3Kδ inhibitor, idelalisib (CAL-101, GS-1101, Calistoga Pharmaceuticals) stimulated apoptosis in primary CLL cells *ex vivo* in a dose- and time-dependent manner, which was shown to be independent of *IGHV* mutational status or recurrent cytogenetic aberrations. Idelalisib-mediated cytotoxicity induced an increase in caspase activity which was not rescued by co-culture on stromal cells. Additionally, idelalisib abolished normal CLL cell protection from spontaneous apoptosis induced by B cell-activating factors like CD40L, or other signals from the microenvironment [70]. Similar to ibrutinib and SYK inhibitors, also idelalisib inhibits CLL cell chemotaxis towards CXCL12 and CXCL13, and induces downregulation of chemokine secretion. CAL-101 was shown to inhibit BCR- and chemokine-receptor-induced AKT phosphorylation, ERK activation and survival signals [140]. In a phase 1 trial idelalisib was evaluated in 54 patients with relapsed/refractory CLL with various adverse prognostic characteristics including unmutated IGHV status (91%), and del17p and/or *TP53* mutations (24%) [141]. The patients were treated with different dosage levels of oral idelalisib and remained on continuous therapy while showing clinical improvement. Similar as was shown *ex vivo* for CLL cells, inhibition of PI3Kδ abolished AKT phosphorylation in patient CLL cells, and significantly reduced serum levels of chemokines related to CLL such as CCL3, CCL4, CCL17 and CCL22. Idelalisib induced clear reductions in lymph node and spleen size and lymphocytosis was observed in the patients already a few hours after the initiation of the treatment. The most commonly observed adverse events

were pneumonia, neutropenic fever and diarrhea. The ORR was 72%, and a median PFS for all patients was 15.8 months, demonstrating the clinical efficacy of idelalisib [141].

In a multicenter phase 3 study, which was performed in a randomized, double-blind, and placebo-controlled manner, the efficacy and safety of idelalisib in combination with rituximab was assessed [142]. Two-hundred-and-twenty CLL patients were included, with advanced stage disease of which a part was in a poor condition with renal dysfunction, previous therapy-induced reduced bone marrow function, or major coexisting diseases. The patients received rituximab and either idelalisib or placebo. PFS at 24 weeks after treatment initiation was ~93% in the idelalisib treated group and ~46% in the placebo group. Because of this large efficacy differences between the two patient groups, the study was stopped before its end point was reached. The observed ORR were ~81% in the patients receiving idelalisib versus ~13% in the placebo group, of which all were considered as partial responses. The idelalisib related lymphocytosis which was seen in the phase 1 study [141], appeared to be less profound and shorter in duration when combined with rituximab [142], parallel to observations in the study in which ibrutinib was combined with rituximab [127].

Taken together, the reported clinical data convincingly show that idelalisib treatment induces an impressive response in CLL patients, without causing significant toxicity, particularly in a combination therapy strategy with rituximab.

So far it is not known whether idelalisib treatment can induce mutations in PI3Kδ or other downstream targets, as it is known for ibrutinib where BTK and PLCγ2 mutations were identified in relapsed patients. *In vitro* data in MCL cell lines and primary CLL cases showed an synergistic effect by the combined usage of ibrutinib and idelalisib in induction of apoptosis, even with lower dosage than used for therapy. This combination might be a good option to prevent relapses as can be seen when used as single agents, whilst the usage of a lower dose could prevent therapy- induced toxicity [143].

A recent publication compared the pan-PI3K-inhibitor, BKM120 (Yamanouchi Pharmaceuticals, Piramed Pharma, and Genentech) with idelalisib with respect to the effect on CLL cell function *in vitro.* It was shown that BKM120 had an almost 4-fold higher toxicity for CLL cells than idelalisib. Furthermore, BKM120 induced apoptosis in CLL cells which could not be prevented by rescue through signals from stromal cells [144]. In line with this, it was recently described that in CLL cells BMK120 decreases PIP3 levels, AKT phosphorylation, and FOXO3a phosphorylation [145]. The latter regulates expression of the pro-apoptotic protein BIM, which is indeed upregulated and goes along with increased apoptosis in CLL cells These results show that BKM120 could have clinical impact for treatment of CLL, which should be further investigated *in vivo*. A phase 1 study for usage of BMK120 in patients with advanced stage solid tumors, showed that the use of this inhibitor is safe and that it is well tolerated, although dose-limiting toxicities were reported; furthermore a clinical response leading to

stable disease was seen in ~58% of included patients [146]. This appears a good starting point to investigate the effect of BMK120 in CLL patients.

Src-family tyrosine kinase inhibitor (dasatinib)

The Src-family tyrosine kinase inhibitor, dasatinib (Sprycell, Bristol-Myers Squibb), is derived from an aminothiazole, and was found to bind Abl tyrosine kinase with an affinity >100-fold that of imatinib mesylate. Therefore it was initially developed as therapeutic agent for chronic myeloid leukemia (CML) [147]. After successful clinical trials, dasatibib received FDA approval in 2006 for the treatment of imatinib-resistant CML. Additionally, dasatinib appeared to be a potent inhibitor of LYN, and was found to induce apoptosis in CLL cells with a higher sensitivity for U-CLL [148]. Targeting LYN with dasatinib in a transplantable mouse model with Eμ-TCL1-derived CLL-lice cells delayed CLL progression [149]. In a phase 2 clinical trial for dasatinib treatment in relapsed/refractory CLL, only partial responses were observed in three of fifteen treated patients, and nodal responses (~50% reduction) together with lymphocyte responses (>50% reduction) were observed in four of these fifteen patients. This indicated some effectiveness in patients with relapsed/refractory CLL, however the majority of patients included showed no clinical response [150]. In another phase 2 study, in which dasatinib was given in combination with fludarabine, twenty fludarabine-refractory CLL patients were enrolled [151]. From these, eighteen patients were assessable for response, and although the ORR was ~18%, three patients showed a partial response, with nodal responses and a reduction in lymphocyte count, and twelve patients showed stable disease. In ~61% of the patients lymphocytosis was observed shortly after treatment initiation. So, in fludarabine refractory CLL patients, a combination of fludarabine with dasatinib shows no obvious clinical efficacy [151]. These results indicate that to date dasatinib is not a very promising treatment option in CLL.

AKT inhibitor (perifosine)

The alkylphospholipid perifosine (Aeterna Zentaris) inhibits the phosphorylation of AKT by preventing membrane localization of AKT and thereby interfering in the interaction between PI3K and AKT [152]. *In vitro* assays showed cytotoxicity in CLL cells induced by perifosine in a dose-dependent manner. In a phase 2 clinical trial perifosine was evaluated as monotherapy for CLL [152]. All sixteen included patients had aggressive form of disease. Although initially perifosine mainly stabilized disease in the heavily pretreated and highly refractory population of CLL patients studied, the median overall survival was 9.7 months. Remarkably, AKT phosphorylation in CLL lymphocytes from treated patients was not correlated with response. Thus, despite the *in vitro* induced cytotoxicity, there was no clinical response observed in CLL patients by perifosine [152]. However, perifosine may have potential as part of combination regimen as was shown in a phase 1/2 study where it was investigated in combination with

proteasome inhibitor, bortezomib, and with or without dexamethasone in patients with relapsed or refractory multiple myeloma. From the 84 patients enrolled in this study, 73 were evaluable for response of which 41% showed an ORR with 4%, 19% and 18% showing a complete response, median response, or partial response, respectively [153]. Taken together, compared with other novel inhibitors perifosine monotherapy currently does not appear a good treatment option for treatment of CLL patients. Nevertheless, it remains possible that subgroups of patients may benefit from perifosine in combination therapy strategies.

CONCLUSIONS AND PERSPECTIVE

Targeting of kinases with essential roles in signaling pathways have shown impressive efficacy as therapeutic option for CLL in various clinical trials. These studies also confirm that BCR and particularly chemokine receptor signaling are critical for CLL. The BTK inhibitor, ibrutinib, SYK inhibitors, fostamatinib and entospletinib, and PI3K inhibitor, idelalisib, have shown significant effects when used as single-agent therapy. Currently a large number of clinical trials are ongoing to investigate efficacy of these inhibitors in combination therapy strategies. This might circumvent the induction of mutations that either alter the binding site of the inhibitor or cause gain-of-function of other signaling molecules, thus leading to resistance, as has already been described for ibrutinib [125]. To date, the frequency of such events is not known but may be quite low inferred from the low number of cases described so far.

Given the various targets of these novel therapies, choosing the right treatment regimen for a given CLL patient has become a task that requires experience, a good clinical assessment of the patients, as well as careful evaluation of the responses and resistance development in CLL patient subgroups in the ongoing clinical trials. Because the individual kinase inhibitors are in different stages of clinical evaluation, it is currently difficult to make fair comparisons and to establish if one inhibitor should be preferred over another. Moreover, the design of new treatment strategies will depend on detailed analyses of clinical responses, resistance development, toxicity and quality of life for individual inhibitors in combination therapies in relation to CLL patient subgroups. Furthermore, effective CLL treatment should take notice of the disease complexity that depends on signaling within the microenvironment, where targeted drugs need to act against. Also, based on the rapidly increasing knowledge on biological variation in human CLL, it will become important to select the correct subgroups of CLL patients that would benefit from a particular drug combination. In this context, next-generation sequencing analyses will provide information about genetic heterogeneity in CLL that is invaluable to optimize treatment regimens for individual CLL patients. Future pre-clinical research, both in animal models and in human *in vitro* cell systems, should provide

the necessary (new) mechanistic insights to understand the interaction of CLL cells with their microenvironment. It is expected that these novel agents will translate into innovative therapies that will eventually take control of a thus far incurable disease.

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REFERENCES

1. DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L. *et al.* Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* **64**, 252-271 (2014).
2. Zenz, T., Mertens, D., Kuppers, R., Dohner, H. & Stilgenbauer, S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer* **10**, 37-50 (2010).
3. Puente, X.S., Pinyol, M., Quesada, V., Conde, L., Ordonez, G.R., Villamor, N. *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* **475**, 101-105 (2011).
4. Quesada, V., Conde, L., Villamor, N., Ordonez, G.R., Jares, P., Bassaganyas, L. *et al.* Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet* **44**, 47-52 (2012).
5. Landau, D.A., Carter, S.L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M.S. *et al.* Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* **152**, 714-726 (2013).
6. Chiorazzi, N. & Ferrarini, M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood* **117**, 1781-1791 (2011).
7. Klein, U., Tu, Y., Stolovitzky, G.A., Mattioli, M., Cattoretti, G., Husson, H. *et al.* Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* **194**, 1625-1638 (2001).
8. Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Durig, J. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* **209**, 2183-2198 (2012).
9. Griffin, D.O., Holodick, N.E. & Rothstein, T.L. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* **208**, 67-80 (2011).
10. DiLillo, D.J., Weinberg, J.B., Yoshizaki, A., Horikawa, M., Bryant, J.M., Iwata, Y. *et al.* Chronic lymphocytic leukemia and regulatory B cells share IL-10 competence and immunosuppressive function. *Leukemia* **27**, 170-182 (2013).
11. Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L. *et al.* Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* **94**, 1840-1847 (1999).
12. Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94**, 1848-1854 (1999).

13. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* **119**, 4467-4475 (2012).
14. Baliakas, P., Agathangelidis, A., Hadzidimitriou, A., Sutton, L.A., Minga, E., Tsanousa, A. *et al.* Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. *Blood* **125**, 856-859 (2015).
15. Baliakas P, H.A., Sutton LA, Minga E, Agathangelidis A, Tsanousa A, Scarfo L, Davis Z, Yan XJ, Shanafelt T, Plevova K, Sandberg Y, Vojdeman FJ, Boudjogra M, Tzenou T, Chatzouli M, Chu CC, Veronese S, Gardiner A, Mansouri L, Smedby KE, Bredo Pedersen L, Moreno D, Van Lom K, Giudicelli V, Shkurova Francova H, Nguyen-Khac F, Panagiotidis P, Juliusson G, Angelis L, Anagnostopoulos A, Lefranc MP, Trentin L, Catherwood M, Montillo M, Geisler G, Langerak AW, Pospisilova S, Chiorazzi N, Oscier D, Jelinek D, Darzentas N, Belessi C, Davi F, Rosenquist R, Ghia P, Stamatopoulos K. B-cell receptor stereotypy defined distinct clinical subgroups of CLL: implications for individualizing treatment. *Lancet Hematol* **in press** (2014).
16. Gauld, S.B., Benschop, R.J., Merrell, K.T. & Cambier, J.C. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* **6**, 1160-1167 (2005).
17. Mockridge, C.I., Potter, K.N., Wheatley, I., Neville, L.A., Packham, G. & Stevenson, F.K. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood* **109**, 4424-4431 (2007).
18. Herishanu, Y., Perez-Galan, P., Liu, D., Biancotto, A., Pittaluga, S., Vire, B. *et al.* The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* **117**, 563-574 (2011).
19. Herve, M., Xu, K., Ng, Y.S., Wardemann, H., Albesiano, E., Messmer, B.T. *et al.* Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* **115**, 1636-1643 (2005).
20. Catera, R., Silverman, G.J., Hatzi, K., Seiler, T., Didier, S., Zhang, L. *et al.* Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol Med* **14**, 665-674 (2008).
21. Chu, C.C., Catera, R., Zhang, L., Didier, S., Agagnina, B.M., Damle, R.N. *et al.* Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin. *Blood* **115**, 3907-3915 (2010).
22. Myhrinder, A.L., Hellqvist, E., Sidorova, E., Soderberg, A., Baxendale, H., Dahle, C. *et al.* A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood* **111**, 3838-3848 (2008).
23. Zwick, C., Fadle, N., Regitz, E., Kemele, M., Stilgenbauer, S., Buhler, A. *et al.* Autoantigenic targets of B-cell receptors derived from chronic lymphocytic leukemias bind to and induce proliferation of leukemic cells. *Blood* **121**, 4708-4717 (2013).
24. Ghia, E.M., Widhopf, G.F., 2nd, Rassenti, L.Z. & Kipps, T.J. Analyses of recombinant stereotypic IGHV3-21-encoded antibodies expressed in chronic lymphocytic leukemia. *J Immunol* **186**, 6338-6344 (2011).
25. Steininger, C., Widhopf, G.F., 2nd, Ghia, E.M., Morello, C.S., Vanura, K., Sanders, R. *et al.* Recombinant antibodies encoded by IGHV1-69 react with pUL32, a phosphoprotein of cytomegalovirus and B-cell superantigen. *Blood* **119**, 2293-2301 (2012).

26. Hoogeboom, R., Wormhoudt, T.A., Schipperus, M.R., Langerak, A.W., Dunn-Walters, D.K., Guikema, J.E. *et al.* A novel chronic lymphocytic leukemia subset expressing mutated IGHV3-7-encoded rheumatoid factor B-cell receptors that are functionally proficient. *Leukemia* **27**, 738-740 (2013).
27. Kostareli, E., Gounari, M., Janus, A., Murray, F., Brochet, X., Giudicelli, V. *et al.* Antigen receptor stereotypy across B-cell lymphoproliferations: the case of IGHV4-59/IGKV3-20 receptors with rheumatoid factor activity. *Leukemia* **26**, 1127-1131 (2012).
28. Hoogeboom, R., van Kessel, K.P., Hochstenbach, F., Wormhoudt, T.A., Reinten, R.J., Wagner, K. *et al.* A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med* **210**, 59-70 (2013).
29. Duhren-von Minden, M., Ubelhart, R., Schneider, D., Wossning, T., Bach, M.P., Buchner, M. *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* **489**, 309-312 (2012).
30. Binder, M., Muller, F., Frick, M., Wehr, C., Simon, F., Leistler, B. *et al.* CLL B-cell receptors can recognize themselves: alternative epitopes and structural clues for autostimulatory mechanisms in CLL. *Blood* **121**, 239-241 (2013).
31. Muggen, A.F., Pillai, S.Y., Kil, L.P., van Zelm, M.C., van Dongen, J.J., Hendriks, R.W. *et al.* Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* **29**, 321-328 (2015).
32. Pao, L.I., Famiglietti, S.J. & Cambier, J.C. Asymmetrical phosphorylation and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. *J Immunol* **160**, 3305-3314 (1998).
33. Rolli, V., Gallwitz, M., Wossning, T., Flemming, A., Schamel, W.W., Zurn, C. *et al.* Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol Cell* **10**, 1057-1069 (2002).
34. O'Rourke, L.M., Tooze, R., Turner, M., Sandoval, D.M., Carter, R.H., Tybulewicz, V.L. *et al.* CD19 as a membrane-anchored adaptor protein of B lymphocytes: costimulation of lipid and protein kinases by recruitment of Vav. *Immunity* **8**, 635-645 (1998).
35. Inabe, K., Ishiai, M., Scharenberg, A.M., Freshney, N., Downward, J. & Kurosaki, T. Vav3 modulates B cell receptor responses by regulating phosphoinositide 3-kinase activation. *J Exp Med* **195**, 189-200 (2002).
36. Inabe, K. & Kurosaki, T. Tyrosine phosphorylation of B-cell adaptor for phosphoinositide 3-kinase is required for Akt activation in response to CD19 engagement. *Blood* **99**, 584-589 (2002).
37. Baracho, G.V., Miletic, A.V., Omori, S.A., Cato, M.H. & Rickert, R.C. Emergence of the PI3-kinase pathway as a central modulator of normal and aberrant B cell differentiation. *Curr Opin Immunol* **23**, 178-183 (2011).
38. So, L., Yea, S.S., Oak, J.S., Lu, M., Manmadhan, A., Ke, Q.H. *et al.* Selective inhibition of phosphoinositide 3-kinase p110alpha preserves lymphocyte function. *J Biol Chem* **288**, 5718-5731 (2013).
39. Saito, K., Scharenberg, A.M. & Kinet, J.P. Interaction between the Btk PH domain and phosphatidylinositol-3,4,5-trisphosphate directly regulates Btk. *J Biol Chem* **276**, 16201-16206 (2001).
40. Rawlings, D.J., Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M. *et al.* Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* **271**, 822-825 (1996).
41. Kurosaki, T. & Kurosaki, M. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *J Biol Chem* **272**, 15595-15598 (1997).
42. Park, H., Wahl, M.I., Afar, D.E., Turck, C.W., Rawlings, D.J., Tam, C. *et al.* Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity* **4**, 515-525 (1996).

43. Fu, C., Turck, C.W., Kurosaki, T. & Chan, A.C. BLNK: a central linker protein in B cell activation. *Immunity* **9**, 93-103 (1998).
44. Oellerich, T., Bremes, V., Neumann, K., Bohnenberger, H., Dittmann, K., Hsiao, H.H. *et al.* The B-cell antigen receptor signals through a preformed transducer module of SLP65 and CIN85. *EMBO J* **30**, 3620-3634 (2011).
45. Kim, Y.J., Sekiya, F., Poulin, B., Bae, Y.S. & Rhee, S.G. Mechanism of B-cell receptor-induced phosphorylation and activation of phospholipase C-gamma2. *Mol Cell Biol* **24**, 9986-9999 (2004).
46. Weber, M., Treanor, B., Depoil, D., Shinohara, H., Harwood, N.E., Hikida, M. *et al.* Phospholipase C-gamma2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med* **205**, 853-868 (2008).
47. Saito, K., Tolias, K.F., Saci, A., Koon, H.B., Humphries, L.A., Scharenberg, A. *et al.* BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity* **19**, 669-678 (2003).
48. Sugawara, H., Kurosaki, M., Takata, M. & Kurosaki, T. Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *EMBO J* **16**, 3078-3088 (1997).
49. Oh-hora, M. & Rao, A. Calcium signaling in lymphocytes. *Curr Opin Immunol* **20**, 250-258 (2008).
50. Hashimoto, A., Okada, H., Jiang, A., Kurosaki, M., Greenberg, S., Clark, E.A. *et al.* Involvement of guanosine triphosphatases and phospholipase C-gamma2 in extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. *J Exp Med* **188**, 1287-1295 (1998).
51. Baracho, G.V., Cato, M.H., Zhu, Z., Jaren, O.R., Hobeika, E., Reth, M. *et al.* PDK1 regulates B cell differentiation and homeostasis. *Proc Natl Acad Sci U S A* **111**, 9573-9578 (2014).
52. Yusuf, I., Zhu, X., Kharas, M.G., Chen, J. & Fruman, D.A. Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood* **104**, 784-787 (2004).
53. Jin, T., Xu, X. & Hereld, D. Chemotaxis, chemokine receptors and human disease. *Cytokine* **44**, 1-8 (2008).
54. Okada, T., Ngo, V.N., Ekland, E.H., Forster, R., Lipp, M., Littman, D.R. *et al.* Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J Exp Med* **196**, 65-75 (2002).
55. Ritter, S.L. & Hall, R.A. Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10**, 819-830 (2009).
56. Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W. & Bourne, H.R. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**, 1037-1040 (2000).
57. Lowry, W.E. & Huang, X.Y. G Protein beta gamma subunits act on the catalytic domain to stimulate Bruton's agammaglobulinemia tyrosine kinase. *J Biol Chem* **277**, 1488-1492 (2002).
58. Tsukada, S., Simon, M.I., Witte, O.N. & Katz, A. Binding of beta gamma subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. *Proc Natl Acad Sci U S A* **91**, 11256-11260 (1994).
59. de Gorter, D.J., Beuling, E.A., Kersseboom, R., Middendorp, S., van Gils, J.M., Hendriks, R.W. *et al.* Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity* **26**, 93-104 (2007).
60. de Rooij, M.F., Kuil, A., Geest, C.R., Eldering, E., Chang, B.Y., Buggy, J.J. *et al.* The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* **119**, 2590-2594 (2012).

61. Byrd, J.C., Furman, R.R., Coutre, S.E., Flinn, I.W., Burger, J.A., Blum, K.A. *et al.* Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *The New England journal of medicine* **369**, 32-42 (2013).
62. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* **168**, 4531-4537 (2002).
63. Kawai, T. & Akira, S. TLR signaling. *Semin Immunol* **19**, 24-32 (2007).
64. Lin, Y.C., Huang, D.Y., Chu, C.L., Lin, Y.L. & Lin, W.W. The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Sci Signal* **6**, ra71 (2013).
65. Jefferies, C.A., Doyle, S., Brunner, C., Dunne, A., Brint, E., Wietek, C. *et al.* Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. *J Biol Chem* **278**, 26258-26264 (2003).
66. Liu, X., Zhan, Z., Li, D., Xu, L., Ma, F., Zhang, P. *et al.* Intracellular MHC class II molecules promote TLR-triggered innate immune responses by maintaining activation of the kinase Btk. *Nat Immunol* **12**, 416-424 (2011).
67. Contri, A., Brunati, A.M., Trentin, L., Cabrelle, A., Miorin, M., Cesaro, L. *et al.* Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest* **115**, 369-378 (2005).
68. Buchner, M., Fuchs, S., Prinz, G., Pfeifer, D., Bartholome, K., Burger, M. *et al.* Spleen tyrosine kinase is overexpressed and represents a potential therapeutic target in chronic lymphocytic leukemia. *Cancer Res* **69**, 5424-5432 (2009).
69. Cesano, A., Perbellini, O., Evensen, E., Chu, C.C., Cioffi, F., Ptacek, J. *et al.* Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica* **98**, 626-634 (2013).
70. Herman, S.E., Gordon, A.L., Wagner, A.J., Heerema, N.A., Zhao, W., Flynn, J.M. *et al.* Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood* **116**, 2078-2088 (2010).
71. Herman, S.E., Gordon, A.L., Hertlein, E., Ramanunni, A., Zhang, X., Jaglowski, S. *et al.* Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* **117**, 6287-6296 (2011).
72. Kil, L.P., de Bruijn, M.J., van Hulst, J.A., Langerak, A.W., Yuvaraj, S. & Hendriks, R.W. Bruton's tyrosine kinase mediated signaling enhances leukemogenesis in a mouse model for chronic lymphocytic leukemia. *Am J Blood Res* **3**, 71-83 (2013).
73. Woyach, J.A., Smucker, K., Smith, L.L., Lozanski, A., Zhong, Y., Ruppert, A.S. *et al.* Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood* **123**, 1810-1817 (2014).
74. Kil, L.P., Yuvaraj, S., Langerak, A.W. & Hendriks, R.W. The role of B cell receptor stimulation in CLL pathogenesis. *Curr Pharm Des* **18**, 3335-3355 (2012).
75. Rassenti, L.Z., Huynh, L., Toy, T.L., Chen, L., Keating, M.J., Gribben, J.G. *et al.* ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* **351**, 893-901 (2004).

76. Huttmann, A., Klein-Hitpass, L., Thomale, J., Deenen, R., Carpinteiro, A., Nuckel, H. *et al.* Gene expression signatures separate B-cell chronic lymphocytic leukaemia prognostic subgroups defined by ZAP-70 and CD38 expression status. *Leukemia* **20**, 1774-1782 (2006).
77. Pepper, C., Brennan, P., Alghazal, S., Ward, R., Pratt, G., Starczynski, J. *et al.* CD38+ chronic lymphocytic leukaemia cells co-express high levels of ZAP-70 and are functionally distinct from their CD38- counter-parts. *Leukemia* **20**, 743-744 (2006).
78. Schroers, R., Griesinger, F., Trumper, L., Haase, D., Kulle, B., Klein-Hitpass, L. *et al.* Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia* **19**, 750-758 (2005).
79. Pepper, C., Ward, R., Lin, T.T., Brennan, P., Starczynski, J., Musson, M. *et al.* Highly purified CD38+ and CD38- sub-clones derived from the same chronic lymphocytic leukemia patient have distinct gene expression signatures despite their monoclonal origin. *Leukemia* **21**, 687-696 (2007).
80. Zupo, S., Isnardi, L., Megna, M., Massara, R., Malavasi, F., Dono, M. *et al.* CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. *Blood* **88**, 1365-1374 (1996).
81. Damle, R.N., Temburni, S., Calissano, C., Yancopoulos, S., Banapour, T., Sison, C. *et al.* CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. *Blood* **110**, 3352-3359 (2007).
82. Blix, E.S., Irish, J.M., Husebekk, A., Delabie, J., Forfang, L., Tierens, A.M. *et al.* Phospho-specific flow cytometry identifies aberrant signaling in indolent B-cell lymphoma. *BMC Cancer* **12**, 478 (2012).
83. Petlickovski, A., Laurenti, L., Li, X., Marietti, S., Chiusolo, P., Sica, S. *et al.* Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood* **105**, 4820-4827 (2005).
84. Gobessi, S., Laurenti, L., Longo, P.G., Sica, S., Leone, G. & Efremov, D.G. ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. *Blood* **109**, 2032-2039 (2007).
85. Muggen, A.F., Pillai, S.Y., Kil, L.P., van Zelm, M.C., van Dongen, J.J., Hendriks, R.W. *et al.* Basal Ca signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* (2014).
86. Apollonio, B., Scielzo, C., Bertilaccio, M.T., Ten Hacken, E., Scarfo, L., Ranghetti, P. *et al.* Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood* **121**, 3879-3888, S3871-3878 (2013).
87. Chatzouli, M., Ntoufa, S., Papakonstantinou, N., Chartomatsidou, E., Anagnostopoulos, A., Kollia, P. *et al.* Heterogeneous functional effects of concomitant B cell receptor and TLR stimulation in chronic lymphocytic leukemia with mutated versus unmutated Ig genes. *J Immunol* **192**, 4518-4524 (2014).
88. Damm, F., Mylonas, E., Cosson, A., Yoshida, K., Della Valle, V., Mouly, E. *et al.* Acquired initiating mutations in early hematopoietic cells of CLL patients. *Cancer Discov* **4**, 1088-1101 (2014).
89. Jebaraj, B.M., Kienle, D., Buhler, A., Winkler, D., Dohner, H., Stilgenbauer, S. *et al.* BRAF mutations in chronic lymphocytic leukemia. *Leuk Lymphoma* **54**, 1177-1182 (2013).
90. Yaktapour, N., Meiss, F., Mastroianni, J., Zenz, T., Andrlova, H., Mathew, N.R. *et al.* BRAF inhibitor-associated ERK activation drives development of chronic lymphocytic leukemia. *J Clin Invest* **124**, 5074-5084 (2014).

91. Mansouri, L., Sutton, L.A., Ljungstrom, V., Bondza, S., Arngarden, L., Bhoi, S. *et al.* Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. *J Exp Med* **212**, 833-843 (2015).
92. Coscia, M., Pantaleoni, F., Riganti, C., Vitale, C., Rigoni, M., Peola, S. *et al.* IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. *Leukemia* **25**, 828-837 (2011).
93. Burkle, A., Niedermeier, M., Schmitt-Graff, A., Wierda, W.G., Keating, M.J. & Burger, J.A. Overexpression of the CXCR5 chemokine receptor, and its ligand, CXCL13 in B-cell chronic lymphocytic leukemia. *Blood* **110**, 3316-3325 (2007).
94. Burger, J.A., Burger, M. & Kipps, T.J. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood* **94**, 3658-3667 (1999).
95. Burger, J.A. & Gandhi, V. The lymphatic tissue microenvironments in chronic lymphocytic leukemia: in vitro models and the significance of CD40-CD154 interactions. *Blood* **114**, 2560-2561; author reply 2561-2562 (2009).
96. Burger, J.A., Tsukada, N., Burger, M., Zvaifler, N.J., Dell'Aquila, M. & Kipps, T.J. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood* **96**, 2655-2663 (2000).
97. Burger, M., Hartmann, T., Krome, M., Rawluk, J., Tamamura, H., Fujii, N. *et al.* Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells. *Blood* **106**, 1824-1830 (2005).
98. O'Hayre, M., Salanga, C.L., Kipps, T.J., Messmer, D., Dorrestein, P.C. & Handel, T.M. Elucidating the CXCL12/CXCR4 signaling network in chronic lymphocytic leukemia through phosphoproteomics analysis. *PLoS One* **5**, e11716 (2010).
99. Burger, J.A., Quiroga, M.P., Hartmann, E., Burkle, A., Wierda, W.G., Keating, M.J. *et al.* High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. *Blood* **113**, 3050-3058 (2009).
100. Sutton, L.A., Ljungstrom, V., Mansouri, L., Young, E., Cortese, D., Navrkalova, V. *et al.* Targeted next-generation sequencing in chronic lymphocytic leukemia: a high-throughput yet tailored approach will facilitate implementation in a clinical setting. *Haematologica* **100**, 370-376 (2015).
101. Puente, X.S., Bea, S., Valdes-Mas, R., Villamor, N., Gutierrez-Abril, J., Martin-Subero, J.I. *et al.* Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature* (2015).
102. Martinez-Trillos, A., Pinyol, M., Navarro, A., Aymerich, M., Jares, P., Juan, M. *et al.* Mutations in TLR/MYD88 pathway identify a subset of young chronic lymphocytic leukemia patients with favorable outcome. *Blood* **123**, 3790-3796 (2014).
103. Poulain, S., Roumier, C., Decambron, A., Renneville, A., Herbaux, C., Bertrand, E. *et al.* MYD88 L265P mutation in Waldenstrom macroglobulinemia. *Blood* **121**, 4504-4511 (2013).
104. Ngo, V.N., Young, R.M., Schmitz, R., Jhavar, S., Xiao, W., Lim, K.H. *et al.* Oncogenically active MYD88 mutations in human lymphoma. *Nature* **470**, 115-119 (2011).
105. Hendriks, R.W., Yuvaraj, S. & Kil, L.P. Targeting Bruton's tyrosine kinase in B cell malignancies. *Nat Rev Cancer* **14**, 219-232 (2014).

106. Simonetti, G., Bertilaccio, M.T., Ghia, P. & Klein, U. Mouse models in the study of chronic lymphocytic leukemia pathogenesis and therapy. *Blood* **124**, 1010-1019 (2014).
107. Bichi, R., Shinton, S.A., Martin, E.S., Koval, A., Calin, G.A., Cesari, R. *et al.* Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6955-6960 (2002).
108. Yan, X.J., Albesiano, E., Zanesi, N., Yancopoulos, S., Sawyer, A., Romano, E. *et al.* B cell receptors in TCL1 transgenic mice resemble those of aggressive, treatment-resistant human chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 11713-11718 (2006).
109. Ponader, S., Chen, S.S., Buggy, J.J., Balakrishnan, K., Gandhi, V., Wierda, W.G. *et al.* The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood* **119**, 1182-1189 (2012).
110. Suljagic, M., Longo, P.G., Bennardo, S., Perlas, E., Leone, G., Laurenti, L. *et al.* The Syk inhibitor fostamatinib disodium (R788) inhibits tumor growth in the Emu- TCL1 transgenic mouse model of CLL by blocking antigen-dependent B-cell receptor signaling. *Blood* **116**, 4894-4905 (2010).
111. Robak, T. & Robak, P. BCR signaling in chronic lymphocytic leukemia and related inhibitors currently in clinical studies. *International reviews of immunology* **32**, 358-376 (2013).
112. ter Brugge, P.J., Ta, V.B., de Bruijn, M.J., Keijzers, G., Maas, A., van Gent, D.C. *et al.* A mouse model for chronic lymphocytic leukemia based on expression of the SV40 large T antigen. *Blood* **114**, 119-127 (2009).
113. Ahuja, D., Saenz-Robles, M.T. & Pipas, J.M. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene* **24**, 7729-7745 (2005).
114. Pan, Z., Scheerens, H., Li, S.J., Schultz, B.E., Sprengeler, P.A., Burrill, L.C. *et al.* Discovery of selective irreversible inhibitors for Bruton's tyrosine kinase. *ChemMedChem* **2**, 58-61 (2007).
115. Chang, B.Y., Francesco, M., De Rooij, M.F., Magadala, P., Steggerda, S.M., Huang, M.M. *et al.* Egress of CD19(+)CD5(+) cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients. *Blood* **122**, 2412-2424 (2013).
116. Saez de Guinoa, J., Barrio, L., Mellado, M. & Carrasco, Y.R. CXCL13/CXCR5 signaling enhances BCR-triggered B-cell activation by shaping cell dynamics. *Blood* **118**, 1560-1569 (2011).
117. Spaargaren, M., Beuling, E.A., Rurup, M.L., Meijer, H.P., Klok, M.D., Middendorp, S. *et al.* The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J Exp Med* **198**, 1539-1550 (2003).
118. Advani, R.H., Buggy, J.J., Sharman, J.P., Smith, S.M., Boyd, T.E., 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* **31**, 88-94 (2013).
119. Cheng, S., Ma, J., Guo, A., Lu, P., Leonard, J.P., Coleman, M. *et al.* BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. *Leukemia* **28**, 649-657 (2014).
120. O'Brien, S., Furman, R.R., Coutre, S.E., Sharman, J.P., Burger, J.A., Blum, K.A. *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* **15**, 48-58 (2014).
121. Herman, S.E., Niemann, C.U., Farooqui, M., Jones, J., Mustafa, R.Z., Lipsky, A. *et al.* Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia* **28**, 2188-2196 (2014).

122. Byrd, J.C., Furman, R.R., Coutre, S.E., Burger, J.A., Blum, K.A., Coleman, M. *et al.* Three-year follow-up of treatment-naive and previously treated patients with CLL and SLL receiving single-agent ibrutinib. *Blood* **125**, 2497-2506 (2015).
123. McMullen, J.R., Boey, E.J., Ooi, J.Y., Seymour, J.F., Keating, M.J. & Tam, C.S. Ibrutinib increases the risk of atrial fibrillation, potentially through inhibition of cardiac PI3K-Akt signaling. *Blood* **124**, 3829-3830 (2014).
124. Dubovsky, J.A., Beckwith, K.A., Natarajan, G., Woyach, J.A., Jaglowski, S., Zhong, Y. *et al.* Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. *Blood* **122**, 2539-2549 (2013).
125. Woyach, J.A., Furman, R.R., Liu, T.M., Ozer, H.G., Zapatka, M., Ruppert, A.S. *et al.* Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med* **370**, 2286-2294 (2014).
126. Fama, R., Bomben, R., Rasi, S., Dal Bo, M., Ciardullo, C., Monti, S. *et al.* Ibrutinib-naive chronic lymphocytic leukemia lacks Bruton tyrosine kinase mutations associated with treatment resistance. *Blood* **124**, 3831-3833 (2014).
127. Burger, J.A., Keating, M.J., Wierda, W.G., Hartmann, E., Hoellenriegel, J., Rosin, N.Y. *et al.* Safety and activity of ibrutinib plus rituximab for patients with high-risk chronic lymphocytic leukaemia: a single-arm, phase 2 study. *Lancet Oncol* **15**, 1090-1099 (2014).
128. Brown, J.R., Barrientos, J.C., Barr, P.M., Flinn, I.W., Burger, J.A., Tran, A. *et al.* The Bruton's tyrosine kinase (BTK) inhibitor, ibrutinib, with chemoimmunotherapy in patients with chronic lymphocytic leukemia. *Blood* (2015).
129. Hallek, M., Kay, N.E., Osterborg, A., Chanan-Khan, A.A., Mahler, M., Salman, M. *et al.* The HELIOS trial protocol: a Phase III study of ibrutinib in combination with bendamustine and rituximab in relapsed/refractory chronic lymphocytic leukemia. *Future Oncol*, 1-9 (2014).
130. Gobessi, S., Laurenti, L., Longo, P.G., Carsetti, L., Berno, V., Sica, S. *et al.* Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia* **23**, 686-697 (2009).
131. Baudot, A.D., Jeandel, P.Y., Mouska, X., Maurer, U., Tartare-Deckert, S., Raynaud, S.D. *et al.* The tyrosine kinase Syk regulates the survival of chronic lymphocytic leukemia B cells through PKCdelta and proteasome-dependent regulation of Mcl-1 expression. *Oncogene* **28**, 3261-3273 (2009).
132. Hoellenriegel, J., Coffey, G.P., Sinha, U., Pandey, A., Sivina, M., Ferrajoli, A. *et al.* Selective, novel spleen tyrosine kinase (Syk) inhibitors suppress chronic lymphocytic leukemia B-cell activation and migration. *Leukemia* **26**, 1576-1583 (2012).
133. Weinblatt, M.E., Kavanaugh, A., Genovese, M.C., Musser, T.K., Grossbard, E.B. & Magilavy, D.B. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. *N Engl J Med* **363**, 1303-1312 (2010).
134. Friedberg, J.W., Sharman, J., Sweetenham, J., Johnston, P.B., Vose, J.M., Lacasce, A. *et al.* Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* **115**, 2578-2585 (2010).
135. Currie, K.S., Kropf, J.E., Lee, T., Blomgren, P., Xu, J., Zhao, Z. *et al.* Discovery of GS-9973, a selective and orally efficacious inhibitor of spleen tyrosine kinase. *J Med Chem* **57**, 3856-3873 (2014).
136. Sharman, J., Hawkins, M., Kolibaba, K., Boxer, M., Klein, L., Wu, M. *et al.* An open-label phase 2 trial of entospletinib (GS-9973), a selective spleen tyrosine kinase inhibitor, in chronic lymphocytic leukemia. *Blood* **125**, 2336-2343 (2015).

137. Burke, R.T., Meadows, S., Loriaux, M.M., Currie, K.S., Mitchell, S.A., Maciejewski, P. *et al.* A potential therapeutic strategy for chronic lymphocytic leukemia by combining Idelalisib and GS-9973, a novel spleen tyrosine kinase (Syk) inhibitor. *Oncotarget* **5**, 908-915 (2014).
138. Coffey, G., DeGuzman, F., Inagaki, M., Pak, Y., Delaney, S.M., Ives, D. *et al.* Specific inhibition of spleen tyrosine kinase suppresses leukocyte immune function and inflammation in animal models of rheumatoid arthritis. *J Pharmacol Exp Ther* **340**, 350-359 (2012).
139. Spurgeon, S.E., Coffey, G., Fletcher, L.B., Burke, R., Tyner, J.W., Druker, B.J. *et al.* The selective SYK inhibitor P505-15 (PRT062607) inhibits B cell signaling and function in vitro and in vivo and augments the activity of fludarabine in chronic lymphocytic leukemia. *J Pharmacol Exp Ther* **344**, 378-387 (2013).
140. Hoellenriegel, J., Meadows, S.A., Sivina, M., Wierda, W.G., Kantarjian, H., Keating, M.J. *et al.* The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* **118**, 3603-3612 (2011).
141. Brown, J.R., Byrd, J.C., Coutre, S.E., Benson, D.M., Flinn, I.W., Wagner-Johnston, N.D. *et al.* Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. *Blood* **123**, 3390-3397 (2014).
142. Furman, R.R., Sharman, J.P., Coutre, S.E., Cheson, B.D., Pagel, J.M., Hillmen, P. *et al.* Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med* **370**, 997-1007 (2014).
143. de Rooij, M.F., Kuil, A., Kater, A.P., Kersten, M.J., Pals, S.T. & Spaargaren, M. Ibrutinib and idelalisib synergistically target BCR-controlled adhesion in MCL and CLL: a rationale for combination therapy. *Blood* **125**, 2306-2309 (2015).
144. Amrein, L., Shawi, M., Grenier, J., Aloyz, R. & Panasci, L. The phosphatidylinositol-3 kinase I inhibitor BKM120 induces cell death in B-chronic lymphocytic leukemia cells in vitro. *Int J Cancer* **133**, 247-252 (2013).
145. Rosich, L., Saborit-Villarroya, I., Lopez-Guerra, M., Xargay-Torrent, S., Montraveta, A., Aymerich, M. *et al.* The phosphatidylinositol-3-kinase inhibitor NVP-BKM120 overcomes resistance signals derived from microenvironment by regulating the Akt/FoxO3a/Bim axis in chronic lymphocytic leukemia cells. *Haematologica* **98**, 1739-1747 (2013).
146. Bendell, J.C., Rodon, J., Burris, H.A., de Jonge, M., Verweij, J., Birle, D. *et al.* Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *J Clin Oncol* **30**, 282-290 (2012).
147. Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D. & Sawyers, C.L. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* **305**, 399-401 (2004).
148. Veldurthy, A., Patz, M., Hagist, S., Pallasch, C.P., Wendtner, C.M., Hallek, M. *et al.* The kinase inhibitor dasatinib induces apoptosis in chronic lymphocytic leukemia cells in vitro with preference for a subgroup of patients with unmutated IgVH genes. *Blood* **112**, 1443-1452 (2008).
149. ten Hacken, E., Scielzo, C., Bertilaccio, M.T., Scarfo, L., Apollonio, B., Barbaglio, F. *et al.* Targeting the LYN/HS1 signaling axis in chronic lymphocytic leukemia. *Blood* **121**, 2264-2273 (2013).
150. Amrein, P.C., Attar, E.C., Takvorian, T., Hochberg, E.P., Ballen, K.K., Leahy, K.M. *et al.* Phase II study of dasatinib in relapsed or refractory chronic lymphocytic leukemia. *Clin Cancer Res* **17**, 2977-2986 (2011).

151. Kater, A.P., Spiering, M., Liu, R.D., Doreen Te Raa, G., Slinger, E., Tonino, S.H. *et al.* Dasatinib in combination with fludarabine in patients with refractory chronic lymphocytic leukemia: a multicenter phase 2 study. *Leuk Res* **38**, 34-41 (2014).
152. Friedman, D.R., Lanasa, M.C., Davis, P.H., Allgood, S.D., Matta, K.M., Brander, D.M. *et al.* Perifosine treatment in chronic lymphocytic leukemia: results of a phase II clinical trial and in vitro studies. *Leuk Lymphoma* **55**, 1067-1075 (2014).
153. Richardson, P.G., Wolf, J., Jakubowiak, A., Zonder, J., Lonial, S., Irwin, D. *et al.* Perifosine plus bortezomib and dexamethasone in patients with relapsed/refractory multiple myeloma previously treated with bortezomib: results of a multicenter phase I/II trial. *J Clin Oncol* **29**, 4243-4249 (2011).

Chapter 3

Basal Ca^{2+} signaling is particularly increased in mutated chronic lymphocytic leukemia

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ABSTRACT

On the basis of somatic hypermutation status of their B-cell antigen receptor (BCR) genes, chronic lymphocytic leukemia (CLL) patients can be divided into unmutated CLL (U-CLL) or mutated CLL (M-CLL). Approximately 30% of CLL patients express a stereotypic BCR, which may indicate that specific antigenic stimulation is driving CLL pathogenesis. Recently it was reported that BCRs from CLL cells are capable of antigen-independent, cell-autonomous signaling, through recognition of an internal framework 2 (FR2) BCR epitope. We hypothesized that the level of cell-autonomous signaling may differ between CLL subgroups. Therefore, we analyzed Ca^{2+} signaling in a series of primary stereotypic or heterogeneous U-CLL and M-CLL (n=68) and healthy controls (n=14). We confirmed that basal Ca^{2+} signaling in CLL cells is higher than in normal B cells. Interestingly, we found that basal signaling was particularly increased in M-CLL. The degree of basal signaling did not correlate with membrane Ig levels, HCDR3 characteristics, or FR2 / FR3 sequence. We conclude that the level of basal Ca^{2+} signaling is not uniformly enhanced in CLL B cells, but is associated with CLL IGHV mutational status, reflecting a distinct cellular origin and possibly a different anergic state induced by repetitive or continuous antigen binding *in vivo*.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by monoclonal expansion of mature $CD5^+$ $CD19^+$ B lymphocytes, and is the most common type of leukemia in the elderly in the Western world[1]. On the basis of the somatic hypermutation status (SHM) of the immunoglobulin heavy chain V (IGHV) genes, CLL can be divided in unmutated CLL (U-CLL) and mutated CLL (M-CLL). This division is clinically relevant because U-CLL has an unfavorable prognosis, with a more aggressive disease course and shorter time to first treatment, while M-CLL represents a more indolent form of the disease with a more favorable prognosis[2, 3].

Over 30% of all CLL cases can be grouped on the basis of their restricted IGHV/IGHD/IGHJ gene usage, similarities of the length and amino acid sequence of the complementarity determining region 3 (CDR3)[4]. These so-called stereotypic B cell receptors (BCR) are found in different CLL patients. This stereotypy indicates involvement of similar antigens, thus implicating antigenic stimulation and BCR specificity in CLL pathogenesis[4]. Indeed, several lines of evidence support the important role of antigen-driven BCR signaling in the pathogenesis of CLL. In general, most U-CLLs express polyreactive, low affinity BCRs, which recognize self- and non-self antigens, such as DNA, insulin, LPS, apoptotic cells, vimentin, myosin heavy chain 2A (MYH2A), and phosphorylcholine-containing antigens like oxidized LDL[5, 6, 7, 8].

For several of the major stereotypic subsets the target antigen has been identified. Many of these are poly- or autoreactive and bind to antigens expressed by apoptotic cells. BCRs from stereotypic subset #1 are found to target oxidized LDL6, 8, 9. Stereotypic subset #2 is able to bind to protein L, a cell-wall protein of the commensal gut bacterium *Peptostreptococcus magnus*[10] and cofilin 1[8]. Recent reports demonstrated that two stereotypic M-CLL subsets have rheumatoid factor activity and show specificity for the Fc-tail of IgG[11, 12]. Stimulation of these stereotypic CLL cells with IgG results in proliferation11. Another recent study describes a stereotypic M-CLL subset with IGHV3-7 usage with an extremely short CDR3, which is highly specific for β-(1,6)-glucan. This is a potent antigenic factor found in yeast and filamentous fungi that induces proliferation of CLL cells expressing this particular stereotypic BCR[13]. Collectively, these data support an important role of antigen-driven BCR signaling in the pathogenesis of CLL.

Contrary to these studies, Dühren – von Minden *et al*. showed that CLL BCR can be stimulated independent of external antigens, because their CDR3 regions recognize an internal epitope in framework 2 (FR2) of IGHV[14]. This recognition induces a higher level of antigen-independent, basal or 'autonomous' signaling of the BCR as demonstrated by increased cytoplasmic Ca^{2+} levels. These observations were primarily made in an *in vitro* assay using triple knockout (TKO) cells, which are BCR-free, conditionally signaling-competent mouse B-lineage cells. Upon transfection of human CLL-derived BCRs (or CDR3s) higher basal

Ca^{2+} signaling was observed, while other B cell lymphoma-derived BCRs needed crosslinking for signaling[14]. Recently, another internal epitope in FR3 of both IGHV and light chain V genes was identified[15].

Collectively, these findings fueled the controversy whether antigen-dependent or autonomous stimulation would be important in CLL pathogenesis. We hypothesized that these apparently contrasting observations may not be mutually exclusive, as the level of cell-autonomous signaling could be different between immunogenetic CLL subgroups. We therefore analyzed Ca^{2+} signaling in a large series of primary CLL samples consisting of U-CLL and M-CLL with stereotypic or heterogeneous BCRs.

MATERIALS & METHODS

Patients and healthy controls

Primary patient material was obtained from CLL patients, whilst the material from healthy controls (mostly >50 years of age) was obtained via Erasmus MC and via Sanquin blood bank (Rotterdam). Diagnostic and control samples were collected upon informed consent and anonymized for further use, following the guidelines of the Institutional Review Board, and in accordance with the declaration of Helsinki.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer's instructions, and were either stored frozen or used freshly. For Ca^{2+} flux assays, CLL cells were isolated by depleting other cell types from PBMCs using the B-CLL isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS (Miltenyi Biotec). When enough PBMCs were obtained from healthy controls, B cells were isolated using the B cell isolation kit (Miltenyi Biotec); alternatively, whole PBMC fractions were used for Ca^{2+} flux analyses. The purity of both the CLL and healthy control B cell samples was >95% as determined by flow cytometry on the LSRII (BD Biosciences, San Jose, California, USA). CLL samples were also evaluated for IgM, IgD, and IgG expression by flow cytometry. Normal B cells were analyzed for CD5 expression.

IGHV sequence analysis

Genomic DNA was extracted from PBMCs with use of spin-column kits and QIAcube (QIAGEN, Valencia, California, USA). Primers and protocols for IGHV mutation status analysis were used according to the BIOMED-2 protocol and following ERIC guidelines[16, 17]. In brief, PCR products were analyzed by electrophoresis on polyacrylamide gels for monoclonality,

followed by direct sequencing. Sequencing results were analyzed online by use of IMGT/V-QUEST on the IMGT website (www.imgt.org version 3.2.32).

Phylogenetic analysis of IGHV gene sequences

The phylogenetic relationships of CDR3, FR2, and FR3 IGHV gene sequences were estimated based on the pairwise matrix of sequence divergences calculated by the online tool Clustal W. using neighbor joining algorithm (http://www.genome.jp/tools/clustalw/)[18]. Visualization of MFI ratio of calcium influx as heatmap was created using the Spotfire analysis software[19].

Ca^{2+} flux assay

Intracellular Ca^{2+} fluxes were measured using the Fluo3-AM and Fura Red-AM fluorogenic probes, both purchased from Molecular Probes (Life technologies). In brief, CLL cells and B cells or PBMCs from healthy controls were incubated with 5 µM Fluo3-AM and 5 µM Fura Red-AM in loading buffer (HBSS medium supplemented with 10 mM HEPES and 5%FCS) at 30°C for 30 minutes in the dark. To gate B cells in PBMCs from healthy controls, we added CD20 APC-H7 and CD3 APC for the final 10 minutes of incubation. Cells were then washed and resuspended in buffer (HBSS medium with 10 mM HEPES, 5%FCS and 1 mM $CaCl_2$) at room temperature. Cells were warmed to 37°C for 5 minutes before acquisition of events. Basal intracellular Ca^{2+} levels were measured for 60 seconds, followed by stimulation of the BCR with 35 µg/ml anti-human IgM (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), or 5 µg/ml anti-human IgD, or 10 µg/ml anti-human IgG (both Southern Biotec, Birmingham, Alabama, USA). At the end of each Ca^{2+} measurement, the cells were stimulated with 2 µg/ml Ionomycin (Life technologies, Carlsbad, Californa, USA) to measure maximum Ca^{2+} signaling. Data were acquired on an LSRII flow cytometer (BD Biosciences) and data analysis was performed with the use of FlowJo software (Tree Star, Ashland, Orlando, USA).

Statistical analyses

Statistical analysis was performed using GraphPad Prism software (San Diego, California, USA). For overall subgroup analysis, we used the Kruskal-Wallis test for nonparametric testing. When 2 groups were compared, the Mann-Whitney U test for nonparametric testing was used. To study the relation between variables we used linear regression analysis.

Table 1. Characteristics of CLL cases

Sample	SHM status (%)	IGHV gene	IGHD gene	IGHJ gene	VH CDR3 length	VH CDR3 sequence AA	Seq. number
CLL #1	100.00	IGHV7-4-1*02	IGHD6-19*01	IGHJ4*02	13	ARKQWLVIPNFDY	1
CLL #1	100.00	IGHV1-3*01	IGHD6-19*01	IGHJ4*02	13	AREQWLDMPSFDY	7
CLL #1	100.00	IGHV1-3*01	IGHD1-7*01	IGHJ4*02	13	ARVQALGLPNFDY	16
CLL #1	99.54	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	13	AREQWLVLSNIDY	40
CLL #1	100.00	IGHV5-a*01	IGHD6-19*01	IGHJ4*02	13	ARVQWLVLPYFDY	55
CLL #2	97.76	IGHV3-21*01	IGHD1-1*01	IGHJ6*02	9	ARDANGMDV	2
CLL #2	99.09	IGHV3-21*01	IGHD6-6*01	IGHJ6*02	9	ARDQNGMDV	9
CLL #2	98.65	IGHV3-21*01	IGHD2-2*01	IGHJ6*02	9	ASDRSGMDV	14
CLL #2	98.21	IGHV3-21*01	IGHD2-2*01	IGHJ6*02	9	ARDANGMDV	24
CLL #2	98.21	IGHV3-21*01	—	IGHJ6*02	9	ARDQTAVDV	32
CLL #2	98.21	IGHV3-21*01	—	IGHJ6*02	9	ARDQNAMDV	41
CLL #2	98.64	IGHV3-21*01	IGHD2-15*01	IGHJ6*02	9	VLPRNGMDV	47
CLL #2	96.40	IGHV3-21*01	—	IGHJ6*02	9	ARDANGMDV	66
CLL #4	96.82	IGHV4-34*01	IGHD3-22*01	IGHJ6*02	20	VRGYPEDTTTRRYYYYGMEV	11
CLL #4	90.00	IGHV4-34*01	IGHD4-23*01	IGHJ6*02	20	ARGYGVDATTRRYYYYGMDV	15
CLL #4	91.82	IGHV4-34*01	IGHD3-16*01	IGHJ6*02	20	VRGYGSTPVtRRYYYYGMDV	22
CLL #4	85.91	IGHV4-34*01	IGHD4-11*01	IGHJ6*02	20	ARGYPTTVTTRRYYYYGIDV	33
CLL #4	92.73	IGHV4-34*01	IGHD3-16*01	IGHJ6*02	20	VRGfPYDYTTRRYYYYGMDV	42
CLL #4	93.18	IGHV4-34*01	IGHD3-22*01	IGHJ6*02	20	ARDYPSDPVIRRYYYYGMDV	49
CLL #4	92.27	IGHV4-34*01	IGHD1-1*01	IGHJ6*02	20	ARGYGTTSTTRRYYYYGMDV	50
CLL #4	90.91	IGHV4-34*01	IGHD5-18	IGHJ6*01	20	VRGYGDTPTLRRYYYYGMEV	67
CLL #5	100.00	IGHV1-69*06	IGHD6-13*01	IGHJ6*03	20	ARAGSSSWYEAYYYYYYMDV	56
CLL #6	100.00	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	21	ARGGGYDYIWGSYRSNDAFDI	12
CLL #6	100.00	IGHV1-69*06	IGHD3-16*02	IGHJ3*02	21	ARGGDYDYIWGSYRTNDAFDI	17
CLL #6	100.00	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	21	ARGGGYDYVWGSYRPNDAFDI	20
CLL #6	100.00	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	21	ARGGNYDYIWGSYRTNDAFDI	37
CLL #6	100.00	IGHV1-69*06	IGHD3-16*02	IGHJ3*02	21	ARGGDYDYIWGSYRPNDAFDI	43
CLL #14	89.24	IGHV4-4*02	IGHD2-21*01	IGHJ4*02	10	ARGGDWRFDS	48
CLL #14	92.38	IGHV4-4*02	IGHD6-19*01	IGHJ4*02	10	ARGGDWKFDS	62
CLL #14	88.98	IGHV4-4*02	IGHD2-21*02	IGHJ5*01	10	ARGGDWRFDS	68
CLL #16	90.40	IGHV4-34*01	—	IGHJ6*02	24	ARRNCSGGSCYLAGYYYYYSGMDV	29
CLL #16	93.18	IGHV4-34*01	IGHD2-15*01	IGHJ6*02	24	AGRFYCSGGSCQDPSYYYYYGIDV	51
CLL #77	89.55	IGHV4-59*03	IGHD3-22*01	IGHJ4*02	14	TRGPDESGWLGLLY	57
CLL #202	95.07	IGHV3-23*01	IGHD4-17*01	IGHJ4*02	14	AKESGDYQGGSFDC	21
M-CLL	97.76	IGHV3-21*01	IGHD1-14*01	IGHJ4*02	12	VRTTQDVTLFDS	10
M-CLL	92.69	IGHV1-2*02	IGHD5-18*01	IGHJ5*01	21	ARAGTALVNRECVGCEFWFDS	3
M-CLL	92.34	IGHV5-51*01	IGHD3-9*01	IGHJ3*01	12	ATGGRLSDVFDL	5
M-CLL	97.59	IGHV2-5	IGHD3-22*01	IGHJ4*02	12	AHMVTGKDYFDY	18
M-CLL	95.25	IGHV3-7*03	IGHD3-10*01	IGHJ3*02	18	ARDRSPKLGSAYFDAFDI	23
M-CLL	87.40	IGHV3-7*01	IGHD4-4*01	IGHJ6*02	7	LTRGIDV	25
M-CLL	94.60	IGHV3-74*01	IGHD2-15*01	IGHJ5*02	18	AREVCIGDNCYSRGWFDP	26
M-CLL	86.75	IGHV2-5*10	IGHD3-16*02	IGHJ3*02	20	AHITITYGGVIAELRDAFDM	30
M-CLL	97.36	IGHV1-2*04	IGHD3-9*01	IGHJ3*02	28	ARSSRPGSRRGLYDILTNYRGEGDAFDI	31
M-CLL	93.09	IGHV4-59*02	IGHD2-15*01	IGHJ4*02	11	AGSPSGTFSWNY	34
M-CLL	96.35	IGHV1-2*02	IGHD6-13*01	IGHJ6*03	18	AREYYTNSWASHDFYMDV	36
M-CLL	97.31	IGHV3-11*03	IGHD2-15*01	IGHJ4*02	13	ARGGEVMVSPLDR	38
M-CLL	90.51	IGHV4-61*02	IGHD1-26*01	IGHJ4*02	18	ARSEGETGGVGATTAVDF	46
M-CLL	94.05	IGHV3-72*01	IGHD2-8*01	IGHJ6*02	20	GRIYCTLSRCSIDQYYGMDV	54
M-CLL	94.62	IGHV3-33*01	IGHD1-26*01	IGHJ2*01	17	ARKQWELLASTYWYFDL	59
M-CLL	93.89	IGHV3-49*03	IGHD3-3*01	IGHJ3*02	16	TRTGWSMTILDDAFDI	60
M-CLL	97.08	IGHV3-11*03	IGHD6-13*01	IGHJ3*02	15	ARVGIAAAAIDAFDI	63
U-CLL	100.00	IGHV3-23*01	IGHD6-13*01	IGHJ6*02	20	AKPLYSSSWYLNYYYYGMDV	13
U-CLL	100.00	IGHV3-30*01	IGHD3-3*01	IGHJ6*02	27	ARASDFWSGYYKYEDPYGYYYYYGMDV	4
U-CLL	100.00	IGHV4-4*05	IGHD3-16*02	IGHJ6*02	26	ARGRRDDYIWGSYRYTDLGYYYGMDV	6
U-CLL	100.00	IGHV1-69*01	IGHD3-3*01	IGHJ4*02	22	ARAAVPYYDFWSGYSLDSGFDY	8
U-CLL	100.00	IGHV1-69*12	IGHD1-7*01	IGHJ2*01	19	ARAEPLNWNYRDLYWYFDL	19
U-CLL	100.00	IGHV4-31*03	IGHD3-22*01	IGHJ6*02	26	ARAKNYYDSSGIGRRGNYYYYYGMDV	27
U-CLL	100.00	IGHV4-34*01/*02	IGHD2-2*01	IGHJ6*02	21	ARQRSSTSCYARYYYYGMDV	28
U-CLL	100.00	IGHV4-38-2*02	IGHD3-9*01	IGHJ6*02	22	ARSRGVLRYFDWPDYYYYGMDV	35
U-CLL	100.00	IGHV1-69*01	IGHD3-3*01	IGHJ2*01	20	AREWGQMGFLEWLSDWYFDL	39
U-CLL	98.72	IGHV3-48*03	IGHD2-8*02	IGHJ4*02	14	ARGGVETTLYYFDY	44
U-CLL	100.00	IGHV1-69*01	IGHD3-16*02	IGHJ4*02	20	ARGGYYVWGSYRYIKDRFDY	45
U-CLL	100.00	IGHV1-69*01	IGHD3-3*01	IGHJ6*02	28	ARDGNEGTRGGDFWSGYYPNYYYYGMDV	52
U-CLL	100.00	IGHV4-59*01	IGHD3-22*01	IGHJ6*03	23	ARGNYYDSSGYYYVGYYYYYMDV	53
U-CLL	100.00	IGHV4-34*01	IGHD3-3*01	IGHJ3*02	19	ARGPYYDFWSGYSRRAFDI	58
U-CLL	100.00	IGHV1-69*01	IGHD3-16*02	IGHJ4*02	20	ARGGYYVWGSYRYIKDRFDY	61
U-CLL	100.00	IGHV1-69*01	IGHD3-3*01	IGHJ4*02	15	AREDSYYDFWSGYDY	64
U-CLL	100.00	IGHV2-5*02	IGHD6-13*01	IGHJ5*02	13	AHSSSWFITGFDP	65

Abbreviations: CDR3, complementarity determining region 3; CLL, chronic lymphocytic leukemia; IGHV, immunoglobulin heavy chain V; M-CLL, mutated CLL; SHM, somatic hypermutation; U-CLL, unmutated CLL; —, unidentified D gene usage.

RESULTS

Basal Ca^{2+} signaling is highest in primary M-CLL

CLL-derived BCRs have been demonstrated to induce higher basal Ca^{2+} signaling than BCRs derived from primary B cells[14]. We wondered if the same phenomenon could be observed in a cohort of primary CLL samples. To this end we selected 68 primary CLL cases (**Table 1**) and compared the Ca^{2+} signaling of the CLL cells with that of peripheral B cells derived from healthy controls (n=14). We chose to use the total normal B cell population for the analyses, because we did not see any difference in basal Ca^{2+} signaling between CD27- naive and CD27+ memory B cells, nor between CD5- and CD5+ B cells (**Figure S1**). Many of the primary CLL samples contained cells that demonstrated high basal Ca^{2+} signaling levels (**Figure 1A**). Within the total CLL cohort, both U-CLL (≥98% IGHV germline identity) and M-CLL (<98% identity) samples demonstrated significantly higher basal Ca^{2+} signaling levels than normal B cells (**Figure 1B**). Importantly, M-CLL had significantly higher average basal Ca^{2+} signaling levels than U-CLL (**Figure 1B,C**).

To identify how basal Ca^{2+} signaling varied within immunogenetic CLL subgroups, we made a further subdivision of U-CLL and M-CLL into stereotypic and non-stereotypic (heterogeneous) subgroups. No significant differences in Ca^{2+} levels were found between stereotypic and heterogeneous subgroups (**Figure 1D**). Basal Ca^{2+} levels in heterogeneous M-CLL samples were significantly higher than in stereotypic U-CLL, whereas the differences seen between heterogeneous U-CLL vs M-CLL (stereotypic or heterogeneous) were not significantly different. Finally, although some CLLs belonging to the same stereotypic subset showed very similar basal Ca^{2+} levels, no clear correlation was found between individual stereotypic CLL subsets and basal Ca^{2+} signaling levels (**Figure 1D**).

Collectively, these results demonstrate that primary CLL cells indeed have higher basal Ca^{2+} signaling levels than B cells from healthy controls, whereby this increase is higher in M-CLL than in U-CLL.

Basal Ca^{2+} levels are not associated with BCR characteristics or cytogenetic aberrations

CLL cells typically show a low level of BCR surface expression, although these levels can vary[20]. Therefore, we analyzed whether cell surface IgM and IgD expression levels correlated with the levels of basal Ca^{2+} signaling in CLL. Despite an overall reduced surface Ig expression when compared with normal B cells, we did not find any relation between high or low basal Ca^{2+} signaling levels in CLL and IgM (**Figure 2A**) or IgD (**Figure 2B**) surface expression levels. Upon exclusion of outliers with relatively high IgM and IgD expression, we did not find any correlations either (data not shown). Furthermore, Ig kappa vs Ig lambda light chain usage also did not correlate with basal Ca^{2+} signaling levels (data not shown).

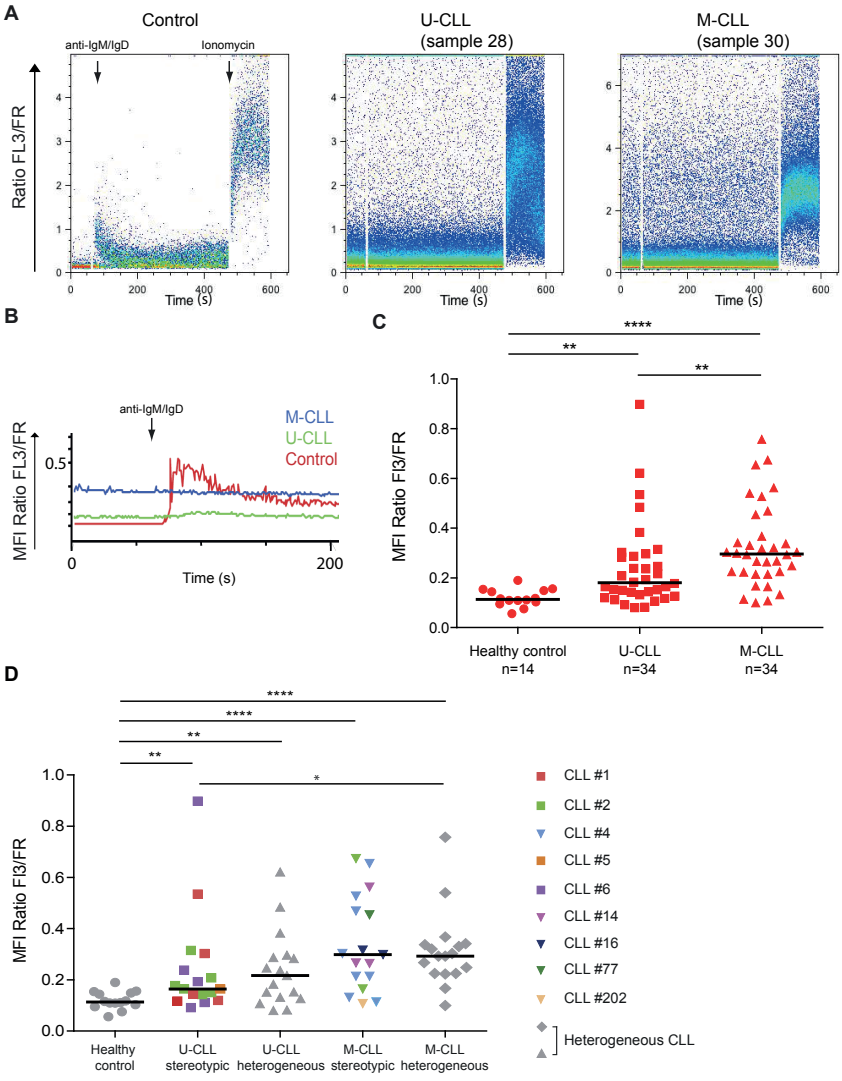

Figure 1. Ca^{2+} flux assays in primary CLL samples and normal human B cells.

A. Representative dot plots of Ca^{2+} flux assays of representative samples from a healthy control, U-CLL (sample 30), and M-CLL (sample 23). BCR stimulation was performed after 60s, and stimulation with ionomycin after 480s (indicated with arrows). The duration of the total Ca^{2+} flux assays was 600s. **B**. Median fluorescence intensity (MFI) kinetics plot of Ca^{2+} signaling in representative healthy control, U-CLL (sample 30) and M-CLL (sample 23) samples. **C**. Basal Ca^{2+} signaling in U-CLL (n=34) and M-CLL (n=34) cases compared with B cells from healthy controls (n=14). **D**. Basal Ca^{2+} signaling in CLL samples from specific immunogenetic subgroups. Overall significance was determined with the Kruskal Wallis test. Significance between two groups was determined with the Mann-Whitney U test. ** $P < 0.01$ **** $P < 0.0001$

We also investigated whether variation in the degree of surface expression of CD5, a transmembrane glycoprotein which down-modulates BCR signaling in B cells, would explain differences in basal Ca^{2+} signaling, but no correlation could be found (**Figure 2C**). In line with this, CD5 surface expression levels did not differ between U-CLL and M-CLL (data not shown).

Several cytogenetic aberrations that have been implicated in CLL pathogenesis and prognosis are associated with IGHV mutation status or stereotypic subsets[21]. However, the variations seen in basal Ca^{2+} signaling levels could not be linked to any specific cytogenetic abnormality (**Supplementary Figure S2**).

Taken together, these results indicate that variation in basal Ca^{2+} signaling levels between CLL subgroups is a phenomenon that cannot be associated to particular BCR characteristics or cytogenetic abnormalities.

CDR3 sequences and FR2 and FR3 motifs do not explain differences in basal Ca^{2+} levels in primary CLL

Since auto- or polyreactive antibodies are associated with long HCDR3 lengths and a positive charge[5, 22], we next analyzed whether these HCDR3 characteristics might influence basal Ca^{2+} signaling levels. We found no correlation between basal Ca^{2+} signaling levels in the CLL cases in our cohort and HCDR3 length (**Figure 3A**) or HCDR3 charge (**Figure 3B**).

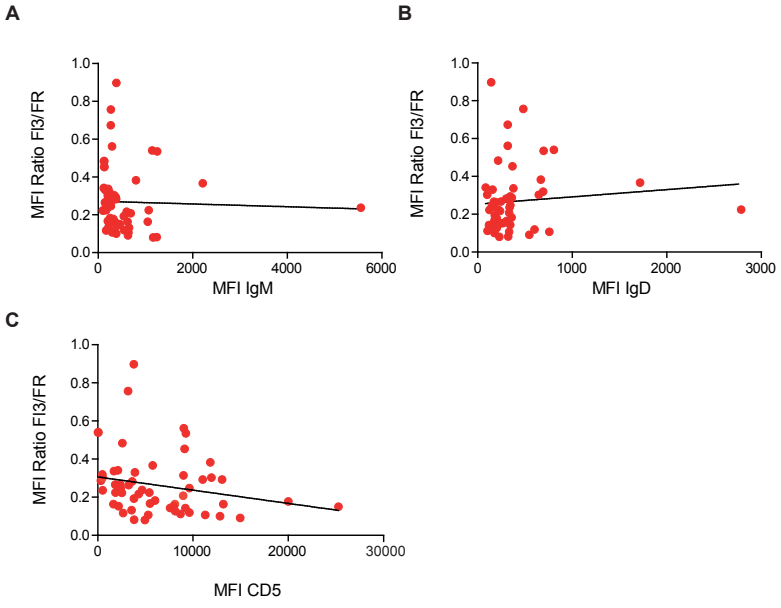

Figure 2. Basal Ca^{2+} signaling levels in CLL do not correlate with IgM, IgD and CD5 expression levels.

Linear regression analysis of basal Ca^{2+} signaling as a function of IgM (**A**), IgD (**B**), and CD5 (**C**) expression levels. Note that IgG expressing CLL cases were excluded from IgM and IgD analysis.

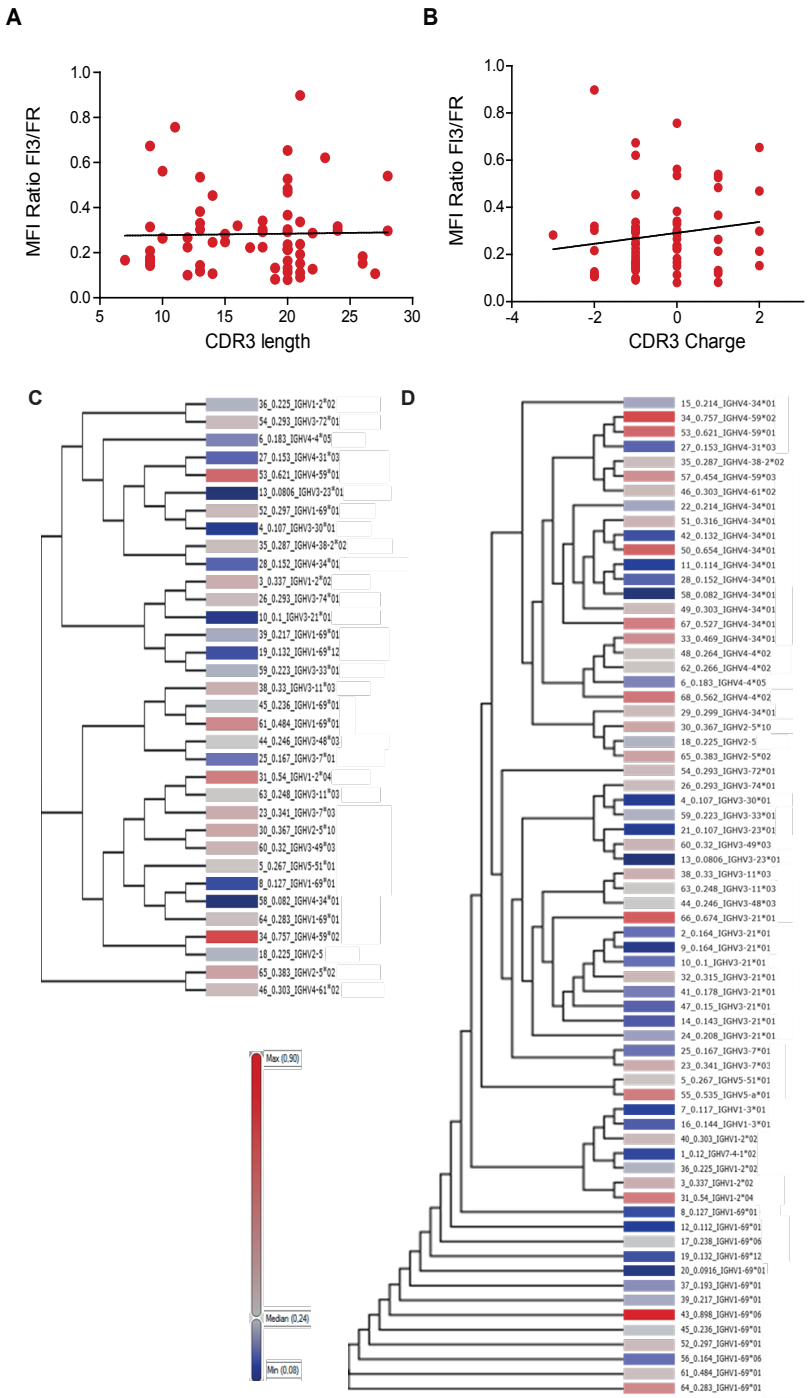
A
MFI Ratio FI3/FR
CDR3 length
B
MFI Ratio FI3/FR
CDR3 Charge
C
D
Max (0.90)
Median (0.24)
Min (0.08)

Figure 3. Basal Ca^{2+} signaling in CLL does not correlate with CDR3 and FR2 composition. ***(see left page)***

A,B. Linear regression analysis of basal Ca^{2+} signaling as a function of CDR3 length (**A**) and CDR3 charge (**B**). Positive and negative charge are defined by amino acids K, R, H and D, E, respectively. **C,D**. Phylogenetic analysis of HCDR3 (**C**) and FR2 (**D**) amino acid sequences combined with heat map analysis of the MFI of basal Ca^{2+} signaling levels of heterogenous CLL samples (C; n= 34) or total CLL samples (D; n=68) depicted in color code, ranging from red (high), via gray (median) to blue (low).

Furthermore, to evaluate a possible effect of the CDR3 amino acid composition, we performed phylogenetic analysis of CDR3 heterogeneity and compared median fluorescence intensity (MFI) Ca^{2+} levels. In accordance with the absence of a correlation of sterereotypic CLL subsets and basal Ca^{2+} signaling values, we also did not observe any correlations between phylogenetic clustering of CDR3 amino acid sequences of the heterogeneous U-CLL or M-CLL and the MFI values of their basal Ca^{2+} signaling (**Figure 3C**).

Furthermore, to investigate if amino acid changes in the IGHV FR2 or FR3 motifs, which has been reported to mediate the cell-autonomous signaling[14, 15], would correlate with the differences in basal Ca^{2+} signaling levels that we observed between CLL cases, we evaluated the IGHV FR2/FR3 sequences in all our 68 CLL patients. Only one of the sequences contained a mutation (V42L in case 29) in this FR2 motif leading to amino acid change with a different charge. In addition, only in a single CLL case the YYC FR3 motif was mutated and resulted in the Y103F amino acid change (case 34). Because these two particular samples with mutated FR2 or FR3 motifs both showed a basal Ca^{2+} signaling level around the median of all CLL samples (data not shown), we conclude that FR2 or FR3 epitopes cannot explain the Ca^{2+} signaling differences we observed between and within CLL subgroups. Moreover, phylogenetic clustering analysis of the FR2 and FR3 sequences from our entire CLL cohort – plus U-CLL, M-CLL, stereotypic or heterogeneous subsets - did not show a significant relation with MFI values (**Figure 3D, Figure S3 and data not shown**).

Collectively, these data show that the variation in basal Ca^{2+} signaling levels within CLL and between M-CLL and U-CLL subgroups cannot be accounted for by differences in the internal epitopes (FR2, FR3) or in the paratopes (CDR3) of their BCR molecules.

Primary CLL are largely unresponsive to BCR stimulation

In the Ca^{2+} flux assays we also evaluated the response of primary CLL samples and B cells of healthy controls to BCR stimulation. To this end we stimulated the cells with either IgM- or IgD-specific antibodies. Whereas the applied antibody concentrations induced a clear Ca^{2+} flux in healthy control B cells, BCR stimulation in primary CLL generally resulted only in a limited increase of cytoplasmic Ca^{2+} levels (**Figure 1, Figure 4A,B**). Even though the response to IgM was mostly low in both CLL subgroups, U-CLL as a whole appeared slightly more responsive towards IgM stimulation than M-CLL (**Figure 4A**), which is exemplified for individual cases in **Figure 4C**.

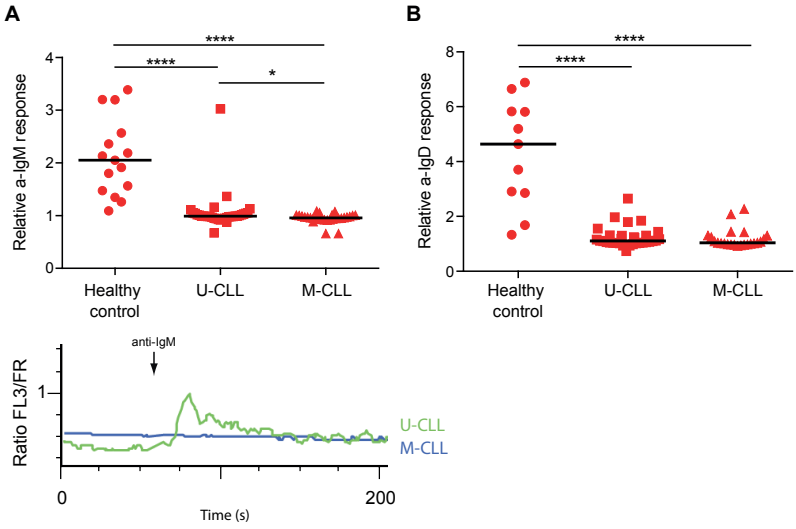

Figure 4. BCR stimulation response in CLL.

Relative response of Ca^{2+} signaling upon IgM (**A**) and IgD (**B**) stimulation. CLL samples were divided into U-CLL and M-CLL. Overall significance was determined with the Kruskal Wallis test (both $P<0.0001$), and comparisons between two groups with the Mann-Whitney U test. * $P < 0.05$ **** $P < 0.0001$. Respresentative kinetics plots showing the higher Ca^{2+} signaling upon IgM stimulation in U-CLL over M-CLL (75 percentiles are depicted) are shown in the lower panel of **A**.

Finally, we analyzed anti-IgG stimulation in CLL cases belonging to subset 4, which are known to have undergone IgG class-switch recombination[23]. Although Ca^{2+} flux kinetics upon IgG stimulation differs from IgM or IgD in healthy controls, the primary IgG CLL cases were also unresponsive to BCR stimulation (data not shown).

Our data demonstrate that CLL cells are largely unresponsive to IgM and IgD stimulation, irrespective of their membrane Ig expression levels. Despite this unresponsiveness to BCR stimulation, the responses to ionomycin demonstrated that Ca^{2+} mobilization per se was not disturbed in primary CLL cases.

DISCUSSION

In this study we provide evidence that primary CLL cells show a generally higher basal Ca^{2+} signaling level than seen in PB B cells derived from healthy controls. Interestingly, when we divide CLL samples on the basis of their IGHV gene SHM status, we found a significantly higher basal Ca^{2+} signaling level in the M-CLL subgroup compared with U-CLL. Because the degree of basal Ca^{2+} signaling did not correlate with other BCR characteristics, including Ig

expression level, HCDR3 length, charge, and composition, or with cytogenetic aberrations, we conclude that difference in basal Ca^{2+} signaling seems directed by the SHM status of the CLL subgroup and that intrinsic differences in basal Ca^{2+} signaling would explain the variation seen between CLL subgroups.

Increased basal Ca^{2+} signaling levels have previously been described in B cells that are in an anergic state[24]. These cells are capable of antigen binding, but are reduced in their ability to signal through their BCR and thus to respond to antigenic stimulation. This is a strategy of the immune system for silencing autoreactive B cells[24]. A constant occupancy of a proportion of BCRs by self-antigen is required for the maintenance of this increased basal signaling[24, 25]. In several publications it has indeed been described that CLL cells are in an anergic state, as indicated by low surface BCR expression and a reduced capability to respond to BCR stimulation[20, 26, 27]. Hence, the observed increased basal signaling in primary CLL might be triggered by constant or repetitive antigenic stimulation of the BCRs and thereby lead to an anergic state. The higher basal signaling that we observed in M-CLL might thus be due to a more efficient route to anergy for M-CLL than for U-CLL, as earlier described by Mockridge et al[20]. They showed that anergy is characterized by a reduced surface IgM signaling capacity, and additionally found that it can be recovered *in vitro* both at the level of surface expression of IgM and signaling capacity[20]. Furthermore, these authors also showed that U-CLL and M-CLL differ in their capacity to respond to IgM stimulation. The latter is in line with our results showing a small but significant increase in response to IgM in U-CLL but not in M-CLL.

The anergic state might thus explain the high level of basal Ca^{2+} signaling seen in the M-CLL cases, and could be induced by antigen-independent recognition of the FR2 or FR3 motif or other unknown intrinsic epitopes, or alternatively by continuous or repetitive occupation of the BCR by a specific antigen. Importantly, we did not find a significant association between FR2 and FR3 amino acid sequences and the level of basal Ca^{2+} signaling. Nevertheless, it is conceivable that the somewhat higher or lower basal signaling observed in some IGHV genes may reflect their occurrence in M-CLL or U-CLL subgroups, respectively. Increased M-CLL BCR affinity for its antigen, as it is induced by SHM, might explain the higher state of anergy in these cells. The U-CLL subsets that carry a heterogeneous or a stereotypic BCR both showed lower basal Ca^{2+} signaling than M-CLLs. This could be explained by the low affinity binding of these unmutated BCRs to their antigen, as most of these are poly- or autoreactive[5]. An additional explanation could be that the U-CLLs actually recognize their cognate antigen within the lymphoid tissues. The U-CLL cells that migrate to peripheral blood will not see their antigen and as a consequence regain their signaling capacity. When this would be accompanied by parallel re-expression of chemokine receptors, this could eventually lead to relocalization to proliferation centers and induction of the more aggressive behavior of U-CLLs.

Several reports have demonstrated that BCR signaling pathways are altered in CLL. For instance, LYN and SYK kinases, are overexpressed and constitutively phosphorylated[28, 29].

SYK expression[29] and phosphorylation[30] are found to correlate with prognosis, being higher in U-CLL than in M-CLL[29, 30]. BTK expression levels are also increased in CLL[31], and even though BTK expression cannot be correlated with prognosis[31], it is now a novel therapeutic target in clinical trials[32]. Furthermore, increased ZAP70 expression is associated with U-CLL and a more aggressive form of the disease[33]. Further downstream in the BCR signaling cascade differences are found in ERK, NF-κB and NFAT activation between M-CLL and U-CLL[27, 30]. Constitutive ERK phosphorylation was found to be associated with NFAT translocation to the nucleus and a higher anergic state[27]. NF-κB activation was found to be higher in U-CLL compared with M-CLL[30]. This factor is associated with increased survival in CLL cells[34]. So it seems likely that downstream BCR signaling is different between U-CLL and M-CLL, and therefore could be associated with the more aggressive behavior of U-CLL and the milder form as induced by the higher anergic state in M-CLL. Thus, it cannot formally be excluded that the observed differences in basal Ca^{2+} signaling between U-CLL and M-CLL are not related to BCR composition or antigenic stimulation, but instead result from differences in BCR downstream signaling pathways between U-CLL and M-CLL.

In summary, we demonstrate here that primary CLL cells show increased basal Ca^{2+} signaling levels compared with PB B cells of healthy controls. However, Ca^{2+} levels differed between CLL samples, with M-CLL showing significantly increased Ca^{2+} levels over U-CLL. We therefore conclude that the level of basal Ca^{2+} signaling is not uniformly enhanced in CLL B cells, but is associated with the CLL IGHV mutational status, reflecting a distinct cellular origin and possibly a different anergic state induced by repetitive or continuous antigen-binding *in vivo*.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

REFERENCES

1. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. & Thun, M.J. Cancer statistics, 2009. *CA Cancer J Clin* **59**, 225-249 (2009).
2. Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L. *et al.* Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* **94**, 1840-1847 (1999).
3. Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94**, 1848-1854 (1999).
4. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* **119**, 4467-4475 (2012).
5. Herve, M., Xu, K., Ng, Y.S., Wardemann, H., Albesiano, E., Messmer, B.T. *et al.* Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* **115**, 1636-1643 (2005).
6. Catera, R., Silverman, G.J., Hatzi, K., Seiler, T., Didier, S., Zhang, L. *et al.* Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol Med* **14**, 665-674 (2008).
7. Chu, C.C., Catera, R., Zhang, L., Didier, S., Agagnina, B.M., Damle, R.N. *et al.* Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin. *Blood* **115**, 3907-3915 (2010).
8. Myhrinder, A.L., Hellqvist, E., Sidorova, E., Soderberg, A., Baxendale, H., Dahle, C. *et al.* A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood* **111**, 3838-3848 (2008).
9. Zwick, C., Fadle, N., Regitz, E., Kemele, M., Stilgenbauer, S., Buhler, A. *et al.* Autoantigenic targets of B-cell receptors derived from chronic lymphocytic leukemias bind to and induce proliferation of leukemic cells. *Blood* **121**, 4708-4717 (2013).
10. Ghia, E.M., Widhopf, G.F., 2nd, Rassenti, L.Z. & Kipps, T.J. Analyses of recombinant stereotypic IGHV3-21-encoded antibodies expressed in chronic lymphocytic leukemia. *J Immunol* **186**, 6338-6344 (2011).
11. Hoogeboom, R., Wormhoudt, T.A., Schipperus, M.R., Langerak, A.W., Dunn-Walters, D.K., Guikema, J.E. *et al.* A novel chronic lymphocytic leukemia subset expressing mutated IGHV3-7-encoded rheumatoid factor B-cell receptors that are functionally proficient. *Leukemia* **27**, 738-740 (2013).
12. Kostareli, E., Gounari, M., Janus, A., Murray, F., Brochet, X., Giudicelli, V. *et al.* Antigen receptor stereotypy across B-cell lymphoproliferations: the case of IGHV4-59/IGKV3-20 receptors with rheumatoid factor activity. *Leukemia* **26**, 1127-1131 (2012).
13. Hoogeboom, R., van Kessel, K.P., Hochstenbach, F., Wormhoudt, T.A., Reinten, R.J., Wagner, K. *et al.* A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med* **210**, 59-70 (2013).
14. Duhren-von Minden, M., Ubelhart, R., Schneider, D., Wossning, T., Bach, M.P., Buchner, M. *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* **489**, 309-312 (2012).

15. Binder, M., Muller, F., Frick, M., Wehr, C., Simon, F., Leistler, B. *et al.* CLL B-cell receptors can recognize themselves: alternative epitopes and structural clues for autostimulatory mechanisms in CLL. *Blood* **121**, 239-241 (2013).
16. van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L. *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* **17**, 2257-2317 (2003).
17. Langerak, A.W., Davi, F., Ghia, P., Hadzidimitriou, A., Murray, F., Potter, K.N. *et al.* Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. *Leukemia* **25**, 979-984 (2011).
18. Thompson, J.D., Higgins, D.G. & Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680 (1994).
19. Chen, X., Hale, G.A., Neale, G.A., Knowles, J., Barfield, R.C., Wang, Y.D. *et al.* A novel approach for the analysis of T-cell reconstitution by using a T-cell receptor beta-based oligonucleotide microarray in hematopoietic stem cell transplantation. *Exp Hematol* **35**, 831-841 (2007).
20. Mockridge, C.I., Potter, K.N., Wheatley, I., Neville, L.A., Packham, G. & Stevenson, F.K. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood* **109**, 4424-4431 (2007).
21. Haferlach, C., Dicker, F., Schnittger, S., Kern, W. & Haferlach, T. Comprehensive genetic characterization of CLL: a study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgV(H) status and immunophenotyping. *Leukemia* **21**, 2442-2451 (2007).
22. Wardemann, H., Yurasov, S., Schaefer, A., Young, J.W., Meffre, E. & Nussenzweig, M.C. Predominant autoantibody production by early human B cell precursors. *Science* **301**, 1374-1377 (2003).
23. Sutton, L.A., Kostareli, E., Hadzidimitriou, A., Darzentas, N., Tsaftaris, A., Anagnostopoulos, A. *et al.* Extensive intraclonal diversification in a subgroup of chronic lymphocytic leukemia patients with stereotyped IGHV4-34 receptors: implications for ongoing interactions with antigen. *Blood* **114**, 4460-4468 (2009).
24. Gauld, S.B., Benschop, R.J., Merrell, K.T. & Cambier, J.C. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* **6**, 1160-1167 (2005).
25. Zikherman, J., Parameswaran, R. & Weiss, A. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* **489**, 160-164 (2012).
26. Muzio, M., Apollonio, B., Scielzo, C., Frenquelli, M., Vandoni, I., Boussiotis, V. *et al.* Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood* **112**, 188-195 (2008).
27. Apollonio, B., Scielzo, C., Bertilaccio, M.T., Ten Hacken, E., Scarfo, L., Ranghetti, P. *et al.* Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood* **121**, 3879-3888, S3871-3878 (2013).
28. Contri, A., Brunati, A.M., Trentin, L., Cabrelle, A., Miorin, M., Cesaro, L. *et al.* Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest* **115**, 369-378 (2005).

29. Buchner, M., Fuchs, S., Prinz, G., Pfeifer, D., Bartholome, K., Burger, M. *et al.* Spleen tyrosine kinase is overexpressed and represents a potential therapeutic target in chronic lymphocytic leukemia. *Cancer Res* **69**, 5424-5432 (2009).
30. Cesano, A., Perbellini, O., Evensen, E., Chu, C.C., Cioffi, F., Ptacek, J. *et al.* Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica* **98**, 626-634 (2013).
31. Herman, S.E., Gordon, A.L., Hertlein, E., Ramanunni, A., Zhang, X., Jaglowski, S. *et al.* Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* **117**, 6287-6296 (2011).
32. Byrd, J.C., Furman, R.R., Coutre, S.E., Flinn, I.W., Burger, J.A., Blum, K.A. *et al.* Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* **369**, 32-42 (2013).
33. Rassenti, L.Z., Huynh, L., Toy, T.L., Chen, L., Keating, M.J., Gribben, J.G. *et al.* ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* **351**, 893-901 (2004).
34. Coscia, M., Pantaleoni, F., Riganti, C., Vitale, C., Rigoni, M., Peola, S. *et al.* IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. *Leukemia* **25**, 828-837 (2011).

SUPPLEMENTARY FILES

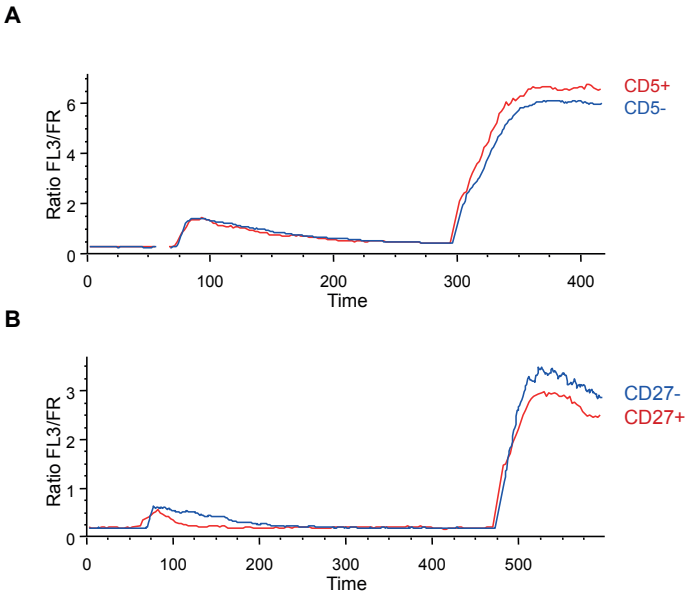

Figure S1. Ca^{2+} flux assays in different normal human B cell fractions.

Representative kinetic plots of $CD5^+$ vs. $CD5^-$ B cells (**A**) and $CD27^+$ vs. $CD27^-$ B cells (**B**) from healthy controls. BCR stimulation was performed after 60s, and stimulation with ionomycin after 480s (indicated with arrows). The duration of the total Ca^{2+} flux assays was 600s.

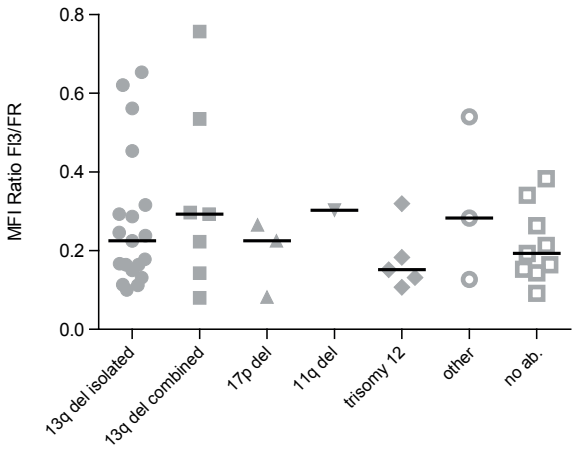

Figure S2. Correlation of basal Ca^{2+} signaling with cytogenetic aberrations.

No significant association with well-known cytogenetic aberrations was established, as determined with the Kruskal Wallis test. Comparisons between 2 groups were performed with the Mann-Whitney U test.

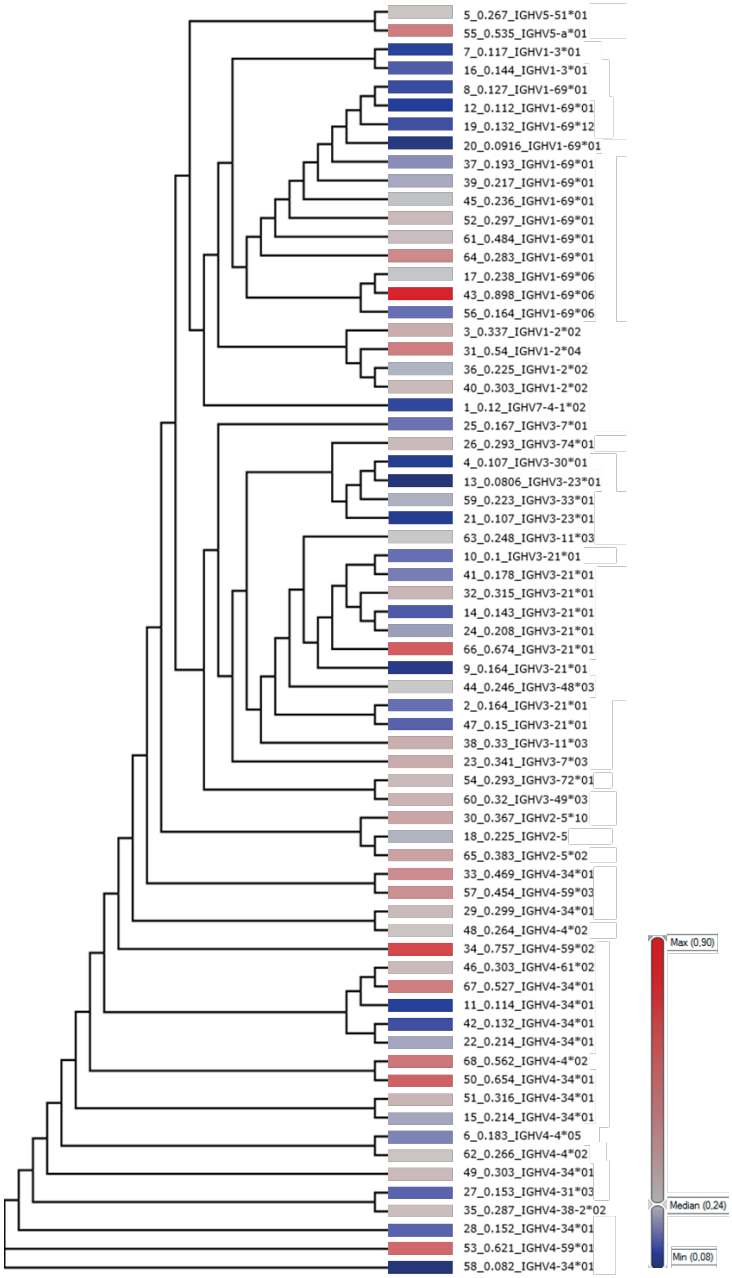

Figure S3.

Phylogenetic analysis of FR3 amino acid sequences from the total group of CLL (n=68) combined with heat map analysis of the MFI of basal Ca^{2+} signaling levels depicted in color code, ranging from red (high), via gray (median) to blue (low).

Chapter 4

Responsiveness of Chronic Lymphocytic Leukemia cells to B cell receptor stimulation is associated with low expression of regulatory molecules of the Nuclear Factor-κB pathway

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[#] RWH and AWL share equal responsibility and share senior authorship

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ABSTRACT

Chronic lymphocytic leukemia is a heterogeneous disease based on clinical and biological characteristics. Differences in Ca^{2+}-levels among cases, both basal and upon B-cell receptor (BCR) stimulation may reflect heterogeneity in the pathogenesis due to cell-intrinsic factors. Our aim is to elucidate cell-intrinsic differences between BCR responsive and unresponsive cases.

Therefore the BCR responsiveness was determined *ex vivo* based on Ca^{2+}-influx upon α-IgM stimulation of purified CLL cell fractions of 52 patients. Phosphorylation levels of various B-cell receptor signaling molecules, and expression of activation markers were assessed by flow cytometry. Transcription profiling of responsive (n=6) and unresponsive cases (n=6) was performed by RNA sequencing. RQ-PCR was used to validate transcript level differences in a larger cohort.

In 24 cases an α-IgM response was visible by Ca^{2+}-influx which was accompanied by higher phosphorylation of PLCγ2 and Akt after α-IgM stimulation in combination with a higher surface expression of IgM, IgD, CD19, CD38 and CD43 compared to the unresponsive cases (n=28). Based on RNA sequencing analysis several components of the canonical NF-κB pathway especially related to NF-κB inhibition were higher expressed in unresponsive cases. Moreover, upon α-IgM stimulation, the expression of these NF-κB pathway genes (especially genes coding for NF-κB pathway inhibitors but also NF-κB subunit REL) are upregulated in B-cell receptor responsive cases while for the unresponsive cases the level did not change compared to basal levels.

These findings suggest cells from chronic lymphocytic leukemia cases with an enhanced NF-κB signaling are associated with a lower responsive capacity towards B-cell receptor stimulation.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy that is characterized by a monoclonal expansion of mature B cells with a homogeneous morphology and a characteristic immunophenotype. [1] CLL is the most common type of leukemia in the Western world and affects mainly the elderly. [1] Based on the somatic hypermutation (SHM) status of the immunoglobulin heavy chain (*IGHV*) gene, CLL can be divided in unmutated CLL (U-CLL) and mutated CLL (M-CLL), with U-CLL generally having a more aggressive form of the disease and M-CLL a more indolent form. [2, 3] Around 30% of all cases can be grouped into subsets based on so-called stereotypic B-cell receptors (BCRs), which are identified by their restricted IGHV/IGHD/IGHJ gene usage plus similarities in length and amino acid sequence of their complementarity-determining region 3 (CDR3). [4]

The BCR stereotypy would be indicative for the involvement of similar specific antigens and underlines the importance of antigenic stimulation and BCR specificity in the pathogenesis. [4] In general, most U-CLLs express a BCR that is polyreactive and recognizes self- and non-self-antigens with low-affinity binding. [5, 6, 7, 8] In addition, for some stereotypic CLL subsets the antigens recognized by their BCRs have been identified. [9, 10, 11, 12, 13]

However, it was previously also shown that the BCR from CLL cells could be stimulated independently of external antigens, as the CDR3 regions are able to recognize an internal epitope in framework 2 (FR2) of the IGHV domain. [14] This induces a higher level of antigen-independent autonomous BCR signaling, since these cells exhibit a higher Ca^{2+} level in their cytoplasm as demonstrated *in vitro* using a triple knock out (TKO) cell system. [14]

We previously demonstrated that primary CLL cells generally have higher basal Ca^{2+} levels compared with peripheral B cells from healthy individuals (HI). [15] Basal Ca^{2+} levels correlated with the IGHV mutational status, as we found on average higher basal Ca^{2+} levels in M-CLL than in U-CLL. [14, 15] However, our data also showed large variation within the subgroups, as cases with high and low basal Ca^{2+} levels could be found in both M-CLL and U-CLL groups. [15] Since there was no correlation with BCR characteristics (i.e. Ig expression level, HCDR3 length, charge and composition) or with cytogenetic aberrations, it is conceivable that high basal Ca^{2+} levels are partly directed by the SHM status and that cell-intrinsic differences caused by cell anergy can explain the variation. [15]

Anergy is an immune state in which the cell is silenced upon low-affinity recognition of self-antigens. [16] Anergy has been linked to CLL based on low surface BCR expression, their reduced responsive capability, [17, 18] and their increased basal Ca^{2+} levels. [15] Especially M-CLL shows this increased basal Ca^{2+} levels in combination with a poorer response to BCR stimulation [15] which is in line with other studies showing that the α-IgM response is associated with the IGHV mutational status and with the surface expression of progression markers, like

CD38. [18, 19] Moreover, high surface IgM is associated with a clinically aggressive form of the disease, which has potential impact as diagnostic parameter for disease progression. [20]

However, Ca^{2+} levels, both basal and upon BCR stimulation vary within the U-CLL and M-CLL groups. We therefore hypothesize that this heterogeneity in BCR responsiveness could reflect a diverse disease pathogenesis involving cell-intrinsic differences. Here, we aim to elucidate potential cell-intrinsic differences underlying the observed differences in Ca^{2+} levels between CLL cases.

METHODS

Study population

Fifty-two patients were included of which 30 patients (58%) are U-CLL and 22 patients (42%) are M-CLL as determined by the IGHV SHM status (Supplementary Methods). All patient characteristics are shown in Supplementary Table 1. The majority of the included patients (n=41, 79%) were treatment-naive. Purified CLL cells were isolated (Supplementary Methods) upon informed consent and anonymized for further use, following the guidelines of the institutional review board (METC-2015-741) and in accordance with the declarations of Helsinki.

Flow cytometry

Flow cytometry was used to assess the responsive capacity upon α-IgM stimulation by measuring Ca^{2+} levels (Supplementary Methods) and to determine the expression of activation markers by using antibodies listed in Supplementary Table 2.

Phospho-flow analysis was done to study the phosphorylation of Spleen tyrosine kinase (Syk) and Phopholipase Cγ2 (PLCγ2) and Protein kinase B (Akt) upon α-IgM stimulation. (Supplementary Methods).

Cell culture and retroviral transduction of TKO cells

Triple knockout (TKO) cells, derived from a signaling-competent mouse pre-B cell line lacking the expression of endogenous pre-BCRs due to inactivation of *RAG2* and *λ5* genes (21), and Phoenix cells (ATCC CRL-3214) were both cultured as described by Meixlsperger et al. [21] The protocol used for the transduction of TKO cells was also documented before by Meixlsperger et al. [21]

RNA sequencing

Twelve cases from our cohort were selected based on their responsiveness upon α-IgM stimulation (6 responsive, 6 unresponsive) and RNA was sequenced. The RNA was

extracted using Allprep DNA/RNA/miRNA Universal (QIAGEN, Hilden, Germany) according to manufacturer's instructions. RNA sequencing was performed on a TruSeq (Illumina, San Diego, CA, USA) platform at the Human Genome Facility (HuGeF), Department of Internal Medicine (Erasmus MC, Rotterdam). Reads were extracted from the raw sequencing data using CASAVA 1.8.2 (Illumina) and aligned to the human reference genome (UCSC's hg19) using the STAR (2.5.0c) splice aware aligner using gencode v19 transcriptome annotations as additional template. The BAM files were processed using various tools from the picard software suite (v1.90), as well as tools from the Genome Analysis ToolKit (GATK, v3.5). QC metrics were collected at various steps using picard and evaluated, along with coverage metrics using GATK. Read counts per exon/gene were then determined by the featureCounts function of the subread package (v1.4.6-p1) using the gencode v19 annotation as markers. The raw read counts were normalized through the FPKM methodology, normalizing for library yield and gene size.

For classification analysis, the calculated Spearman correlation as a distance (1/similarity) measurement and Ward.D2 for the unsupervised clustering was applied to the used samples. The R (version 3.4.4) packages were used for the differential expression analysis and to create plots for visualization. We analyzed the samples fitting, with edgeR, the gene wise negative binomial generalized linear models for contrast.

To validate transcript level differences in a larger cohort, RNA was synthesized to cDNA and RQ-PCR was performed (Supplementary Methods and Supplementary Table 2).

RESULTS

U-CLL cases are generally more responsive to α-IgM stimulation than M-CLL cases

To determine whether high basal Ca^{2+} levels are BCR dependent or caused by cell-intrinsic factors, we selected a small series of CLL samples with known high (n=3) or low (n=6) basal Ca^{2+} levels from our previous study of 2015 [15], and cloned their BCR into TKO cells as described by Dühren-von Minden et al. [14]

Even though we could detect Ca^{2+} signaling by the BCR in TKO cells for all analyzed CLL-derived BCR expressed as IgM, we could not detect any correlation (R^2=0.014, p=0.764) between the Ca^{2+} signal in CLL and that in TKO cells (**Figure 1B**) indicating that high basal Ca^{2+} levels as seen in some CLL samples would result from cell-intrinsic changes rather than from BCR-dependent autonomous signaling.

To determine which cell-intrinsic differences might cause the heterogeneity in Ca^{2+} signaling at basal level and upon BCR stimulation, we established a new cohort of patients (n=52, Supplementary Table 1). CLL cells were isolated from peripheral blood and immediately

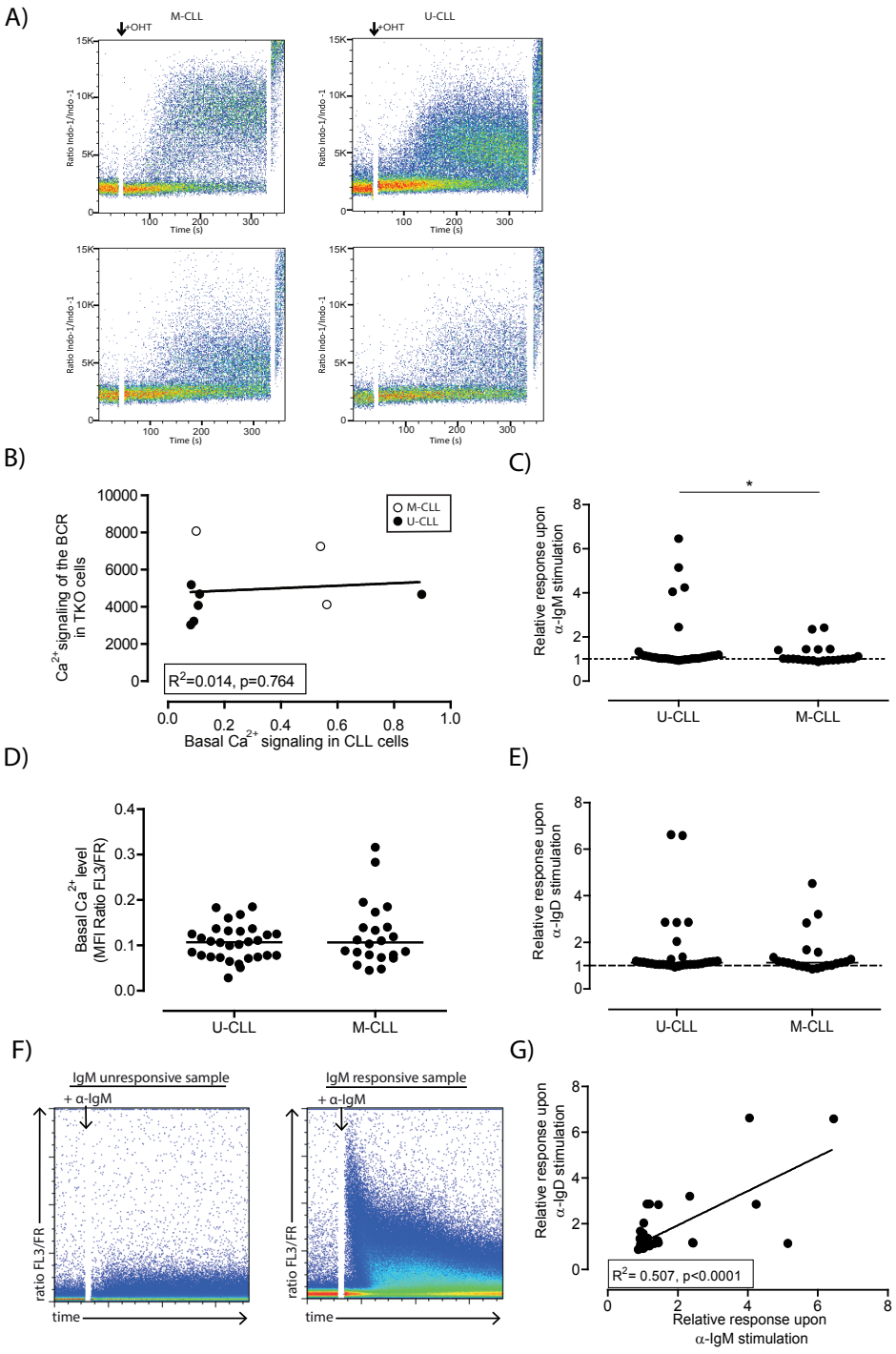
A)
M-CLL
U-CLL
+OHT
Ratio Indo-1/Indo-1
Time (s)
B)
M-CLL
U-CLL
Ca^{2+} signaling of the BCR in TKO cells
R^2=0.014, p=0.764
Basal Ca^{2+} signaling in CLL cells
C)
*
Relative response upon α-IgM stimulation
U-CLL
M-CLL
D)
Basal Ca^{2+} level (MFI Ratio FL3/FR)
U-CLL
M-CLL
E)
Relative response upon α-IgD stimulation
U-CLL
M-CLL
F)
IgM unresponsive sample
IgM responsive sample
+ α-IgM
ratio FL3/FR
time
G)
Relative response upon α-IgD stimulation
R^2= 0.507, p<0.0001
Relative response upon α-IgM stimulation

Figure 1. Ca^{2+} signaling in CLL cells

A. Flowcytometric analysis of Ca^{2+} flux (ratio Indo-1/Indo-1) after the addition of 4-hydroxytamoxifen (4-OHT) to TKO cells expressing the BCR from two representative M-CLL (left) and two U-CLL (right) samples. **B.** From 9 CLL cases (6 U-CLL, black dots and 3 M-CLL, open dots) of which the basal Ca^{2+} level (x-axis) was assessed earlier, the BCR was cloned into TKO cells to determine the autonomous Ca^{2+} signal (y-axis). Linear regression was performed and the R^2 and p-value is shown. **C**. Basal Ca^{2+} level (MFI ratio FI3/FR) was determined in a new cohort of 52 CLL samples (freshly isolated) consisting of 30 U-CLL and 22 M-CLL cases. **D.** Responsive capacity upon BCR stimulation. Flowcytometric analysis of a representative CLL sample showing no Ca^{2+} influx (ratio FL3/FR) upon α-IgM stimulation (left) and a representative CLL sample with an increase in Ca^{2+} influx (ratio FL3/FR) upon α-IgM stimulation (right). Based on this analysis the responsive capacity upon α-IgM stimulation (**E**) and α-IgD stimulation (**F**) was determined in the 30 U-CLL and 22 M-CLL cases. Individual plots and medians (grey bars) are shown. Mann-Whitney U-test was performed for statistical analysis between the groups of patients (* = $p<0.05$). **G**. Linear regression analysis between the relative response after α-IgM and α-IgD stimulation and both R^2 and p values are shown.

used for further analysis. First, basal Ca^{2+} levels were assessed (**Figure 1B**). Similar to the previous study, basal Ca^{2+} levels were heterogeneous for both U-CLL and M-CLL. [15]

Next, we examined the responsive capacity of the CLL samples upon BCR stimulation. In **Figure 1D**, two flowcytometric examples are provided. In line with our previous study [15], we found that U-CLL cells in general respond significantly ($p=0.049$) better upon α-IgM stimulation compared with M-CLL cells (**Figure 1E**). Although no differences were found in the response after α-IgD stimulation (**Figure 1F**), a strong correlation existed between the relative response towards α-IgM and α-IgD stimulation (**Figure 1G**; $R^2=0.508$, $p<0.0001$). Based on this, we further define CLL subgroups based on BCR responsiveness upon α-IgM stimulation. Twenty-four cases were classified as responsive (MFI ratio response/basal signal: 1.1-6.5; n=17 U-CLL and n=7 M-CLL) and 28 cases were unresponsive (MFI ratio response/basal signal: <1.1; n=13 U-CLL and n=15 M-CLL).

Higher phosphorylation of PLCγ2 and Akt in CLL correlated with responsiveness upon BCR stimulation

In order to better understand BCR responsive capacity, as defined by Ca^{2+}-influx, we examined phosphorylation of the Syk, PLCγ2 and Akt upon α-IgM stimulation. First, we evaluated differences basal phosphorylation levels of Syk (pSyk), PLCγ2 (pPLCγ2) and Akt (pAkt) (**Figure 2A).** The responsive cases showed a significantly ($p=0.0013$) higher basal pPLCγ2 level than unresponsive cases but no differences were found in basal pSyk and pAKT levels (**Figure 2B**). Next we examined the relative response of kinase phosphorylation upon BCR stimulation. Even though no difference in relative response of pSYK after α-IgM stimulation was found, the responsive patients had a higher relative response of pPLCγ2 and pAkt upon α-IgM stimulation (**Figure 2C**).

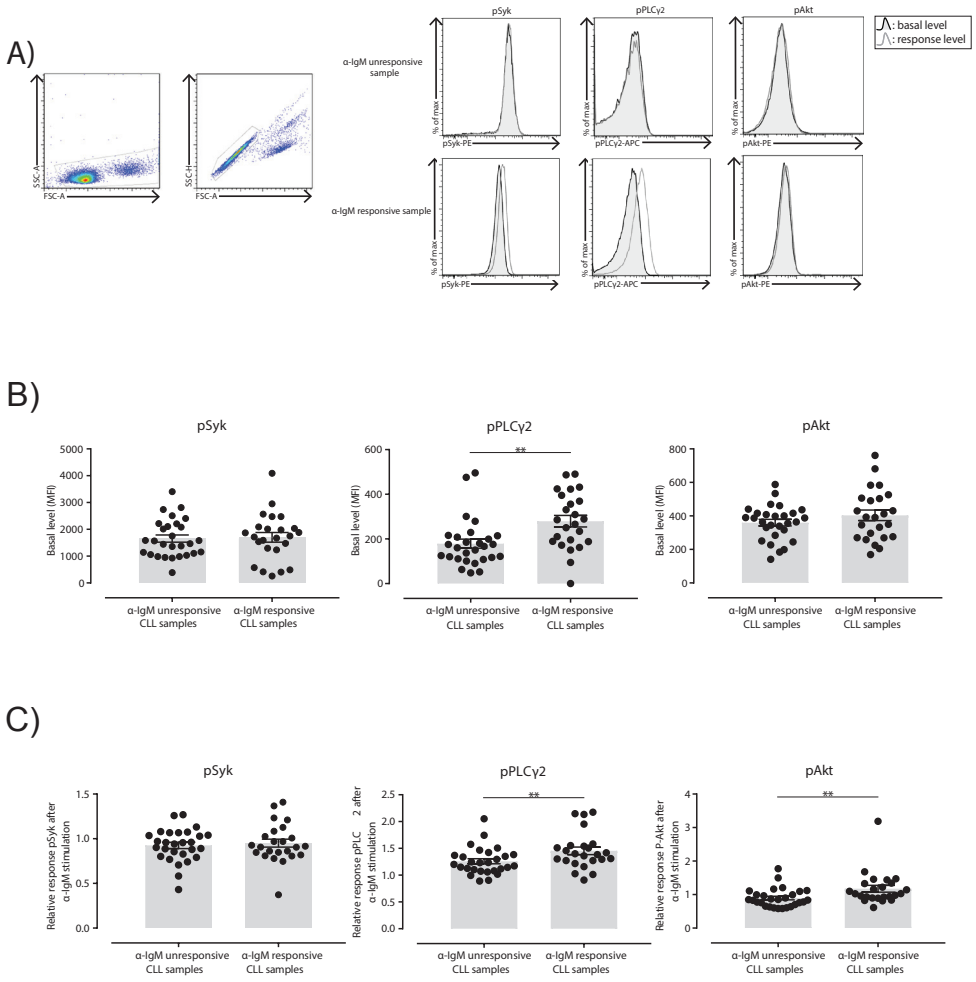

Figure 2. Phosphorylation of Syk, PLCγ2 and Akt.

A. The level of pSyk, pPLCγ2 and pAkt was determined at basal level and upon stimulation with α-IgM and correlated with the α-IgM response as determined by Ca^{2+} signaling. Representative examples of the analysis in an unresponsive CLL case (upper histograms) and a responsive CLL case (lower histograms) are shown. After single viable cell selection, the level of pSyk, pPLCγ2 and pAkt was determined, both as basal level (black line) and upon stimulation with α-IgM (grey line). **B.** Differences in basal level of pSYK, pPLCγ2 and pAKT (x-axis) between α-IgM unresponsive and responsive samples. **C.** The relative response after α-IgM stimulation for pSYK, pPLCγ2 and pAKT (x-axis) in the two patient groups. Individual plots and medians (grey bars) are shown. Mann-Whitney U-test was performed for statistical analysis between the groups of patients (** = $p<0.01$).

Taken together, the α-IgM response as determined by Ca^{2+}-influx is consistent with a higher phosphorylation of pPLCγ2 and pAkt upon α-IgM stimulation

CLL cases showing good BCR responsiveness have a more activated phenotype

Next we examined whether the expression of activation markers associates with the response to α-IgM. As expected, CLL cells from responsive cases displayed a significantly (p=0.0002) higher expression of surface IgM compared to the unresponsive cases, IgD (p=0.036), CD19 (p=0.029), CD38 (p=0.035), and CD43 (p=0.047) expression levels were also higher compared to unresponsive cases (**Figure 3A**). No differences were found in CD20, CD21, CD27, CD69, CD80, CD86 and CXCR4 expression. (**Supplementary Figure S1**).

To determine if the α-IgM responsiveness within the responsive cases correlates with the expression level of these markers, we compared surface expression and relative response. The relative response did correlate with surface IgM (R^2=0.322, p=0.0038) and CD21 (R^2=0.469, p=0.0002) expression levels (**Figure 3B**).

Particularly IκB-related genes are differentially expressed between BCR responsive and unresponsive cases

Twelve cases from our cohort were selected to evaluate cell-intrinsic differences based on RNA sequencing of total RNA from MACS-purified (>95%) CLL cells. Six patients were classified based on Ca^{2+} levels as responders upon α-IgM stimulation and were compared to another 6 patients who were unresponsive. (Supplementary Table 3) First, RNA expression profiles of the 12 cases were compared to each other via Spearman's correlation (**Figure 4A**). Based on these results the patients could be divided into three major clusters, which did not correlate with BCR responsiveness (blue label) or SHM status (green label). In addition, when comparing the variation in total gene expression levels between the samples, as shown by Z-scores in a heat map (**Supplementary Figure S2**), no clear division of responsive and unresponsive cases was found either, probably reflecting the biological heterogeneity of CLL samples, even when classified as BCR responsive and unresponsive.

Next, we therefore focused on genes involved in BCR signaling using QIAGEN's Ingenuity Pathway Analysis (IPA). As illustrated by the volcano plot, responsive cases demonstrated a significantly higher *EBF1, FCGR2A, SYK* and *FYN* expression (positive logFC values), whereas the non-responders showed a significantly higher N*FKBID, NFKB2, CAM2KA, NFKBIE, RAF1, NFKBIB, NFKB1, RPS6K1, PLCG1* and *BCL3* expression (negative logFC values) (**Figure 4B** and **Supplementary Figure S2**). Interestingly, the *NFKBIB, NFKBID* and *NFKBIE* genes are all encoding inhibitors of NF-κB (IκBs), while *NFKB1, NFKB2* and *BCL3* are genes coding for NF-κB components that are associated with inhibition. [22]

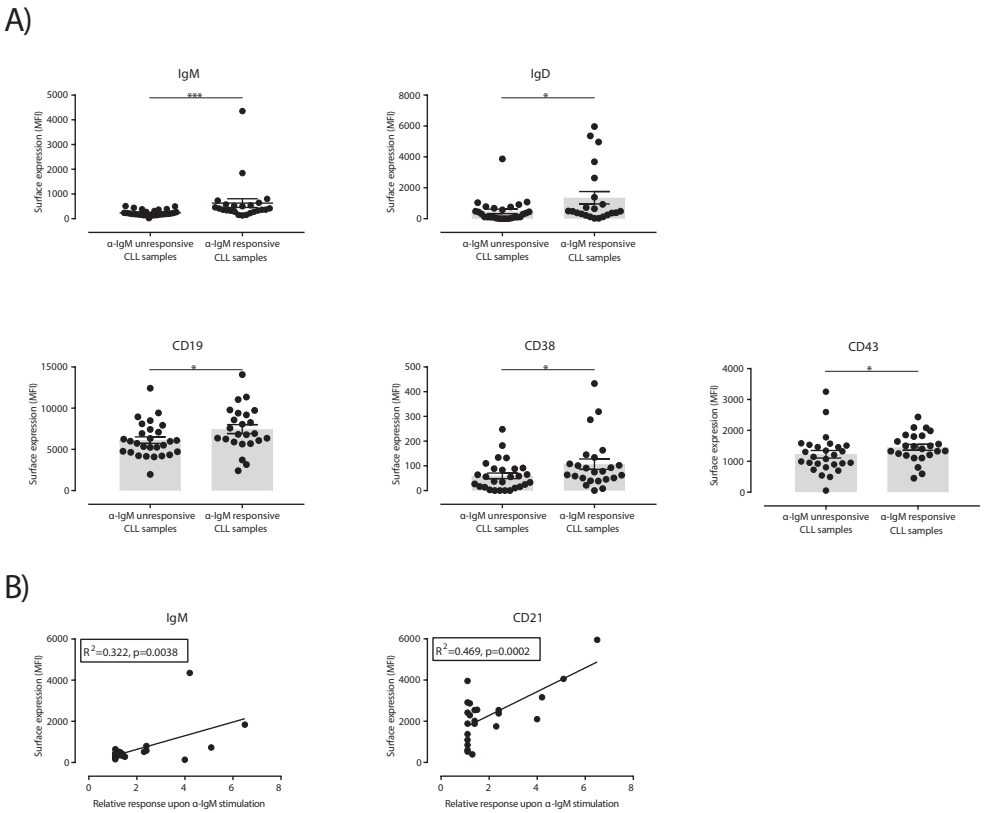

Figure 3. Surface expression of activation-associated markers.

The surface expression of several activation-associated markers were measured in 52 patients with CLL.

A. Surface expression level (X-axis) of IgM, IgD, CD19, CD38 and CD43 in α-IgM unresponsive and α-IgM responsive cases. Individual plots and medians (grey bars) are shown. Mann-Whitney U-test was performed for statistical analysis between the groups of patients (* = $p<0.05$). **B**. Relative response upon α-IgM stimulation (x-axis) is plotted against the surface expression (Median Fluorescence Intensity (MFI)) expression level of IgM and CD21. Linear regression analysis was performed and R^2 and p values are shown.

BCR unresponsive CLL have a higher expression of genes expressing regulatory molecules of NF-κB signaling

Additional samples were selected (n=13 unresponsive, n=15 responsive) to validate the differences in transcript levels NF-κB genes (*NFKB1, NFKB2, BCL3, NFKBIB, NFKBID* and *NFKBIE*) using RQ-PCR. RQ-PCR results (displayed as $2^{-deltaCT}$ values) indeed confirmed that responding cases have a significantly lower expression of *NFKB1* and *NFKB2* (**Figure 5A**) *NFKBIB* and *NFKBIE* (**Figure 5B**). Furthermore, we found a trend towards a lower *NFKBID* expression, but no difference in *BCL3* expression between the subgroups (**Figure 5B**).

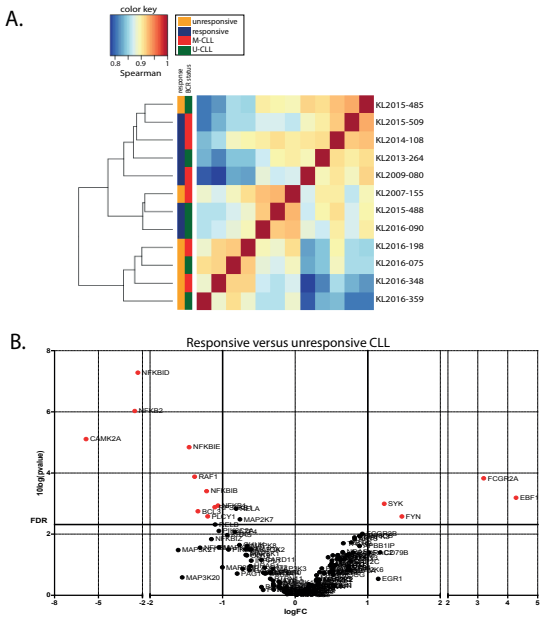

Figure 4. Differential expression analysis based on RNA sequencing data.

A. Results of the Spearman correlation of the RNA expression analysis in different CLL samples (n=6 responsive vs. n=6 unresponsive CLL cases). The color scale indicates the degree of correlation varying from blue (low correlation) to red (high correlation). The two panels at the left end indicate the responsiveness (orange=unresponsive, blue=-responsive) and the IGHV SHM status (red=M-CLL, green=U-CLL) of the selected CLL cases. **B**. Volcano plot showing differences in transcript levels of genes involved in the BCR signaling pathway as determined using QIAGEN's Ingenuity Pathway Analysis (IPA). A negative logFC value indicates a higher expression of certain genes in unresponsive CLL cases, while a positive logFC value is indicative of a higher expression in responsive CLL cases. The logFC value was plotted against the 10log(pvalue). False Discovery Rate (FDR) was calculated and transcriptional differences of genes with a logFC value of 1 or -1 in combination with a 10log(pvalue) above the FDR are indicated in red.

In addition, we further examined if the transcriptional levels of these NF-κB pathway genes also correlated with basal Ca^{2+} levels (**Supplementary Figure S3**). A significant correlation could only be found between basal Ca^{2+} levels and *NFKB1* (R^2=0.163, p=0.033) and *NFKBIE* (R^2=0.234, p=0.0091) transcript levels (**Supplementary Figure S3**).

Since loss of IκBε (encoded by *NFKBIE* as caused by an identical 4-bp frameshift deletion in the first exon), has been associated with a progressive form of CLL [23], we examined if patients in our cohort with a low *NFKBIE* expression would carry this identical deletion. Upon sequencing of the first exon of *NFKBIE*, this 4-bp deletion was not observed (data not shown).

Expression levels of genes coding for NF-κB regulators (*NFKB1* and *NFKB2*) and coding for IκBs that were lower expressed in responsive cases appeared to correlate with each other (**Supplementary Figure S4**), implying that unresponsive patients show higher expression of

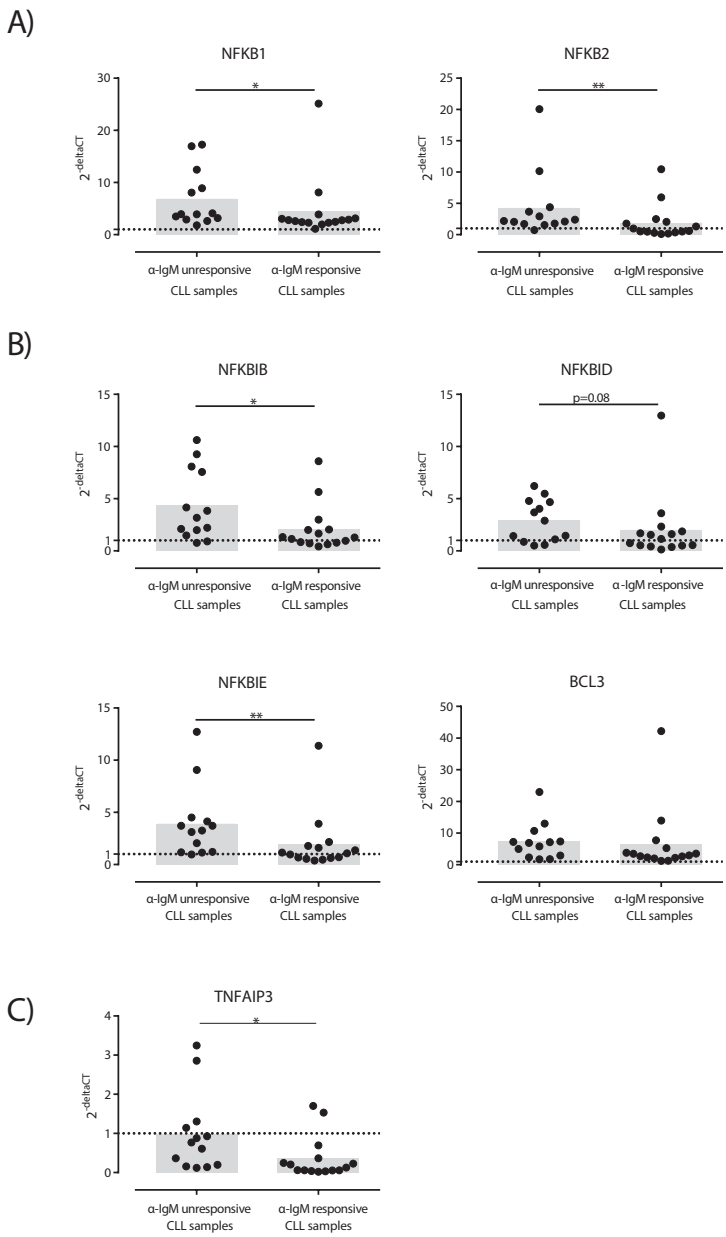

Figure 5. Validation of transcriptional differences of NF-κB related genes

RQ-PCR validation of *NFKB1*, *NFKB2* (A) *NFKBIB*, *NFKBID*, *NFKBIE, BCL3* (B) and *TNFAIP3* (C) expression in an extended cohort of unresponsive (n=13) and responsive (n=15) CLL cases. $2^{-deltaCT}$ values were determined for each sample and individual data plots and the median are shown. Comparison between the two groups was done using the Mann-Whitney U-test (* = p<0.05, ** = p<0.01).

multiple NF-κB inhibitors. Even though we could not detect statistically significant differences in expression levels of genes coding for the NF-κB subunits *RELA, RELB* and *REL* between the two subgroups (data not shown), we did observe clear correlations between expression levels of genes associated with NF-κB inhibition and expression levels of *RELA* and *REL* (**Supplementary Figure S5**), both involved in the canonical NF-κB. No correlations between inhibitor levels and levels of the non-canonical NF-κB subunit *RELB* were found (data not shown).

Besides the IκBs genes, we also found a difference in expression of tumor necrosis factor-α induced protein 3 (*TNFAIP3*; logFC=-1.70, 10log(Pvalue)=2.24) based on RNA sequencing analysis. *TNFAIP3* encodes for protein A20 that is induced by TNF-α and functions as a negative regulator through inhibition of NF-κB signaling. [24] In addition, RQ-PCR showed a significantly (**Figure 5C**; p=0.017) higher expression of *TNFAIP3* in unresponsive cases compared to responsive patients.

Collectively, these results illustrate that unresponsive cases have higher basal gene expression of several regulatory molecules of canonical NF-κB pathway signaling.

Upregulation of NF-κB pathway genes upon α-IgM stimulation in BCR responsive cases.

To further study expression of the NF-κB genes upon stimulation, frozen PBMCs of 21 cases (unresponsive CLL; n=11 and responsive CLL; n=10) were thawed, after which CLL cells were MACS-isolated and stimulated for 2.5 hours with α-IgM (optimal stimulation was defined using normal B-cells, data not shown). The $2^{-deltaCT}$ values obtained after incubation (α-IgM stimulated and unstimulated) were normalized by subtraction of the basal $2^{-deltaCT}$ value to calculate the fold differences in expression between the patient groups (**Figure 6**). BCR responsive cases showed a significant upregulation of *NFKB2, REL, NFKBID, NFKBIE* and *TNFAIP3* after stimulation compared with unresponsive cases for which the expression of the NF-κB genes remained roughly equal.

In summary, α-IgM unresponsive cases had a high basal transcription of especially NF-κB inhibitory components, whereas the responsive cases showed a clear upregulation of NF-κB inhibitory components, including *TNFAIP3* and NF-κB subunit *REL,* upon stimulation.

DISCUSSION

Here we aimed to study cell intrinsic differences between unresponsive and responsive CLL that may underlie differences in Ca^{2+} levels upon α-IgM stimulation. Based on RNA sequencing analysis several components of the canonical NF-κB pathway, especially related to NF-κB inhibition, were higher expressed in unresponsive cases. Besides these inhibitors,

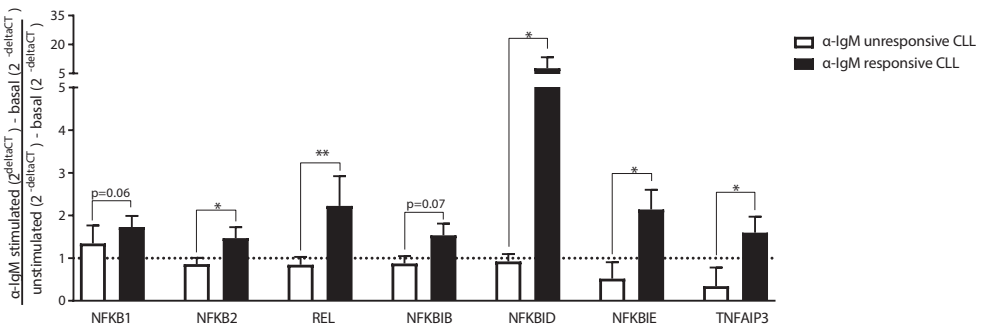

Figure 6. Transcriptional levels of NF-κB related genes upon α-IgM stimulation

Upon α-IgM stimulation, RQ-PCRs were performed to examine the transcriptional level of multiple NF-κB pathway genes (*NFKB1*, *NFKB2*, *REL*, *NFKBIB*, *NFKBID*, *NFKBIE* and *TNFAIP3*) that were differently expressed at basal level between responsive and unresponsive CLL cases. The $2^{-deltaCT}$ value upon α-IgM stimulation was subtracted by the $2^{-deltaCT}$ value at basal level and divided by the $2^{-deltaCT}$ value of the unstimulated condition corrected by the $2^{-deltaCT}$ value at basal level to calculate the net increase or decrease upon α-IgM stimulation. α-IgM unresponsive CLL cases (n=11, white bar) were compared to α-IgM responsive CLL cases (n=10, black bar) and statistics was performed using the Mann-Whitney U-test (* = p<0.05, **p<0.01).

the TNFα-induced NF-κB inhibitor A20 was also significantly higher expressed in the BCR unresponsive cases. Lastly we showed that upon α-IgM stimulation, the expression of these NF-κB pathway genes (especially genes coding for NF-κB pathway inhibitors but also NF-κB component *REL*) are upregulated in BCR responsive cases while for the unresponsive cases the transcriptional level did not change compared to basal levels, indicating that NF-κB signaling is an important pathway for CLL cells in their ability to respond upon BCR stimulation.

Based on the lack of correlation between basal Ca^{2+} levels and autonomous signaling in TKO cells (14),we then aimed to gain more insight into possible cell-intrinsic differences, although formally we cannot exclude that Ca^{2+} levels could also (partly) be high due to previous antigenic stimulation in our ex-vivo samples. Using a new cohort, Ca^{2+} signaling was now determined in freshly isolated cells instead of thawed cells, which on average resulted in lower basal Ca^{2+} levels (data not shown). This might be, in combination with the heterogeneity in basal Ca^{2+} levels, an underlying explanation for the fact that in this cohort the basal Ca^{2+} levels were not different between M- and U-CLL cases. Further building on the study of Mockridge et al. [18], who also showed differences in responsiveness to BCR stimulation between CLL cases, we therefore divided our patient cohort based on the responsive capacity to BCR stimulation. In both M- and U-CLL groups, there were cases showing a clear α-IgM response based on Ca^{2+} influx, while others did not show such a response, indicating that the level of anergy is independent of the IGHV SHM status of the BCR.

The anergic nature of unresponsive CLL was partly confirmed by the marker profile. IgM responders co-express higher surface IgM and IgD expression, which explains the response to α-IgM as well as α-IgD stimulation. The higher expression of the prognostic marker CD38 by the responsive cases is also in line with findings of Mockridge et al [18] suggesting that responsive patients in general have a poor prognosis. [2] Striking is the sharp correlation between CD21 expression and the responsive capacity upon α-IgM stimulation. In other immune related diseases like rheumatoid arthritis, common variable immunodeficiency [25] and Sjögren syndrome [26], patients had increased populations of $CD21^{low}$ B cells compared to HI. [25] These $CD21^{low}$ B cells were found to represent unresponsive cells expressing autoreactive BCRs which failed to respond based on Ca^{2+} levels upon BCR stimulation. [25, 26] $CD21^{low}$ CLL cells were not found to be autoreactive and are associated with a poor prognosis. [27] Unfortunately we had no access to longitudinal patient data and were therefore unable to evaluate progression of the CLL.

RNA sequencing analysis showed that especially genes coding for regulatory molecules involved in NF-κB inhibition are differentially expressed between BCR responsive and unresponsive cases. Several studies have reported that CLL cells have higher basal NF-κB levels compared to normal B cells and are continuously activated. [28] In addition, it has been shown that NF-κB signaling is important for preventing apoptosis by multiple mechanisms, including CD40L mediated signaling. [28, 29, 30]

We found that the unresponsive cases have higher basal gene expression of several components of the canonical NF-κB pathway, especially those involved in the inhibition. Genes coding for the p105/p50 (*NFKB1*) and p100/p52 (*NFKB2*) subunits are higher expressed in unresponsive CLL. Both are potential inhibitors and allow functional NF-κB activation in which p105/p50 is involved in the canonical NF-κB pathway and p100/p52 in the alternative (non-canonical) NF-κB pathway. [31] In addition, we found that genes coding for IκBs were higher expressed in unresponsive cases. IκBε (coded by *NFKBIE*), which is an important regulator of B-cell proliferation [32], was found to be mutated in patients with CLL. [23, 33] Especially a recurrent 4-basepair-frameshift deletion resulting in functional loss of IκBε and leading to continuous NF-κB activation was detected in progressive CLL forms [23] as well as in other B-cell malignancies. [34] However, we could not identify this identical deletion as a possible cause for the lower *NFKBIE* gene expression in the responsive cases.

Besides BCR stimulation, the canonical NF-κB pathway can be activated by TNF receptor stimulation. [31] It might thus be that NF-κB signaling in BCR unresponsive cases is more dependent on TNF-mediated activation. Higher *TNFAIP3* expression, a negative feedback regulator of NF-κB signaling induced by TNFα, as we noticed in unresponsive cases provides basis for this theory. From B-cell lymphoma patients it is known that increased and sustained NF-κB activation of especially proto-oncogene c-REL, promotes TNFα-induced cell survival. [35] Through this feedback loop mechanism, secretion and uptake of TNFα might result in

NF-κB induced survival of (anergic) CLL cells, independently from BCR signaling. Foa et al. [36] reported that CLL cells continuously produce TNFα, especially cells from patients with an indolent form compared to patients with a progressive form. [36]

Genomic aberrations in the *TNFAIP3* gene resulting in the loss of A20 are linked with autoimmune disease with a humoral component as well as several B-cell lymphomas. [37] In B cells from aged mice it was demonstrated that selective loss of A20 increases the activation threshold and enhances proliferation and survival of B cells causing an inflammatory condition and inducing autoimmunity. [38] Such loss of A20 caused by genetic aberrations of *TNAIP3* has not been associated with human CLL. [39]

Even though the focus of our study has mostly been on those genes that were higher expressed in unresponsive cases, multiple genes were found to be higher expressed in responsive cases, such as *SYK*. Although *SYK* was differently expressed based on the RNA sequencing analysis between the two groups of patients in the extended cohort of patients with CLL, we could not confirm this difference on SYK protein level (by phospho-Flow analysis; data not shown). Another gene that strikingly came out of our analysis to BCR signaling genes is Early B-cell Factor 1 (*EBF1*), a transcription factor important in B-cell differentiation that was higher expressed by the responsive cases. [40] Earlier it was shown by Seifert et al. [41] that compared to conventional B cells, *EBF1* was significantly downregulated in patients with CLL. [41] It was suggested that the low expression of *EBF1* might result in reduced levels of B-cell signaling and might contribute to an anergic phenotype of CLL cells. [41] Our results showing a lower level of *EBF1* transcripts for unresponsive cases would support this theory. Future studies would have to elucidate the importance of *EBF1* in CLL pathogenesis.

In summary, our results are showing that responsive CLL cases, irrespective of the IGHV SHM status, have a more activated phenotype and a reduced basal expression of several regulatory molecules of the canonical NF-κB pathway including those associated NF-κB inhibition. Upon α-IgM stimulation these responsive cases showed upregulation of NF-κB, including NF-κB inhibitors, whereas transcriptional levels of NF-κB signaling pathway components remained unaltered in unresponsive cases. Our findings suggest that enhanced basal NF-κB inhibition may be strongly associated with a lower responsive capacity of CLL cells to BCR stimulation and for the survival of anergic CLL cells.

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REFERENCES

1. Chiorazzi, N., Rai, K.R. & Ferrarini, M. Chronic lymphocytic leukemia. *N Engl J Med* **352**, 804-815 (2005).
2. Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L. *et al.* Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* **94**, 1840-1847 (1999).
3. Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94**, 1848-1854 (1999).
4. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* **119**, 4467-4475 (2012).
5. Catera, R., Silverman, G.J., Hatzi, K., Seiler, T., Didier, S., Zhang, L. *et al.* Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol Med* **14**, 665-674 (2008).
6. Chu, C.C., Catera, R., Zhang, L., Didier, S., Agagnina, B.M., Damle, R.N. *et al.* Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin. *Blood* **115**, 3907-3915 (2010).
7. Herve, M., Xu, K., Ng, Y.S., Wardemann, H., Albesiano, E., Messmer, B.T. *et al.* Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* **115**, 1636-1643 (2005).
8. Lanemo Myhrinder, A., Hellqvist, E., Sidorova, E., Soderberg, A., Baxendale, H., Dahle, C. *et al.* A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood* **111**, 3838-3848 (2008).
9. Ghia, E.M., Widhopf, G.F., 2nd, Rassenti, L.Z. & Kipps, T.J. Analyses of recombinant stereotypic IGHV3-21-encoded antibodies expressed in chronic lymphocytic leukemia. *J Immunol* **186**, 6338-6344 (2011).
10. Hoogeboom, R., van Kessel, K.P., Hochstenbach, F., Wormhoudt, T.A., Reinten, R.J., Wagner, K. *et al.* A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med* **210**, 59-70 (2013).
11. Hoogeboom, R., Wormhoudt, T.A., Schipperus, M.R., Langerak, A.W., Dunn-Walters, D.K., Guikema, J.E. *et al.* A novel chronic lymphocytic leukemia subset expressing mutated IGHV3-7-encoded rheumatoid factor B-cell receptors that are functionally proficient. *Leukemia* **27**, 738-740 (2013).

12. Kostareli, E., Gounari, M., Janus, A., Murray, F., Brochet, X., Giudicelli, V. *et al.* Antigen receptor stereotypy across B-cell lymphoproliferations: the case of IGHV4-59/IGKV3-20 receptors with rheumatoid factor activity. *Leukemia* **26**, 1127-1131 (2012).
13. Zwick, C., Fadle, N., Regitz, E., Kemele, M., Stilgenbauer, S., Buhler, A. *et al.* Autoantigenic targets of B-cell receptors derived from chronic lymphocytic leukemias bind to and induce proliferation of leukemic cells. *Blood* **121**, 4708-4717 (2013).
14. Duhren-von Minden, M., Ubelhart, R., Schneider, D., Wossning, T., Bach, M.P., Buchner, M. *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* **489**, 309-312 (2012).
15. Muggen, A.F., Pillai, S.Y., Kil, L.P., van Zelm, M.C., van Dongen, J.J., Hendriks, R.W. *et al.* Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* **29**, 321-328 (2015).
16. Gauld, S.B., Benschop, R.J., Merrell, K.T. & Cambier, J.C. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* **6**, 1160-1167 (2005).
17. Apollonio, B., Scielzo, C., Bertilaccio, M.T., Ten Hacken, E., Scarfo, L., Ranghetti, P. *et al.* Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood* **121**, 3879-3888, S3871-3878 (2013).
18. Mockridge, C.I., Potter, K.N., Wheatley, I., Neville, L.A., Packham, G. & Stevenson, F.K. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood* **109**, 4424-4431 (2007).
19. Lanham, S., Hamblin, T., Oscier, D., Ibbotson, R., Stevenson, F. & Packham, G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. *Blood* **101**, 1087-1093 (2003).
20. D'Avola, A., Drennan, S., Tracy, I., Henderson, I., Chiecchio, L., Larrayoz, M. *et al.* Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood* **128**, 816-826 (2016).
21. Meixlsperger, S., Kohler, F., Wossning, T., Reppel, M., Muschen, M. & Jumaa, H. Conventional light chains inhibit the autonomous signaling capacity of the B cell receptor. *Immunity* **26**, 323-333 (2007).
22. Jost, P.J. & Ruland, J. Aberrant NF-kappaB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood* **109**, 2700-2707 (2007).
23. Mansouri, L., Sutton, L.A., Ljungstrom, V., Bondza, S., Arngarden, L., Bhoi, S. *et al.* Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. *J Exp Med* **212**, 833-843 (2015).
24. Verstrepen, L., Verhelst, K., van Loo, G., Carpentier, I., Ley, S.C. & Beyaert, R. Expression, biological activities and mechanisms of action of A20 (TNFAIP3). *Biochem Pharmacol* **80**, 2009-2020 (2010).
25. Isnardi, I., Ng, Y.S., Menard, L., Meyers, G., Saadoun, D., Srdanovic, I. *et al.* Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood* **115**, 5026-5036 (2010).
26. Saadoun, D., Terrier, B., Bannock, J., Vazquez, T., Massad, C., Kang, I. *et al.* Expansion of autoreactive unresponsive CD21-/low B cells in Sjogren's syndrome-associated lymphoproliferation. *Arthritis Rheum* **65**, 1085-1096 (2013).
27. Nichols, E.M., Jones, R., Watson, R., Pepper, C.J., Fegan, C. & Marchbank, K.J. A CD21 low phenotype, with no evidence of autoantibodies to complement proteins, is consistent with a poor prognosis in CLL. *Oncotarget* **6**, 32669-32680 (2015).

28. Furman, R.R., Asgary, Z., Mascarenhas, J.O., Liou, H.C. & Schattner, E.J. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* **164**, 2200-2206 (2000).
29. Cuni, S., Perez-Aciego, P., Perez-Chacon, G., Vargas, J.A., Sanchez, A., Martin-Saavedra, F.M. *et al.* A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. *Leukemia* **18**, 1391-1400 (2004).
30. Yu, M., Chen, Y., He, Y., Podd, A., Fu, G., Wright, J.A. *et al.* Critical role of B cell lymphoma 10 in BAFF-regulated NF-kappaB activation and survival of anergic B cells. *J Immunol* **189**, 5185-5193 (2012).
31. Gasparini, C., Celeghini, C., Monasta, L. & Zauli, G. NF-kappaB pathways in hematological malignancies. *Cell Mol Life Sci* **71**, 2083-2102 (2014).
32. Alves, B.N., Tsui, R., Almaden, J., Shokhirev, M.N., Davis-Turak, J., Fujimoto, J. *et al.* IkappaBepsilon is a key regulator of B cell expansion by providing negative feedback on cRel and RelA in a stimulus-specific manner. *J Immunol* **192**, 3121-3132 (2014).
33. Domenech, E., Gomez-Lopez, G., Gzlez-Pena, D., Lopez, M., Herreros, B., Menezes, J. *et al.* New mutations in chronic lymphocytic leukemia identified by target enrichment and deep sequencing. *PLoS One* **7**, e38158 (2012).
34. Mansouri, L., Noerenberg, D., Young, E., Mylonas, E., Abdulla, M., Frick, M. *et al.* Frequent NFKBIE deletions are associated with poor outcome in primary mediastinal B-cell lymphoma. *Blood* **128**, 2666-2670 (2016).
35. Feuerhake, F., Kutok, J.L., Monti, S., Chen, W., LaCasce, A.S., Cattoretti, G. *et al.* NFkappaB activity, function, and target-gene signatures in primary mediastinal large B-cell lymphoma and diffuse large B-cell lymphoma subtypes. *Blood* **106**, 1392-1399 (2005).
36. Foa, R., Massaia, M., Cardona, S., Tos, A.G., Bianchi, A., Attisano, C. *et al.* Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: a possible regulatory role of TNF in the progression of the disease. *Blood* **76**, 393-400 (1990).
37. Das, T., Chen, Z., Hendriks, R.W. & Kool, M. A20/Tumor Necrosis Factor alpha-Induced Protein 3 in Immune Cells Controls Development of Autoinflammation and Autoimmunity: Lessons from Mouse Models. *Front Immunol* **9**, 104 (2018).
38. Chu, Y., Vahl, J.C., Kumar, D., Heger, K., Bertossi, A., Wojtowicz, E. *et al.* B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice. *Blood* **117**, 2227-2236 (2011).
39. Frenzel, L.P., Claus, R., Plume, N., Schwamb, J., Konermann, C., Pallasch, C.P. *et al.* Sustained NF-kappaB activity in chronic lymphocytic leukemia is independent of genetic and epigenetic alterations in the TNFAIP3 (A20) locus. *Int J Cancer* **128**, 2495-2500 (2011).
40. Nechanitzky, R., Akbas, D., Scherer, S., Gyory, I., Hoyler, T., Ramamoorthy, S. *et al.* Transcription factor EBF1 is essential for the maintenance of B cell identity and prevention of alternative fates in committed cells. *Nat Immunol* **14**, 867-875 (2013).
41. Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Durig, J. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* **209**, 2183-2198 (2012).

SUPPLEMENTARY METHODS

IGHV sequence analysis

Genomic DNA was extracted from PBMCs via spin-column kits and QIAcube (Qiagen, Valencia, CA, USA). Primers and protocols for IGHV mutation status analysis were according to the BIOMED-2 protocol and following ERIC guidelines. [1, 2] In brief, PCR products were analyzed by electrophoresis on polyacrylamide gels for monoclonality, followed by direct sequencing. Sequencing results were analyzed online by use of IMGT/V-QUEST (www.imgt.org).

Isolation of CLL cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque (GE, Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. The obtained PBMC fractions were sorted via magnetic-activated cell sorting (MACS), using the B-CLL isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For RNA sequencing, a minimum of $5x10^6$ isolated CLL cells was resuspended in RLT buffer (Qiagen) supplemented with 1% 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and stored at -80°C. To check purities, a small fraction of the isolated cells was stained with phycoerythrin (PE)-Cy7 labelled CD19, Allophycocyanin (APC)-H7 labelled CD3, together with APC-labelled CD5 (CLL cases only). All fractions had a purity of more than 95% $CD19^+$ cells.

Ca^{2+} flux assay

Intracellular Ca^{2+} fluxes were measured using Fluo-3AM and Fura Red-AM probes (both from Life Technologies, Carlsbad, CA, USA). Briefly, isolated CLL cells were incubated with 5 mM Fluo3-AM and 5 mM Fura Red-AM in loading buffer (Hank's balanced salt solution medium (HBSS) supplemented with 10 mM HEPES and 5% fetal calf serum) at 37°C for 30 min in the dark. Cells were washed and resuspended in 1 ml loading buffer. Before FACS measurements, cells were warmed to 37°C for 10 min. Basal intracellular Ca^{2+} levels were measured for 60 seconds, followed by stimulation of the BCR with 20 µg/ml anti-human IgM $F(Ab`)_2$ (Southern Biotech, Birmingham, USA) or with 20 µg/ml anti-human IgD $F(Ab`)_2$ (Southern Biotech). After stimulation cells were acquired for 360 seconds on the LSRII flow cytometer (BD Biosciences, Erembodegem, Belgium). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

For Ca^{2+} influx analysis in TKO cells, 1×10^6 transduced TKO cells were loaded with Indo-1 (Invitrogen, Carlsbad, California, USA) with use of Pluronic (Invitrogen, Carlsbad, California, USA). Induction of ERT2–SLP65 to allow signaling was performed by the addition of 2 µM 4-hydroxy tamoxifen (4-OHT; Sigma-Aldrich, Saint Louis, Missouri, USA). As controls for BCR

crosslinking 10 µg/ml Goat anti-mouse IgM F(Ab`)$_2$ (10 µg/ml; Southern Biotech) and 10 µg/ml Goat anti-mouse IgD F(Ab`)$_2$ (Southern Biotech) were used.

Phospho-flow cytometry

Isolated CLL cells with 20 µg/ml anti-human IgM F(Ab`)$_2$ (Southern Biotech) at 37°C for 1 minute to measure phosphorylation of Syk and PLCγ2. To measure phosphorylation of Akt, stimulation was done for 10 minutes. After stimulation, the cells were fixated for 10 minutes using cytofix (BD Biosciences). Before intracellular staining, cells were permeabilized by adding Perm III buffer (BD) at -20°C for 30 minutes. After washing, the cells were stained with either PE labelled pSyk (pY348; clone: I120-722), APC-Alexa fluor (AF)-647 labelled pPLCγ2 (pY759; clone: K86-689.37) or with PE-labelled pAkt (pY473; clone: M89-61). Data was acquired on the LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Gene mutation sequencing

To examine a previously reported 4-bp deletion in the first exon of the *NFKBIE* gene, as described by Mansouri et al. [3], this region was sequenced. [3] In brief, first DNA (10 ng/µl) was amplified by hot-start PCR (35 cycles, Tm=60°C) by addition of Gold buffer (10x; Thermo Fisher), $MgCl_2$ (25 mM; Applied Biosystems Life Technologies), dNTPs (20 mM; GE Healthcare), Taq Gold polymerase (5U/µl; Thermo Fisher), forward primer (10 pmol/µl; 5'-CCTCAAAAGTGGGCTGAG-3') and reverse primer (10 pmol/µl; 5'-CAAGGAACCACAGGAGAAGG-3'). Two µl of the amplified DNA was used and mixed with Big Dye® Terminator (BDT; Thermo Fisher), 5x sequencing buffer (Thermo Fisher) together with the forward and reverse primers (separately). After amplification, the sequence run was performed on an ABI Prism® 3130*xl* Genetic Analyzer (Thermo Fisher). Analysis was done using the program CLC Main Workbench 7 (Qiagen).

cDNA synthesis and Real-time Quantitative PCR (RQ-PCR)

cDNA was synthesized from isolated RNA by adding reverse transcriptase Superscript II (Invitrogen Life Technologies, Waltham, MA, USA), 10x CA buffer (0.2 M Tris pH 8.3, 0.5 M KCl), dNTP (GE Healthcare, Cleveland, OH, USA), dithiotreitol (Invitrogen Life Technologies), $MgCl_2$ (Applied Biosystems Life Technologies), recombinant RNAsin (Promega, Fitchburg, WI, USA) and random primers (Invitrogen Life Technologies). The reaction mixture was incubated for 45 minutes at 42°C and the reaction was stopped by a 2 minute incubation at 99°C.

Real-time Quantitative PCR (RQ-PCR) assays were designed with the Roche Universal Probe Library (Roche, Basel, Switzerland) (Supplementary Table 4). TaqMan Universal PCR master mix (2x; Applied Biosystems) was used for the reaction mix and the run was performed on the StepOne Plus instrument (Thermo Fisher, Waltham, MA, USA). Ct values

of the samples of each target gene were normalized to the Ct value of the ABL housekeeping gene (ΔCT) and the relative quantification ($2^{-\Delta CT}$) was performed. [4]

References

1. Langerak, A.W., Davi, F., Ghia, P., Hadzidimitriou, A., Murray, F., Potter, K.N. *et al.* Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. *Leukemia* **25**, 979-984 (2011).

2. van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L. *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* **17**, 2257-2317 (2003).

3. Mansouri, L., Sutton, L.A., Ljungstrom, V., Bondza, S., Arngarden, L., Bhoi, S. *et al.* Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. *J Exp Med* **212**, 833-843 (2015).

4. Beillard, E., Pallisgaard, N., van der Velden, V.H., Bi, W., Dee, R., van der Schoot, E. *et al.* Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia* **17**, 2474-2486 (2003).

SUPPLEMENTARY FILES

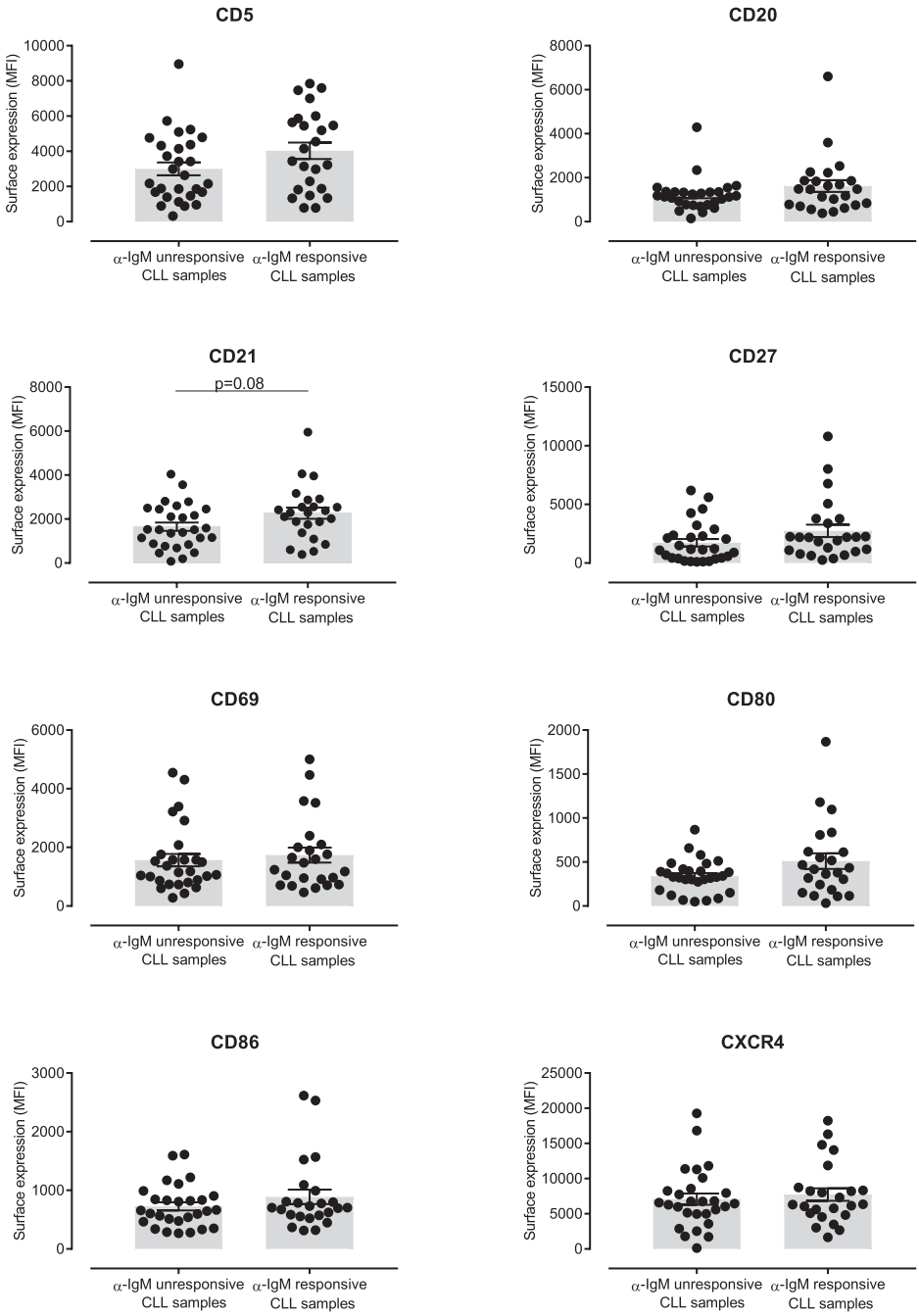

Figure S1.

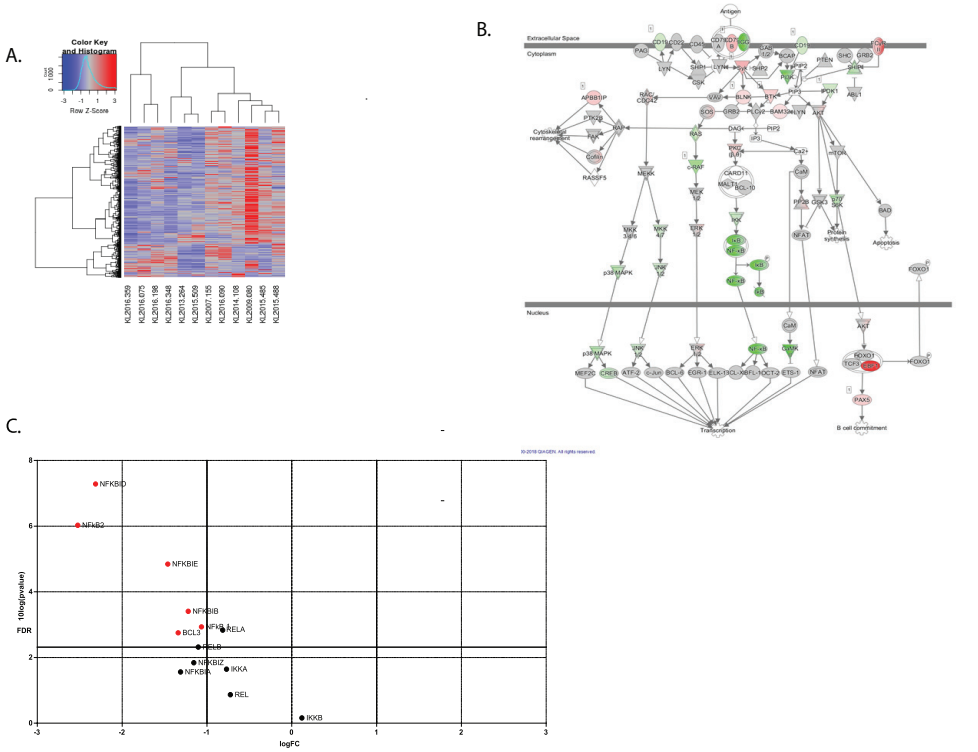

Figure S2.

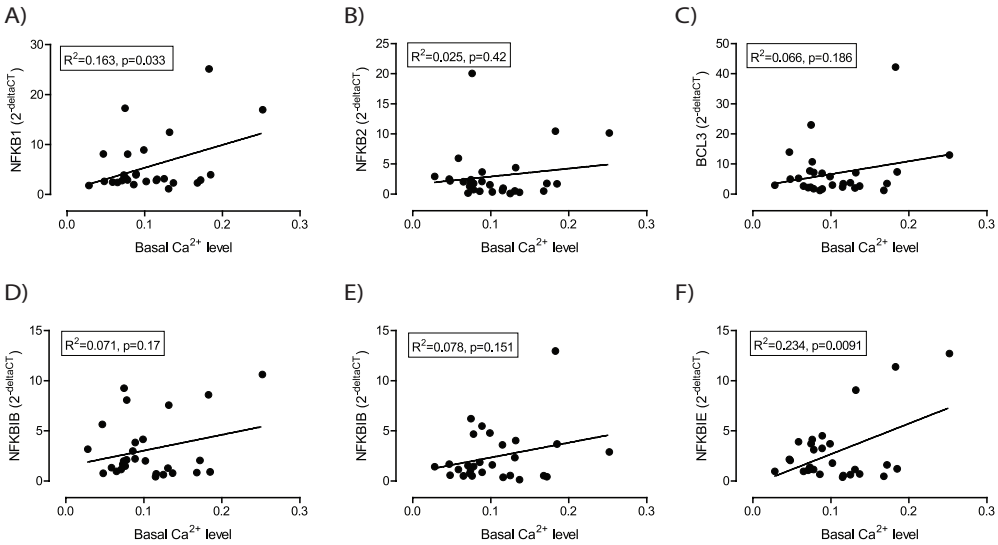

Figure S3.

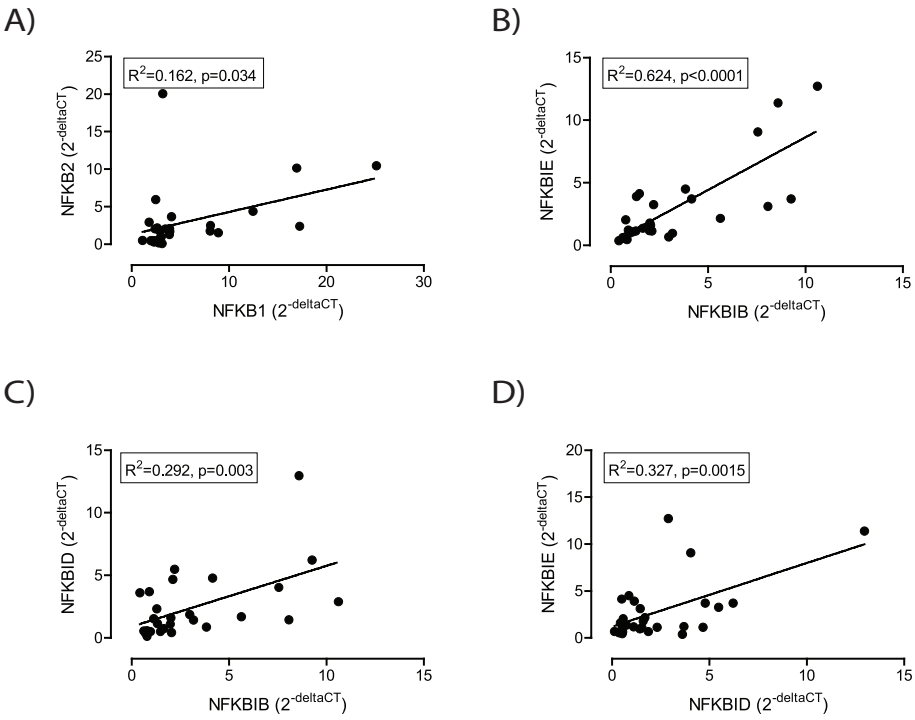
A)
R2=0.162, p=0.034
NFKB2 (2-deltaCT)
NFKB1 (2-deltaCT)
B)
R2=0.624, p<0.0001
NFKBIE (2-deltaCT)
NFKBIB (2-deltaCT)
C)
R2=0.292, p=0.003
NFKBID (2-deltaCT)
NFKBIB (2-deltaCT)
D)
R2=0.327, p=0.0015
NFKBIE (2-deltaCT)
NFKBID (2-deltaCT)

Figure S4.

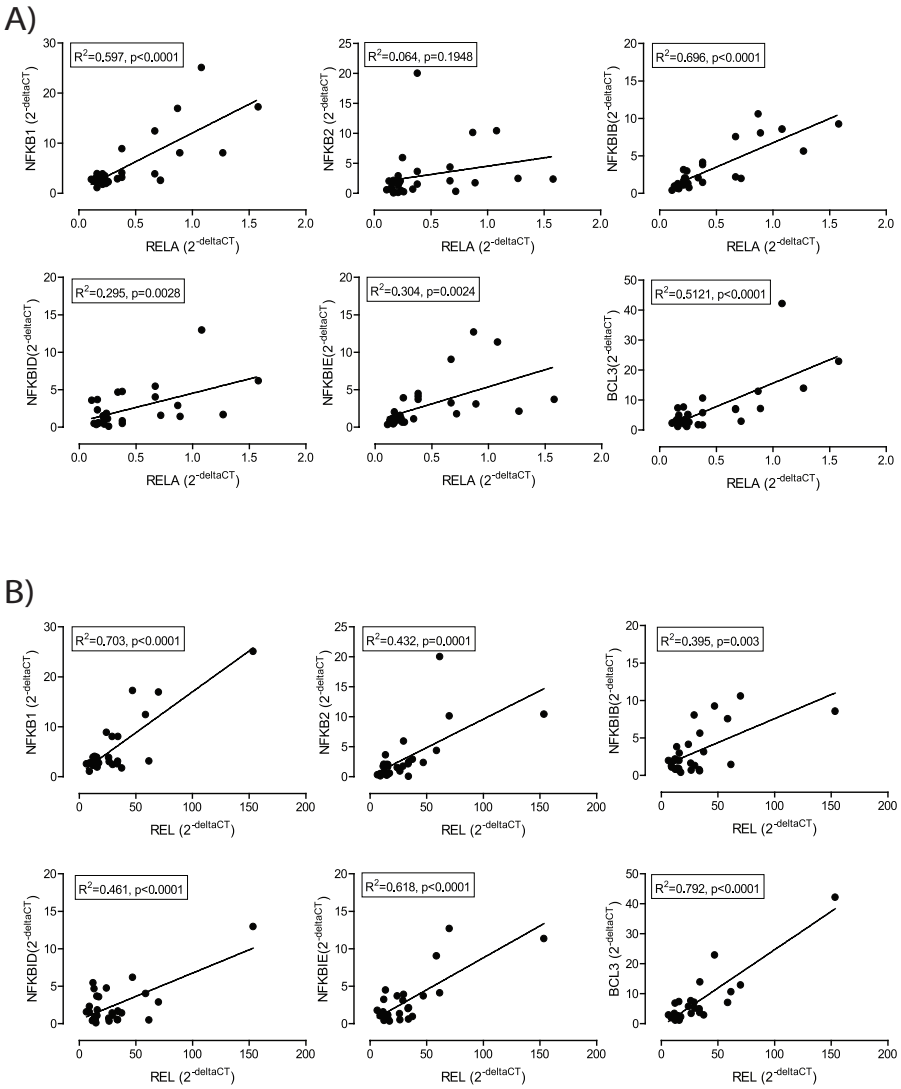
A)
R²=0.597, p<0.0001
NFKB1 (2-deltaCT)
RELA (2-deltaCT)
R²=0.064, p=0.1948
NFKB2 (2-deltaCT)
RELA (2-deltaCT)
R²=0.696, p<0.0001
NFKBIB(2-deltaCT)
RELA (2-deltaCT)
R²=0.295, p=0.0028
NFKBID(2-deltaCT)
RELA (2-deltaCT)
R²=0.304, p=0.0024
NFKBIE(2-deltaCT)
RELA (2-deltaCT)
R²=0.5121, p<0.0001
BCL3(2-deltaCT)
RELA (2-deltaCT)
B)
R²=0.703, p<0.0001
NFKB1 (2-deltaCT)
REL (2-deltaCT)
R²=0.432, p=0.0001
NFKB2 (2-deltaCT)
REL (2-deltaCT)
R²=0.395, p=0.003
NFKBIB(2-deltaCT)
REL (2-deltaCT)
R²=0.461, p<0.0001
NFKBID(2-deltaCT)
REL (2-deltaCT)
R²=0.618, p<0.0001
NFKBIE(2-deltaCT)
REL (2-deltaCT)
R²=0.792, p<0.0001
BCL3 (2-deltaCT)
REL (2-deltaCT)

Figure S5.

Table S1. Patient characteristics

Total number of CLL patients	52
• U-CLL	30 (58%)
• M-CLL	22 (42%)
Heterogeneous CLL	44 (85%)
Stereotypic CLL	8 (15%)
• U-CLL #1	2
• U-CLL #3	1
• U-CLL #5	1
• U-CLL #6	1
• U-CLL #64B	2
• M-CLL #2	1
Cytogenic abberations	
• none	9 (17%)
• trisomy 12	8 (15%)
• del 11q (including partial)	8 (15%)
• del 17p	5 (10%)
• del13q isolated	15 (29%)
• del13q combined	11 (21%)
• unknown	5 (10%)
Age in years	66.3 (38-88)#
Female	20 (38%)
Male	32 (62%)

= mean with range

Table S2. Antibodies used for phenotyping

Antibody	Clone	Conjugate	Supplier
CD3	Sk7	APC-H7	BD Biosciences
CD5	UCHT2	APC	BD Biosciences
CD19	J3-119	PC7	Beckman Coulter
CD20	2H7	BV606	BioLegend
CD21	B-ly4	BV711	BD Biosciences
CD27	M-T271	BV421	BD Biosciences
CD38	HB7	AF750	BD Biosciences
CD43	IG10	FITC	BD Biosciences
CD69	FN50	BV711	BD Biosciences
CD80	L307.4	PE	BD Biosciences
CD86	HA5.2B7	PE	Beckman Coulter
CD184 (CXCR4)	12G5	APC	BD Biosciences
IgD	1A6-2	PE-CF594	BD Biosciences
IgM	MHM-88	BV-510	BD Biosciences

Table S3. Selected CLL cases for RNA sequencing

	Patient ID	SHM status	α-IgM response (FL3/FR ratio)
Unresponsive CLL cases	KL2007-155	M-CLL	0.95
	KL2016-348	M-CLL	0.94
	KL2016-198	M-CLL	1.00
	KL2016-359	U-CLL	0.98
	KL2015-485	U-CLL	0.98
	KL2016-075	U-CLL	0.96
Responsive CLL cases	KL2014-108	M-CLL	1.40
	KL2009-080	M-CLL	1.43
	KL2015-509	M-CLL	1.44
	KL2016-090	U-CLL	5.15
	KL2013-264	U-CLL	6.46
	KL2015-488	U-CLL	4.24

Table S4. Primers and probes from Roche Universal Probe Library for RQ-PCR design.

Gene	Sequence forward primer	Sequence reverse primer[1]	Probe[2]
NFKB1	CCTGCTCCTTCCAAAACACT	CGGTGTAGCCCATTTGTCTC	22
NFKB2	CTCGTGTCTGTCCACCTGTC	GAGCATCTGCGAGCATACAG	74
BCL3	ACATCCTGAGGGGGAAGG	TGATGCGGAGAGAAGACCA	38
NFKBIB	CCCTTCATTTGGCAGTGG	CTGCCCTCAGGAGAAGCTC	27
NFKBID	ATTTCTCCCTCCCCCAGA	GAACCCACAGTCTCCGAGTG	90
NFKBIE	TGCTGTGTACCGACTGAAGC	CCAGACTGGCTCTCTTCCAC	63
REL	TGAACATGGTAATTTGACGACTG	ACACGACAAATCCTTAATTCTGC	69
RELA	CTGGGAATCCAGTGTGTGAA	AAGGGGTTGTTGTTGGTCTG	1
RELB	GCTCTACTTGCTCTGCGACA	GGCCTGGGAGAAGTCAGC	79
TNFAIP3	AATTTCCTCCAGGTCACCTAAAC	GGGGACCTTGGTTCTAGCTT	68
TNFα	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	40

1. Reverse complementary primers.

2. Probe numbers according to the Roche Universal Probe Library.

Chapter 5

Both related and diverse BCR usage across siblings within CLL families

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Manuscript in preparation

ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common type of hematological malignancy in elderly in the Western world. As key processes in the onset of disease chronic antigenic activation via the B cell receptor (BCR), signals from the microenvironment, and genetic alterations in mature B cells are mentioned. Known risk factors for CLL are aging and European ancestry, but the etiology of CLL is still largely unknown. Previous epidemiological studies indicated that inherited factors play a role in disease onset, and relatives of CLL patients were found to have an 8.5-fold increased risk of developing CLL. Familial CLL is defined by having at least two affected relatives within one family. Here, we describe three families with two or more siblings affected with CLL. We hypothesized that similarities BCR composition and/or chromosomal aberrations cause familial CLL occurrence. Therefore, we determined whether there are parallels in the BCR heavy and light chains in the CLL clones of the siblings of these families. Furthermore, we also investigated if similarities in known CLL-associated chromosomal aberrations are present within the three families by using single nucleotide polymorphism (SNP) array analysis.

We found that the three families represented three main CLL subgroup, with family 1 consisting of two brothers and two sisters being M-CLL cases, and family 2 consisting of two brothers being U-CLL cases. Additionally, family 3 consisting of two brothers and two sisters form a CLL#2-like family with subject 3C being a CLL#2 case and subject 3B a CLL#2-related CLL#169 case, and all four having a CLL#2-light chain. Furthermore, based on SNP array analysis several known chromosomal aberrations, which have prognostic significance, were detected for all siblings of a CLL family. In families 1 and 3 11q14.2-11q14.3 deletions were detected, and both family 2 members had trisomy 12. Also, other minor losses, previously associated with CLL, were detected for all siblings in these respective families.

With the exception of family 3 being a CLL#2-like family, we found no relationship in BCR gene usage in our CLL families. As we found similarities in known CLL-associated chromosomal aberrations within our families, this might indicate a shared path of CLL development, which requires further investigation.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of hematological malignancy in elderly in the Western world, with a prevalence of 4.2 per 100000 between 1975 and 2009[1, 2]. Chronic antigenic activation, either self or non-self, via the B cell receptor (BCR), signals from the microenvironment, and genetic alterations of mature B cells are mentioned as key processes in the onset of disease[3].

The BCR has an important role in the development of CLL, which is why its downstream signaling pathway is nowadays an attractive target for therapies. In addition, the BCR is of significant prognostic value, since the presence of somatic hypermutations (SHM) in the immunoglobulin heavy variable (IGHV) gene is generally associated with an indolent form of the disease, known as mutated CLL (M-CLL), while the absence of SHM is associated with a more aggressive form, known as unmutated CLL (U-CLL)[4]. The importance of the BCR in CLL is corroborated by the fact that about one-third of the CLL cases carry a very restricted BCR repertoire, leading to further segregation of CLL into so-called stereotypic BCR subsets. Stereotypic BCR are defined by a restricted IGHV, IGH diversity (IGHD), and IGH joining (IGHJ) gene usage, with restricted complementarity determining region 3 (HCDR3) length and amino acid usage[5]. In addition, also the immunoglobulin light chain (IGL) shows stereotypy. This restricted usage of BCRs in CLL suggests a selection for particular BCR that might be causal in the development of the disease. Apart from the biological implications, these stereotypic subsets also have clinical impact. This is probably best exemplified by the largest stereotypic CLL subset, CLL#2, (characterized by IGHV3-21 in combination with IGHJ6, a short HCDR3 of 9 amino acids, and a light chain consisting of IGLV3-21 in combination with IGLJ3 with a LCDR3 length of 12 amino acids), which defines a prognostically poor CLL subgroup[5].

Next to the BCR configuration, several genetic aberrations are found to have prognostic value. Deletions of chromosomal regions 17p, 11q, or 13q, and trisomy of chromosome 12 can be detected in about 80% of CLL cases. These chromosomal deletions are associated with the loss of the tumor suppressor genes *TP53* and *ATM*, and with *miR15a* and *miR16-1* microRNA genes, respectively [6, 7 8]. Deletion of 17p and deletion of 11q are associated with an aggressive form of CLL, while 13q deletion as a single event is associated with a milder form and thereby a more favorable prognosis. Trisomy 12 was recently found to be associated with altered nuclear factor of activated T cells (NFAT) signaling and immune checkpoint molecule CD73[9], and additionally has an intermediate disease outcome[8].

Although advanced age and European ancestry are known risk factors, the etiology of CLL is still largely unknown. Epidemiological studies indicate that inherited factors would play a role in disease onset[10]. Relatives of CLL patients have an 8.5-fold increased risk of developing CLL, and additionally have an increased risk in developing other types of B cell malignancies [11]. Familial CLL is defined by the presence of at least two affected relatives within one family.

It was estimated in a Swedisch cohort that first degree relatives of CLL have a 3.05% life time risk to develop CLL, while this is 0.41% in the normal population, as was based on a 7.52 increased relative risk for relatives of CLL cases[12]. Previous linkage studies in familial CLL did not reveal major susceptibility loci. However, GWAS studies in CLL identified many single nucleotide polymorphisms (SNPs) with small effects, which can add up in the etiology of familial CLL [13, 14, 15]. Twin studies for lymphomas showed that monozygotic twins have a 100-fold higher risk for Hodgkin lymphoma, with no increased risk in dizygotic twins, comparable with normal population[16]. For non-Hodgkin lymphomas, monozygotic twins of a patient were found to have a 23-fold increased risk in developing NHL, while dizygotic twins have a 14-fold increased risk[17].

In this report, we describe three families with two or more siblings affected with CLL. We hypothesize that similarities in chromosomal aberrations and/or immunogenic configuration (i.e. BCR composition) would underlie familial CLL occurrence. To this end, we determined whether there are parallels in the heavy and light chains of the BCR in the CLL clones of the siblings of these families. Furthermore, we also investigated if similarities in known CLL-associated chromosomal aberrations are present within the three families by using SNP array analysis.

MATERIALS & METHODS

Samples

Peripheral blood was obtained from CLL patients of three families (for details see **Figure 1**) in accordance with the declaration of Helsinki. The study was approved by the hospital medical ethics committee under number METC2015-741. By use of STR analysis, the familial connection was confirmed for the siblings of these three families.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque (GE Healthcare, Little Chalfont, UK) gradient centrifugation. CLL cells and T cells were sorted from PBMC using a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA). Immediately after sorting, cells were lysed in RLT+ buffer (Qiagen, Valencia, CA, USA) complemented with β-mercapto-ethanol and stored in -80°C until further processing. DNA was isolated with the DNA/RNA/miRNA easy kit (Qiagen) according to the manufacturer's protocol. In case DNA was isolated from total PBMC, spin-column kits and the QIAcube (Qiagen) were used.

IGHV-IGHD-IGHJ gene rearrangement analysis

IGH and IGV gene rearrangements were amplified from 100 ng DNA of total PBMC in a multiplex PCR using BIOMED-2 IGH primers and Vκ-Jκ and Vλ-Jλ light chain consensus primers, following ERIC guidelines[18, 19]. Clonal PCR products were separated by heteroduplex

gel electrophoresis, and were purified by gel extraction. Rearrangements were determined through Sanger sequencing by an ABI 3130xl instrument (ThermoFischer, Waltham, MA, USA). Sequencing results were analyzed using the IMGT/V-QUEST tool on the IMGT website (www.imgt.org, version 3.3.1).

SNP array analysis

Single nucleotide polymorphism (SNP) array analysis was performed using the Illumina Human OmniExpress Beadchip (Illumina, San Diego, CA, USA). Data were analyzed with Beadstudio software (Illumina). The log R ratio and B allele frequency data were analyzed with the software program Nexus Copy Number (Nexus BioDiscovery, El Segundo, CA, USA). The results were compared with a database of known copy number variations (Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands) and a public copy number variations dataset containing approximately 3500 healthy controls (dataset of genomic variants). The affected locations detected, were analyzed in Ensembl Genome Browser 95 (https://www.ensembl.org/index.html).

RESULTS AND DISCUSSION

The BCR configuration of CLL cells is highly similar between siblings within each CLL family

In family 1 (**Figure 1A**) four (out of the total ten) siblings, i.e. two brothers and two sisters, suffered from CLL, were diagnosed at advanced age (85 (1A), 86 (1B), 79 (1C), and 60 (1D)) and were followed until late age (98, 91, 84 and 82 years, respectively)[20]. Patient 1A was treated with chlorambucil due to increased leukocytosis and thrombocytopenia, twelve years after diagnosis[20]. Further follow-up data were unfortunately not available.

Based on the IGHV sequence analysis all four siblings in this family appeared to carry a somatically hypermutated BCR (below 98% identity cut-off with germline IGHV gene) and thus should be considered as M-CLL, which are mostly associated with an indolent form of the disease. IGHV-IGHD-IGHJ and IGKV-IGKJ/IGLV-IGLJ gene usage were very diverse in this family (**Figure 1A**). Family members 1A and 1C both showed only one IGH rearrangement, in addition to two in-frame Ig light chain rearrangements. In family member 1B we could detect two productive IGH rearrangements, but only one in-frame rearranged IGL gene plus an unproductive (out-of-frame) IGK rearrangement. Family member 1D appeared to have bi-clonal CLL, as evidenced from the Igκ positive and Igλ positive CLL clones in flow cytometry. Indeed, we could detect the Ig light chain rearrangements for both CLL clones, and found three productive IGH rearrangements. Although, CLL is generally characterized by a monoclonal lymphoproliferation, multiple productive IGH rearrangements have been

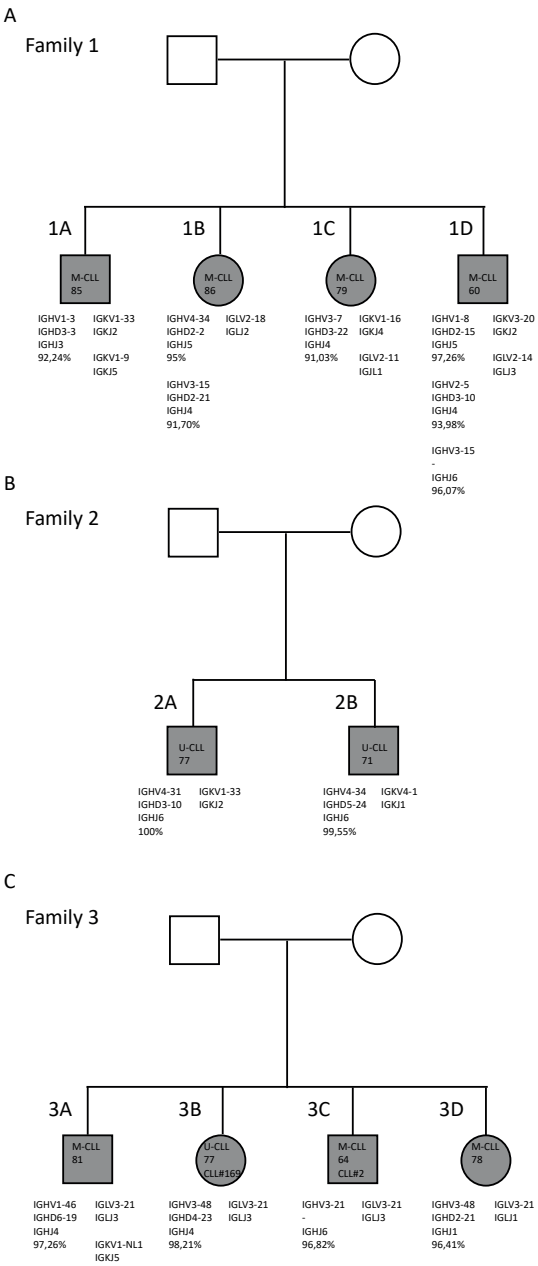

Figure 1. Family trees and BCR characteristics of familial CLL cases.

A. Family 1 consisting of two brothers and two sisters being M-CLL cases. **B.** Family 2 consisting of two brothers being U-CLL cases. C. Family 3 consisting of two brothers and two sisters and forming a CLL#2-like family with subject 3C being a true CLL#2 case and subject 3B a CLL#2-related CLL#169 case, and all four having a CLL#2-light chain.

observed in around 2% of CLL cases [18]. These can arise from a single CLL clone (so-called biallelic IGH configuration) caused by a failure of allelic exclusion, or reflect biclonal CLL disease. Additionally, CLL with multiple partner clones were also reported in around 2% of cases (5 of 198), being all M-CLL cases, as based on single cell PCR analysis [21]. Interestingly, within M-CLL family 1, member 1D appeared to have biclonal CLL consisting of a SmIgκ+ and a SmIgλ+ CLL clone in flow cytometry (data not shown). Since we detected 3 productive IGH rearrangements, one of these CLL clones seems biallelically rearranged. In addition, family member 1B also appeared to have biallelic productive IGH rearrangements with only one rearranged Ig light chain gene. Previously, multiple additional IGH bands were reported for these family members in Southern blot analysis[20], but these were now all found to be unproductive.

The two brothers of family 2 were diagnosed with CLL at age 77 (2A) and 71 (2B) years. They both carried an unmutated IGHV gene in their CLL clone (U-CLL), and used an IGHV4 gene member in combination with the IGHJ6 gene, and a rearranged IGK light chain gene (**Figure 1B**).

Family 3 also consisted of two brothers and two sisters with CLL, being diagnosed in the age range from 64 until 81 years. Three siblings showed M-CLL, with members 3C and 3D carrying a BCR with a clearly mutated IGHV gene, while member 3A showed a borderline mutated IGHV gene. The fourth sibling, family member 3B, was found to have a borderline U-CLL with a SHM status of 98.2%. Of note, family member 3C showed a stereotypic #2 BCR, consisting of IGHV3-21 in combination with IGHJ6, with the typical 9 amino acids short HCDR3. This particular stereotypic receptor is generally combined with an IGL light chain, consisting of IGLV3-21 and IGLJ3 with a LCDR3 length of 12 amino acids. Remarkably, subject 3B was found to have a stereotypic CLL#2-like receptor, CLL#169, with use of IGHV3-48, IGHD4-23, and IGHJ4, with a CDR3 length of 9 amino acids. Interestingly, this CLL#2-like IGH chain was found in combination with the typical CLL#2 IGL light chain in this case. The respective IGH genes of these stereotypic CLL subsets, IGHV3-21 and IGHV3-48, are 97% identical, and both show an aspartic acid at position 107 in the HCDR3 and use similar IGL chains[5]. Stereotypic CLL#2 is the most common stereotypic subset, with almost 3% of all CLL cases expressing this BCR[5]. This subset is associated with an aggressive type of disease with a time to first treatment (TTFT) of 1 to 6 years after diagnosis[22]. Preliminary data from the European Research Initiative on CLL (ERIC) comparing CLL#2 with CLL#169, illustrate striking similarities in cytogenetic aberrations and TTFT for both subsets. For CLL#169 family member 3B, we know that treatment with Fludarabine was needed. Unfortunately, we do not have additional follow-up data from this patient, nor from the other family 3 siblings.

Furthermore, also family members 3A and 3D carried a CLL#2 IGL light chain, albeit with distinct IGH chains (**Figure 1C** and **Table 1**). Existence of stereotypic BCR usage in CLL is thought to be induced by chronic stimulation involving a common antigen. Up to now there

Table 1. HCDR3 and LCDR3 amino acid composition in members of CLL family 3

Family member	Stereotypic BCR	HCDR3	LCDR3
3A	-	ARAWSSAWKYYFDY	QVWDSGSDHPWV
3B	CLL#169	ARDGVGAPY	QVWDSGTDHPWV
3C	CLL#2	ARDQNGMDV	QVWDSSSDHPWV
3D	-	ARDGGPCGDCYQ	LVWDSGSDHPYV

is no clear evidence for a common antigen in CLL#2 cases, but Menici et al. showed with a crystal structure of the CLL#2-BCR that inter-BCR interactions play a role and that these are mediated by this particular IGL chain, similar to a BCR binding its antigen. The IGLV3-21 light chain gene encodes two Asp residues at position 50 and 52 in the VL CDR2 regions, which interacts with an Arg110 light chain residue produced by a G to C nucleotide substitution caused by SHM in the linker region between the variable and constant region of the light chain. This Arg110 L was detected in the CLL#2 BCR in a cohort of 17 patients[23]. We noticed the presence of the Asp 50 and 52 residues in the CLL clones of our family 3 members, but we could not detect the Arg110 residue as it is outside the analyzed region (data not shown).

With the exception of the BCR usage of family 3, which have remarkable similarities, the other families are alike in being all M-CLL cases in family 1 and both family 2 cases being U-CLL. In a large cohort of 327 CLL patients from 214 families the IGHV gene usage was analyzed and compared with sporadic CLL cases[24]. In this study, it was found that there was no relationship in IGHV gene usage between CLL patients of a family. This suggests that familial CLL is generally comparable with sporadic CLL based on the BCR, and makes a genetic predisposition more supportive for being the basis of familial CLL [24].

SNP array-based chromosome alterations are overlapping between and within families with CLL

Several genetic alterations are common in CLL disease onset or clonal evolution. Accumulation of passenger mutations in normal counterpart cells can be an initiating event in leukemogenesis [25], where after subsequent driver aberrations can induce the next steps in clonal evolution and are generally found throughout the CLL patient population. Deletion of the short arm of chromosome 13 (del13q), trisomy 12, and mutations in the *MYD88* gene, encoding a signaling molecule acting downstream of Toll-like receptors, are examples of such driver aberrations in CLL[25, 26]. Clonal evolution is frequently directed by subclonal mutations which result in increased proliferation and decreased apoptosis over other CLL cells [25, 27]. Such alterations include gain-of-function mutations in *NOTCH1*, *SF3B1*, and *RAS* genes and loss-of-function mutations in *ATM,* a protein that functions in DNA double strand break (DSB) repair, and *TP53* involved in inducing apoptosis, or loss of chromosomal arms where these genes are located, i.e. del11q and del17p respectively [25, 26].

Table 2. Chromosomal aberrations as observed in the different members of the three families with CLL

Chromosomal aberration	Family member
Family 1	
13q14.2-13q14.3 del	1A, 1B, 1C, 1D
2q34-2q35 del	1C
Family 2	
Trisomy 12	2A, 2B
Family 3	
13q14.2-13q14.3 del	3A, 3B, 3C, 3D
11q21-11q24 del	3C

To see whether there are common genetic alterations at the chromosomal and allelic level uniformly present in familial CLL, all our familial CLL cases were analyzed by means of a SNP array. Notably, the most common chromosomal aberration in CLL, the 13q14.2-13q14.3 allelic deletion, was detected in all siblings of families 1 and 3 (**Table 2**). In contrast, the siblings of family 2 did not show this deletion, but both were found to contain the trisomy 12 aberration, albeit that in family member 2B this aberration was only found in part of the CLL cells (**Table 2** and data not shown). So we could detect the CLL–associated driver genetic aberrations, i.e. del13q in families 1 and 3, and trisomy 12 in family 2. Interestingly, in family member 3C an additional deletion of 11q21-11q24 was found (**Table 2**). Previously CLL#2 subset has been associated with a higher incidence of 11q22-q23 deletions and *SF3B1* mutations [28, 29]. Since family member 3C was found to have this particular 11q deletion, additional mutation analysis might shed light on the existence of *SF3B1* mutations in the CLL clone of this individual.

We then focused on less known and smaller chromosomal alterations (reviewed in [30]) occurring in all CLL family members within one family (**Table 3 + Supplementary tables**). Deletion of one allele or loss of heterozygosity (LOH) of chromosome region 11q22.3, where the *ATM* gene is located, was detected in the members of family 1. 11q22.3 LOH was also seen in family 2, albeit that this was in an adjacent location, not including *ATM*. Interestingly, in both families 2 and 3 also 11q23.1 LOH was seen. Chromosome 17p13.1 deletion, which is associated with loss of the *TP53* gene, we did not identify this loss in one of our families, although in family 1 we did detect LOH of the adjacent cytoband 17p13.2. Besides looking at these most common deletions, we also evaluated whether other known chromosomal aberrations for CLL[30] could be found in these CLL families. Although we could not detect other known copy number gains for CLL in our families, we did observe losses that have earlier been described in CLL. LOH in chromosome region 3p21.31 was seen in families 2 and 3, LOH of 4p15.2 (associated with del11q and del17p) in family 3, and LOH of 9q12-q21.11 (earlier described as the 9q13-q21.11 region) in families 1 and 2. We detected LOH of the 10q24.33

Table 3. Loss of heterozygosity observed in three families with CLL

Chromosomal aberration - Loss of heterozygosity	Family
3p21.31	1, 2
4p15.2	3
9q12-q21.11	1, 2
10q24.33	3 (associated with NFκB2 gene)
11q22.3	1 (region incl ATM), 2 (region not including ATM)
14q24.1-32.3	2
15q15.1	1 (region incl TP53BP), 2 (region incl RAD51)

region in family 3, which is adjacent to the 10q24.32 region that was earlier described in association with the *NFKB2* gene. Loss of 14q24.1-32.3 was previously found in association with trisomy 12 in CLL, and in line with this finding we found LOH of 14q32.2-q32.33 in family 2. In addition we detected 14q31.1 LOH for family 3. Finally, loss of 15q15.1 with designated candidate gene MGA, was detected in family 1 (15q15.1-15.3) and family 2 (15q15.1). Of note, the affected location did not contain the MGA gene. In fact in family 1, the LOH region involved the DNA damage repair gene *TP53BP*, whereas in family 2 it involved the *RAD51* gene, also known to be important for DSB repair.

Finally, it would be of interest to determine using whole genome or whole exome next-generation-sequencing approaches whether there are shared mutations in the non-CLL cells within each of these families, as this could point to predisposing events in CLL development. Additionally, using next-generation-sequencing methodology it would be relevant to evaluate whether comparable patterns of clonal evolution can be detected in the CLL clones within each family.

SUMMARY

The three families with siblings with CLL, as described in this study, illustrate that both immunogenetic and chromosomal similarities exist within these families in a disease that is known to be quite heterogeneous. Interestingly, from the immunogenetic standpoint these families represent three completely different groups of CLL, i.e. M-CLL (family 1), U-CLL (family 2) and CLL#2-like CLL (family 3), with member 3C being a true CLL#2 case and member 3B a closely related CLL#169 case. Additionally, several common chromosomal aberrations, which have been shown to be of prognostic significance, could be detected for all siblings of a CLL family. In families 1 and 3 this concerned 11q14.2-11q14.3 deletions, whereas both family 2 members had trisomy 12. Also, other minor losses, which have previously been associated with CLL, could be detected for all siblings within the respective families. Further analysis at

the gene level (e.g. whole exome or whole genome sequencing) is required to identify the relevant genes that are involved in CLL pathogenesis in the members of each of these three families.

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REFERENCES

1. DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L. *et al.* Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* (2014).
2. Slager, S.L., Caporaso, N.E., de Sanjose, S. & Goldin, L.R. Genetic susceptibility to chronic lymphocytic leukemia. *Semin Hematol* **50**, 296-302 (2013).
3. DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L. *et al.* Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* **64**, 252-271 (2014).
4. Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94**, 1848-1854 (1999).
5. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* **119**, 4467-4475 (2012).
6. Marinelli, M., Peragine, N., Di Maio, V., Chiaretti, S., De Propris, M.S., Raponi, S. *et al.* Identification of molecular and functional patterns of p53 alterations in chronic lymphocytic leukemia patients in different phases of the disease. *Haematologica* **98**, 371-375 (2013).
7. Guarini, A., Marinelli, M., Tavolaro, S., Bellacchio, E., Magliozzi, M., Chiaretti, S. *et al.* ATM gene alterations in chronic lymphocytic leukemia patients induce a distinct gene expression profile and predict disease progression. *Haematologica* **97**, 47-55 (2012).
8. Zenz, T., Mertens, D., Kuppers, R., Dohner, H. & Stilgenbauer, S. From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nat Rev Cancer* **10**, 37-50 (2010).
9. Abruzzo, L.V., Herling, C.D., Calin, G.A., Oakes, C., Barron, L.L., Banks, H.E. *et al.* Trisomy 12 chronic lymphocytic leukemia expresses a unique set of activated and targetable pathways. *Haematologica* (2018).
10. Goldin, L.R., Landgren, O., Marti, G.E. & Caporaso, N.E. Familial Aspects of Chronic Lymphocytic Leukemia, Monoclonal B-Cell Lymphocytosis (MBL), and Related Lymphomas. *European J Clin Med Oncol* **2**, 119-126 (2010).
11. Goldin, L.R., Bjorkholm, M., Kristinsson, S.Y., Turesson, I. & Landgren, O. Elevated risk of chronic lymphocytic leukemia and other indolent non-Hodgkin's lymphomas among relatives of patients with chronic lymphocytic leukemia. *Haematologica* **94**, 647-653 (2009).

12. Goldin, L.R., Pfeiffer, R.M., Li, X. & Hemminki, K. Familial risk of lymphoproliferative tumors in families of patients with chronic lymphocytic leukemia: results from the Swedish Family-Cancer Database. *Blood* **104**, 1850-1854 (2004).
13. Sellick, G.S., Goldin, L.R., Wild, R.W., Slager, S.L., Ressenti, L., Strom, S.S. *et al.* A high-density SNP genome-wide linkage search of 206 families identifies susceptibility loci for chronic lymphocytic leukemia. *Blood* **110**, 3326-3333 (2007).
14. Slager, S.L., Rabe, K.G., Achenbach, S.J., Vachon, C.M., Goldin, L.R., Strom, S.S. *et al.* Genome-wide association study identifies a novel susceptibility locus at 6p21.3 among familial CLL. *Blood* **117**, 1911-1916 (2011).
15. Di Bernardo, M.C., Crowther-Swanepoel, D., Broderick, P., Webb, E., Sellick, G., Wild, R. *et al.* A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia. *Nat Genet* **40**, 1204-1210 (2008).
16. Mack, T.M., Cozen, W., Shibata, D.K., Weiss, L.M., Nathwani, B.N., Hernandez, A.M. *et al.* Concordance for Hodgkin's disease in identical twins suggesting genetic susceptibility to the young-adult form of the disease. *N Engl J Med* **332**, 413-418 (1995).
17. Cerhan, J.R. & Slager, S.L. Familial predisposition and genetic risk factors for lymphoma. *Blood* **126**, 2265-2273 (2015).
18. Langerak, A.W., Davi, F., Ghia, P., Hadzidimitriou, A., Murray, F., Potter, K.N. *et al.* Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. *Leukemia* **25**, 979-984 (2011).
19. van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L. *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* **17**, 2257-2317 (2003).
20. Fernhout, F., Dinkelaar, R.B., Hagemeijer, A., Groeneveld, K., van Kammen, E. & van Dongen, J.J. Four aged siblings with B cell chronic lymphocytic leukemia. *Leukemia* **11**, 2060-2065 (1997).
21. Kriangkum, J., Motz, S.N., Mack, T., Beiggi, S., Baigorri, E., Kuppusamy, H. *et al.* Single-Cell Analysis and Next-Generation Immuno-Sequencing Show That Multiple Clones Persist in Patients with Chronic Lymphocytic Leukemia. *PLoS One* **10**, e0137232 (2015).
22. Baliakas, P., Agathangelidis, A., Hadzidimitriou, A., Sutton, L.A., Minga, E., Tsanousa, A. *et al.* Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. *Blood* **125**, 856-859 (2015).
23. Minici, C., Gounari, M., Ubelhart, R., Scarfo, L., Duhren-von Minden, M., Schneider, D. *et al.* Distinct homotypic B-cell receptor interactions shape the outcome of chronic lymphocytic leukaemia. *Nat Commun* **8**, 15746 (2017).
24. Crowther-Swanepoel, D., Wild, R., Sellick, G., Dyer, M.J., Mauro, F.R., Cuthbert, R.J. *et al.* Insight into the pathogenesis of chronic lymphocytic leukemia (CLL) through analysis of IgVH gene usage and mutation status in familial CLL. *Blood* **111**, 5691-5693 (2008).
25. Landau, D.A., Carter, S.L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M.S. *et al.* Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* **152**, 714-726 (2013).
26. Puente, X.S., Pinyol, M., Quesada, V., Conde, L., Ordonez, G.R., Villamor, N. *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* **475**, 101-105 (2011).

27. Zhao, Z., Goldin, L., Liu, S., Wu, L., Zhou, W., Lou, H. *et al.* Evolution of multiple cell clones over a 29-year period of a CLL patient. *Nat Commun* **7**, 13765 (2016).
28. Marincevic, M., Cahill, N., Gunnarsson, R., Isaksson, A., Mansouri, M., Goransson, H. *et al.* High-density screening reveals a different spectrum of genomic aberrations in chronic lymphocytic leukemia patients with 'stereotyped' IGHV3-21 and IGHV4-34 B-cell receptors. *Haematologica* **95**, 1519-1525 (2010).
29. Baliakas, P., Hadzidimitriou, A., Sutton, L.A., Minga, E., Agathangelidis, A., Nichelatti, M. *et al.* Clinical effect of stereotyped B-cell receptor immunoglobulins in chronic lymphocytic leukaemia: a retrospective multicentre study. *Lancet Haematol* **1**, e74-84 (2014).
30. Chun, K., Wenger, G.D., Chaubey, A., Dash, D.P., Kanagal-Shamanna, R., Kantarci, S. *et al.* Assessing copy number aberrations and copy-neutral loss-of-heterozygosity across the genome as best practice: An evidence-based review from the Cancer Genomics Consortium (CGC) working group for chronic lymphocytic leukemia. *Cancer Genet* **228-229**, 236-250 (2018).

SUPPLEMENTARY DATA

SNP analysis

Family 1

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr5:104,431,051-104,505,444	74393	q21.2 - q21.3	CN Loss	1	0	100.0
chr13:50,594,028-50,977,946	383918	q14.2 - q14.3	CN Loss	6	2	100.0
chrY:2,655,180-3,274,356	619176	p11.31 - p11.2	CN Loss	4	0	100.0
chrY:6,088,397-6,453,668	365271	p11.2	CN Loss	14	0	100.0
chrY:6,614,711-9,974,735	3360024	p11.2	CN Loss	41	0	100.0
chrY:13,504,884-19,567,718	6062834	q11.21 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0
chr1:35,379,026-36,172,748	793722	p34.3	LOH	10	0	100.0
chr1:49,217,100-50,594,083	1376983	p33	LOH	5	0	100.0
chr1:72,554,899-74,232,322	1677423	p31.1	LOH	2	0	100.0
chr1:102,892,857-103,489,911	597054	p21.1	LOH	1	0	100.0
chr1:185,560,304-186,155,434	595130	q25.3 - q31.1	LOH	3	0	100.0
chr2:27,997,621-28,600,002	602381	p23.2	LOH	7	1	100.0
chr2:74,468,875-74,969,186	500311	p13.1	LOH	24	0	100.0
chr2:110,471,451-111,450,478	979027	q13	LOH	27	3	100.0
chr2:175,584,886-176,424,238	839352	q31.1	LOH	5	1	100.0
chr2:198,143,796-198,956,120	812324	q33.1	LOH	12	0	100.0
chr2:203,634,425-204,414,782	780357	q33.2	LOH	8	0	100.0
chr3:52,269,021-52,817,501	548480	p21.2 - p21.1	LOH	25	2	100.0
chr3:74,696,073-75,246,290	550217	p12.3	LOH	0	0	100.0
chr3:138,121,037-138,843,786	722749	q22.3 - q23	LOH	12	0	100.0
chr4:33,464,268-34,721,840	1257572	p15.1	LOH	1	0	100.0
chr4:47,707,595-48,228,024	520429	p12 - p11	LOH	8	0	100.0
chr4:48,353,699-50,400,000	2046301	p11 - q11	LOH	7	0	100.0
chr4:52,697,856-53,310,666	612810	q11 - q12	LOH	4	0	100.0
chr5:61,459,189-61,990,246	531057	q12.1	LOH	5	0	100.0
chr5:109,640,022-110,385,094	745072	q22.1	LOH	2	1	100.0
chr5:121,046,612-121,490,403	443791	q23.1 - q23.2	LOH	4	0	100.0
chr5:176,900,534-177,083,853	183319	q35.3	LOH	8	0	100.0
chr6:27,812,754-28,325,491	512737	p22.1	LOH	23	0	100.0
chr6:34,488,013-35,366,938	878925	p21.31	LOH	12	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr6:63,145,180-63,856,600	711420	q11.1 - q12	LOH	0	0	100.0
chr6:83,969,883-84,940,602	970719	q14.2 - q14.3	LOH	7	0	100.0
chr6:121,193,332-121,721,042	527710	q22.31	LOH	1	0	100.0
chr6:128,530,979-129,060,850	529871	q22.33	LOH	1	0	100.0
chr8:42,468,283-43,570,212	1101929	p11.21 - p11.1	LOH	10	1	100.0
chr8:47,002,900-49,782,743	2779843	q11.1 - q11.21	LOH	10	0	100.0
chr8:50,041,266-50,281,380	240114	q11.21	LOH	0	0	100.0
chr9:40,714,011-44,887,313	4173302	p13.1 - p11.2	LOH	29	0	100.0
chr9:65,629,772-69,410,435	3780663	q12 - q21.11	LOH	19	3	100.0
chr10:22,097,219-22,778,784	681565	p12.31 - p12.2	LOH	8	0	100.0
chr11:107,836,378-108,377,055	540677	q22.3	LOH	7	0	100.0
chr12:88,346,583-89,141,109	794526	q21.32 - q21.33	LOH	5	0	100.0
chr12:112,090,689-112,870,613	779924	q24.12 - q24.13	LOH	15	3	100.0
chr13:19,967,131-20,479,141	512010	q12.11	LOH	4	0	100.0
chr15:42,628,517-44,360,693	1732176	q15.1 - q15.3	LOH	39	1	100.0
chr15:44,506,807-45,093,361	586554	q15.3 - q21.1	LOH	9	0	100.0
chr15:50,035,265-50,193,311	158046	q21.2	LOH	1	0	100.0
chr16:21,980,261-22,674,107	693846	p12.2	LOH	11	0	100.0
chr16:66,914,076-67,031,763	117687	q22.1	LOH	7	0	100.0
chr17:4,838,934-5,399,205	560271	p13.2	LOH	25	2	100.0
chr17:58,047,835-58,826,484	778649	q23.1 - q23.2	LOH	14	1	100.0
chr18:18,556,505-19,485,452	928947	q11.1 - q11.2	LOH	10	3	100.0
chrX:18,940,301-19,514,138	573837	p22.13 - p22.12	LOH	4	0	100.0
chrX:27,457,880-28,033,736	575856	p21.3	LOH	4	0	100.0
chrX:35,866,731-36,448,757	582026	p21.1	LOH	5	0	100.0
chrX:36,867,611-37,648,093	780482	p21.1 - p11.4	LOH	6	0	100.0
chrX:41,238,580-41,814,194	575614	p11.4	LOH	4	0	100.0
chrX:47,515,770-48,056,381	540611	p11.23	LOH	13	0	100.0
chrX:53,158,285-55,189,679	2031394	p11.22 - p11.21	LOH	28	5	100.0
chrX:70,879,864-71,616,164	736300	q13.1	LOH	16	0	100.0
chrX:78,609,014-80,019,624	1410610	q21.1	LOH	5	0	100.0
chrX:84,031,249-85,101,938	1070689	q21.1 - q21.2	LOH	6	1	100.0
chrX:93,231,600-95,058,136	1826536	q21.32 - q21.33	LOH	0	1	100.0
chrX:96,413,763-96,929,550	515787	q21.33	LOH	2	0	100.0
chrX:100,866,801-102,191,205	1324404	q22.1	LOH	22	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chrX:104,464,158-104,950,968	486810	q22.3	LOH	2	0	100.0
chrX:109,569,422-111,390,962	1821540	q23	LOH	13	0	100.0
chrX:113,769,528-114,561,241	791713	q23	LOH	12	6	100.0
chrX:116,605,263-117,608,772	1003509	q24	LOH	3	1	100.0
chrX:120,222,656-122,317,172	2094516	q24 - q25	LOH	0	1	100.0
chrX:129,624,961-130,779,855	1154894	q26.1 - q26.2	LOH	5	0	100.0
chrX:135,586,894-136,176,088	589194	q26.3	LOH	9	1	100.0
chrX:138,457,383-139,132,961	675578	q27.1	LOH	6	1	100.0
chrX:153,183,036-154,918,789	1735753	q28	LOH	92	8	100.0

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Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr12:31,240,839-31,407,801	166962	p11.21	Allelic Imbalance	1	0	100.0
chrY:7,464,715-10,077,460	2612745	p11.2	Allelic Imbalance	38	0	100.0
chr12:0-256,127	256127	p13.33	CN Gain	4	0	100.0
chr12:3,302,395-3,340,081	37686	p13.32	CN Gain	1	0	100.0
chr12:9,219,050-9,272,773	53723	p13.31	CN Gain	2	0	100.0
chr12:17,525,916-17,639,517	113601	p12.3	CN Gain	0	0	100.0
chr12:19,414,992-20,922,051	1507059	p12.3 - p12.2	CN Gain	5	0	100.0
chr12:21,447,001-21,464,915	17914	p12.1	CN Gain	1	0	100.0
chr12:31,240,839-31,407,801	166962	p11.21	CN Gain	1	0	100.0
chr12:33,846,842-35,800,000	1953158	p11.1 - q11	CN Gain	1	0	100.0
chr12:52,960,073-53,010,650	50577	q13.13	CN Gain	4	0	100.0
chr12:54,385,273-54,687,878	302605	q13.13	CN Gain	19	3	100.0
chr12:59,459,881-62,137,864	2677983	q14.1	CN Gain	2	0	100.0
chr12:64,324,996-64,409,482	84486	q14.2	CN Gain	1	0	100.0
chr12:69,513,557-69,542,929	29372	q15	CN Gain	0	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr12:71,249,238-71,388,234	138996	q15	CN Gain	1	0	100.0
chr12:71,482,919-71,570,918	87999	q15 - q21.1	CN Gain	1	0	100.0
chr12:72,728,822-73,077,229	348407	q21.1	CN Gain	1	0	100.0
chr12:73,343,040-74,402,940	1059900	q21.1	CN Gain	1	0	100.0
chr12:74,850,958-75,015,653	164695	q21.1	CN Gain	1	0	100.0
chr12:76,636,821-76,941,675	304854	q21.2	CN Gain	2	0	100.0
chr12:77,215,437-77,398,066	182629	q21.2	CN Gain	2	0	100.0
chr12:77,722,882-78,269,390	546508	q21.2	CN Gain	1	0	100.0
chr12:78,508,794-81,089,543	2580749	q21.2 - q21.31	CN Gain	8	1	100.0
chr12:82,537,785-82,976,126	438341	q21.31	CN Gain	2	0	100.0
chr12:83,147,536-85,410,579	2263043	q21.31	CN Gain	3	0	100.0
chr12:85,920,682-87,533,262	1612580	q21.31 - q21.32	CN Gain	4	0	100.0
chr12:98,207,772-98,487,513	279741	q23.1	CN Gain	1	2	100.0
chr12:99,196,800-99,229,824	33024	q23.1	CN Gain	1	0	100.0
chr12:101,194,298-101,379,071	184773	q23.1	CN Gain	1	0	100.0
chr12:126,738,610-126,881,735	143125	q24.32	CN Gain	0	0	100.0
chr12:127,850,708-128,208,987	358279	q24.32	CN Gain	1	0	100.0
chr12:128,406,449-128,522,391	115942	q24.32	CN Gain	1	0	100.0
chr12:129,171,957-129,247,690	75733	q24.32	CN Gain	1	0	100.0
chr12:129,459,964-129,530,498	70534	q24.33	CN Gain	1	0	100.0
chr12:129,808,003-129,870,681	62678	q24.33	CN Gain	1	0	100.0
chr12:130,808,601-130,911,986	103385	q24.33	CN Gain	2	0	100.0
chr12:131,557,897-131,654,487	96590	q24.33	CN Gain	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chrY:5,893,803-6,088,397	194594	p11.2	CN Gain	0	0	100.0
chr1:111,927,724-111,939,188	11464	p13.2	CN Loss	1	0	100.0
chr8:9,054,811-9,063,535	8724	p23.1	CN Loss	1	0	100.0
chr15:21,903,815-22,701,317	797502	q11.2	CN Loss	15	4	100.0
chr19:24,378,791-24,629,285	250494	p12 - p11	CN Loss	0	0	100.0
chrX:2,699,012-58,483,247	55784235	p22.33 - p11.1	CN Loss	460	34	100.0
chrX:61,719,290-154,918,789	93199499	q11.1 - q28	CN Loss	802	82	100.0
chrY:2,655,180-3,205,097	549917	p11.31 - p11.2	CN Loss	4	0	100.0
chrY:6,088,397-10,077,460	3989063	p11.2	CN Loss	55	0	100.0
chrY:12,500,000-19,567,718	7067718	q11.1 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0
chrY:27,390,536-28,817,458	1426922	q11.23 - q12	CN Loss	7	0	100.0
chr1:4,016,259-4,717,365	701106	p36.32	LOH	2	0	100.0
chr1:5,786,633-7,123,793	1337160	p36.31	LOH	26	2	100.0
chr1:35,380,394-36,653,079	1272685	p34.3	LOH	20	0	100.0
chr1:52,444,299-53,314,329	870030	p32.3	LOH	14	0	100.0
chr1:102,715,763-103,228,130	512367	p21.1	LOH	0	0	100.0
chr1:108,754,355-109,260,195	505840	p13.3	LOH	6	0	100.0
chr1:146,982,244-150,273,115	3290871	q21.1 - q21.2	LOH	72	2	100.0
chr1:155,198,085-155,856,049	657964	q22	LOH	20	1	100.0
chr1:184,919,131-185,321,452	402321	q25.3	LOH	6	0	100.0
chr1:189,878,840-189,917,632	38792	q31.1	LOH	0	0	100.0
chr1:196,931,277-197,833,592	902315	q31.3	LOH	6	0	100.0
chr1:248,578,156-249,250,621	672465	q44	LOH	18	1	100.0
chr2:72,400,279-73,127,289	727010	p13.2	LOH	2	0	100.0
chr2:83,038,741-83,445,915	407174	p12 - p11.2	LOH	1	0	100.0
chr2:115,582,560-116,803,204	1220644	q14.1	LOH	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr2:120,415,435-121,073,997	658562	q14.2	LOH	5	0	100.0
chr2:122,321,522-122,501,276	179754	q14.2 - q14.3	LOH	3	0	100.0
chr2:135,596,163-137,047,657	1451494	q21.3 - q22.1	LOH	15	2	100.0
chr2:144,680,114-145,603,608	923494	q22.3	LOH	6	0	100.0
chr2:155,651,456-156,421,270	769814	q24.1	LOH	1	0	100.0
chr2:162,934,383-163,694,830	760447	q24.2	LOH	7	0	100.0
chr2:188,885,530-189,739,668	854138	q32.1 - q32.2	LOH	3	1	100.0
chr2:189,767,583-189,845,650	78067	q32.2	LOH	3	2	100.0
chr2:189,881,782-190,945,244	1063462	q32.2	LOH	11	1	100.0
chr2:196,447,589-197,139,484	691895	q32.3	LOH	5	0	100.0
chr2:197,302,186-197,467,881	165695	q32.3 - q33.1	LOH	1	0	100.0
chr2:209,680,376-211,378,875	1698499	q34	LOH	10	0	100.0
chr2:229,364,088-229,901,642	537554	q36.3	LOH	1	0	100.0
chr2:235,193,356-235,982,045	788689	q37.1 - q37.2	LOH	2	0	100.0
chr3:17,217,661-17,919,247	701586	p24.3	LOH	1	0	100.0
chr3:37,814,046-38,362,894	548848	p22.2	LOH	12	1	100.0
chr3:46,703,041-47,652,726	949685	p21.31	LOH	20	0	100.0
chr3:47,940,266-49,361,616	1421350	p21.31	LOH	53	10	100.0
chr3:50,442,701-52,012,422	1569721	p21.31 - p21.2	LOH	27	1	100.0
chr3:57,214,270-57,963,559	749289	p14.3	LOH	8	0	100.0
chr3:58,698,805-59,262,425	563620	p14.2	LOH	2	0	100.0
chr3:74,874,137-75,688,849	814712	p12.3	LOH	4	2	100.0
chr3:81,189,382-82,269,480	1080098	p12.2	LOH	1	0	100.0
chr3:84,962,866-85,519,268	556402	p12.1	LOH	2	1	100.0
chr3:89,144,116-91,000,000	1855884	p11.1 - q11.1	LOH	1	0	100.0
chr3:93,537,290-95,152,163	1614873	q11.1 - q11.2	LOH	6	0	100.0
chr3:96,453,404-97,451,705	998301	q11.2	LOH	1	0	100.0
chr3:98,769,252-99,361,262	592010	q12.1	LOH	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr3:123,211,813-123,963,648	751835	q21.1 - q21.2	LOH	8	1	100.0
chr3:126,569,921-127,473,759	903838	q21.3	LOH	11	2	100.0
chr3:128,693,984-129,687,483	993499	q21.3 - q22.1	LOH	22	1	100.0
chr3:130,562,656-131,149,371	586715	q22.1	LOH	6	0	100.0
chr3:140,221,000-141,453,382	1232382	q23	LOH	8	0	100.0
chr3:145,338,487-149,232,963	3894476	q24 - q25.1	LOH	20	0	100.0
chr3:152,307,615-153,017,777	710162	q25.2	LOH	2	0	100.0
chr3:159,254,869-159,905,509	650640	q25.33	LOH	6	0	100.0
chr3:160,406,601-161,139,545	732944	q25.33 - q26.1	LOH	4	0	100.0
chr3:162,439,405-163,241,531	802126	q26.1	LOH	1	0	100.0
chr3:175,115,958-175,670,087	554129	q26.31	LOH	3	0	100.0
chr3:191,064,673-191,594,905	530232	q28	LOH	3	0	100.0
chr3:197,316,962-198,022,430	705468	q29	LOH	11	1	100.0
chr4:35,689,092-36,299,203	610111	p15.1 - p14	LOH	3	0	100.0
chr4:43,195,130-43,767,034	571904	p13	LOH	0	0	100.0
chr4:48,333,953-50,400,000	2066047	p11 - q11	LOH	7	0	100.0
chr4:62,631,861-63,335,182	703321	q13.1	LOH	2	0	100.0
chr4:65,630,867-66,146,740	515873	q13.1	LOH	1	0	100.0
chr4:66,344,312-67,206,875	862563	q13.1 - q13.2	LOH	2	1	100.0
chr4:74,980,499-75,654,445	673946	q13.3	LOH	5	0	100.0
chr4:81,205,180-81,957,891	752711	q21.21	LOH	3	0	100.0
chr4:86,692,130-88,310,459	1618329	q21.23 - q22.1	LOH	12	2	100.0
chr4:88,431,819-89,285,296	853477	q22.1	LOH	9	0	100.0
chr4:96,733,414-97,648,466	915052	q22.3	LOH	1	0	100.0
chr4:109,875,222-110,630,257	755035	q25	LOH	5	1	100.0
chr4:131,100,989-131,641,353	540364	q28.3	LOH	0	0	100.0
chr5:23,723,195-24,810,874	1087679	p14.2 - p14.1	LOH	1	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr5:26,389,096-27,810,897	1421801	p14.1	LOH	2	0	100.0
chr5:32,650,154-33,521,774	871620	p13.3	LOH	3	0	100.0
chr5:45,484,737-46,399,093	914356	p12 - p11	LOH	1	0	100.0
chr5:54,320,927-54,862,001	541074	q11.2	LOH	14	4	100.0
chr5:70,305,696-70,970,864	665168	q13.2	LOH	7	0	100.0
chr5:74,285,356-74,925,970	640614	q13.3	LOH	6	0	100.0
chr5:79,007,849-79,602,200	594351	q14.1	LOH	6	0	100.0
chr5:87,783,396-87,789,234	5838	q14.3	LOH	0	0	100.0
chr5:100,083,616-100,606,712	523096	q21.1	LOH	1	1	100.0
chr5:102,102,192-102,829,017	726825	q21.1 - q21.2	LOH	4	0	100.0
chr5:109,614,961-110,388,292	773331	q22.1	LOH	2	1	100.0
chr5:151,905,383-152,471,313	565930	q33.1	LOH	1	0	100.0
chr5:162,737,230-174,289,546	11552316	q34 - q35.2	LOH	67	9	100.0
chr6:27,643,618-27,874,755	231137	p22.1	LOH	18	0	100.0
chr6:46,150,913-47,007,505	856592	p21.1 - p12.3	LOH	12	0	100.0
chr6:63,757,593-64,355,373	597780	q12	LOH	3	0	100.0
chr6:69,303,495-69,864,787	561292	q12	LOH	2	0	100.0
chr7:33,017,558-33,554,811	537253	p14.3	LOH	4	0	100.0
chr7:83,947,969-84,714,347	766378	q21.11	LOH	2	0	100.0
chr7:97,657,477-98,297,659	640182	q21.3 - q22.1	LOH	6	0	100.0
chr7:98,784,554-100,218,724	1434170	q22.1	LOH	70	5	100.0
chr7:118,350,126-120,161,811	1811685	q31.31	LOH	1	0	100.0
chr7:127,977,707-128,521,335	543628	q32.1	LOH	15	0	100.0
chr7:132,895,740-133,740,061	844321	q33	LOH	3	1	100.0
chr8:33,198,980-33,976,120	777140	p12	LOH	5	0	100.0
chr8:50,400,061-51,371,902	971841	q11.21	LOH	1	0	100.0
chr8:57,801,924-58,414,726	612802	q12.1	LOH	5	0	100.0
chr8:60,941,817-61,544,883	603066	q12.1	LOH	3	0	100.0
chr8:71,466,636-72,016,193	549557	q13.3	LOH	4	0	100.0
chr8:91,245,848-91,831,782	585934	q21.3	LOH	4	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr8:104,452,240-105,466,077	1013837	q22.3	LOH	4	0	100.0
chr8:106,768,766-107,381,279	612513	q23.1	LOH	3	0	100.0
chr8:111,232,782-112,194,918	962136	q23.2 - q23.3	LOH	2	0	100.0
chr8:112,612,536-114,668,761	2056225	q23.3	LOH	2	1	100.0
chr8:120,030,797-120,562,718	531921	q24.12	LOH	3	1	100.0
chr9:30,118,918-30,824,061	705143	p21.1	LOH	1	0	100.0
chr9:40,714,011-44,887,313	4173302	p13.1 - p11.2	LOH	29	0	100.0
chr9:65,629,772-69,410,435	3780663	q12 - q21.11	LOH	19	3	100.0
chr9:75,135,868-75,708,218	572350	q21.13	LOH	3	0	100.0
chr10:57,392,918-58,341,005	948087	q21.1	LOH	1	0	100.0
chr10:73,795,084-75,737,378	1942294	q22.1 - q22.2	LOH	37	1	100.0
chr10:86,442,005-86,501,917	59912	q23.1	LOH	0	0	100.0
chr11:109,811,640-109,865,601	53961	q22.3	LOH	0	0	100.0
chr11:111,340,514-111,968,864	628350	q23.1	LOH	20	2	100.0
chr12:29,907,850-30,500,007	592157	p11.22	LOH	1	0	100.0
chr12:42,351,506-42,874,797	523291	q12	LOH	5	1	100.0
chr12:43,974,715-44,929,120	954405	q12	LOH	5	0	100.0
chr12:75,048,084-75,666,423	618339	q21.1	LOH	1	0	100.0
chr12:79,922,622-80,624,751	702129	q21.2 - q21.31	LOH	3	0	100.0
chr12:81,344,005-81,863,060	519055	q21.31	LOH	4	1	100.0
chr12:87,806,746-88,346,583	539837	q21.32	LOH	1	0	100.0
chr12:88,816,482-89,327,106	510624	q21.32 - q21.33	LOH	1	0	100.0
chr12:90,267,231-91,258,064	990833	q21.33	LOH	0	0	100.0
chr12:92,064,462-92,680,637	616175	q21.33 - q22	LOH	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr13:65,384,770-66,338,879	954109	q21.31 - q21.32	LOH	0	0	100.0
chr13:71,977,167-72,478,934	501767	q21.33	LOH	1	0	100.0
chr13:77,540,443-78,197,615	657172	q22.3	LOH	5	0	100.0
chr13:87,041,530-87,732,973	691443	q31.1 - q31.2	LOH	0	0	100.0
chr14:37,447,823-38,100,171	652348	q13.3 - q21.1	LOH	4	0	100.0
chr14:40,341,881-42,311,608	1969727	q21.1	LOH	2	0	100.0
chr14:42,557,388-43,094,022	536634	q21.1	LOH	0	0	100.0
chr14:56,527,831-57,247,713	719882	q22.3	LOH	2	0	100.0
chr14:63,291,869-63,898,582	606713	q23.2	LOH	4	0	100.0
chr14:98,891,530-107,349,540	8458010	q32.2 - q32.33	LOH	195	70	100.0
chr15:28,179,087-29,424,997	1245910	q13.1	LOH	20	2	100.0
chr15:29,734,810-30,296,926	562116	q13.1	LOH	2	0	100.0
chr15:35,445,410-35,952,899	507489	q14	LOH	4	1	100.0
chr15:40,641,754-41,146,399	504645	q15.1	LOH	19	0	100.0
chr15:42,993,637-43,542,417	548780	q15.2	LOH	8	0	100.0
chr15:50,035,265-50,726,076	690811	q21.2	LOH	8	1	100.0
chr15:52,356,004-53,014,853	658849	q21.2 - q21.3	LOH	9	1	100.0
chr15:53,904,718-54,591,907	687189	q21.3	LOH	2	0	100.0
chr16:25,071,876-25,594,081	522205	p12.1	LOH	5	0	100.0
chr17:18,910,214-19,595,331	685117	p11.2	LOH	22	1	100.0
chr17:27,923,227-28,562,673	639446	q11.2	LOH	8	2	100.0
chr17:58,042,824-58,907,819	864995	q23.1 - q23.2	LOH	14	1	100.0
chr18:36,365,993-36,985,148	619155	q12.2	LOH	1	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr18:61,126,522-61,655,577	529055	q21.33 - q22.1	LOH	11	0	100.0
chr18:72,077,495-72,739,391	661896	q22.3	LOH	5	0	100.0
chr19:21,166,576-22,086,094	919518	p12	LOH	11	0	100.0
chr19:22,687,065-23,378,095	691030	p12	LOH	10	0	100.0
chr19:42,740,369-43,427,508	687139	q13.2 - q13.31	LOH	20	1	100.0
chr19:55,412,185-56,428,148	1015963	q13.42 - q13.43	LOH	54	5	100.0
chr19:56,776,801-57,604,668	827867	q13.43	LOH	17	0	100.0
chr20:21,754,925-22,266,532	511607	p11.22	LOH	2	0	100.0
chr20:24,792,689-26,293,985	1501296	p11.21 - p11.1	LOH	21	1	100.0
chr20:30,631,997-31,187,852	555855	q11.21	LOH	10	1	100.0
chr20:32,848,215-33,418,434	570219	q11.22	LOH	9	1	100.0
chr22:22,556,516-23,267,314	710798	q11.22	LOH	10	2	100.0
chr22:32,026,214-32,550,766	524552	q12.2 - q12.3	LOH	8	0	100.0
chrX:2,699,012-58,483,247	55784235	p22.33 - p11.1	LOH	460	34	100.0
chrX:61,719,290-88,460,879	26741589	q11.1 - q21.31	LOH	151	14	100.0
chrX:92,364,640-154,918,789	62554149	q21.32 - q28	LOH	647	68	100.0
chrY:14,368,629-19,567,718	5199089	q11.21 - q11.221	LOH	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	LOH	55	0	100.0

Family 3

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr2:117,777,567-117,937,434	159867	q14.1	Allelic Imbalance	0	0	100.0
chr2:117,777,567-117,942,955	165388	q14.1	CN Gain	0	0	100.0
chr4:64,131,761-64,152,984	21223	q13.1	CN Loss	0	0	100.0
chr13:50,565,275-50,977,946	412671	q14.2 - q14.3	CN Loss	8	3	100.0
chr13:69,250,062-69,279,507	29445	q21.33	CN Loss	0	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	mi-RNAs	Frequency %
chrY:2,655,180-2,915,938	260758	p11.31	CN Loss	4	0	100.0
chrY:6,088,397-6,453,668	365271	p11.2	CN Loss	14	0	100.0
chrY:6,614,711-9,974,735	3360024	p11.2	CN Loss	41	0	100.0
chrY:13,504,884-19,567,718	6062834	q11.21 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0
chrY:27,390,536-28,817,458	1426922	q11.23 - q12	CN Loss	7	0	100.0
chr1:12,867,459-13,894,497	1027038	p36.21	LOH	47	0	100.0
chr1:35,380,394-36,583,527	1203133	p34.3	LOH	18	0	100.0
chr1:44,011,963-44,541,978	530015	p34.2 - p34.1	LOH	13	1	100.0
chr1:52,420,352-52,948,569	528217	p32.3	LOH	9	0	100.0
chr1:87,017,898-87,586,244	568346	p22.3	LOH	5	0	100.0
chr1:88,988,945-89,520,321	531376	p22.2	LOH	7	0	100.0
chr1:92,884,038-93,471,407	587369	p22.1	LOH	6	0	100.0
chr1:151,791,836-152,310,313	518477	q21.3	LOH	14	0	100.0
chr1:155,198,085-156,023,132	825047	q22	LOH	30	2	100.0
chr2:72,267,907-74,122,284	1854377	p13.2 - p13.1	LOH	22	0	100.0
chr2:125,743,710-126,273,043	529333	q14.3	LOH	0	0	100.0
chr2:135,279,341-136,833,450	1554109	q21.3 - q22.1	LOH	15	2	100.0
chr2:152,675,099-152,869,334	194235	q23.3	LOH	2	0	100.0
chr2:197,309,620-198,041,313	731693	q32.3 - q33.1	LOH	7	0	100.0
chr3:46,754,908-47,652,726	897818	p21.31	LOH	18	0	100.0
chr3:48,626,312-48,655,179	28867	p21.31	LOH	2	0	100.0
chr3:51,977,348-52,538,540	561192	p21.2 - p21.1	LOH	27	2	100.0
chr3:58,202,231-58,491,150	288919	p14.3	LOH	6	0	100.0
chr3:96,423,966-96,696,356	272390	q11.2	LOH	1	0	100.0
chr3:96,926,055-97,232,093	306038	q11.2	LOH	1	0	100.0
chr4:8,677,677-9,825,907	1148230	p16.1	LOH	90	1	100.0
chr4:60,857,979-61,358,488	500509	q13.1	LOH	0	0	100.0
chr4:81,208,622-81,877,198	668576	q21.21	LOH	2	0	100.0
chr4:93,607,879-94,116,170	508291	q22.1 - q22.2	LOH	1	0	100.0
chr4:131,595,687-131,791,265	195578	q28.3	LOH	0	0	100.0
chr4:170,151,499-170,647,296	495797	q33	LOH	3	0	100.0
chr5:12,010,729-12,593,544	582815	p15.2	LOH	1	0	100.0
chr5:54,523,599-55,036,332	512733	q11.2	LOH	8	1	100.0
chr5:63,165,056-63,742,557	577501	q12.2 - q12.3	LOH	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	mi-RNAs	Frequency %
chr5:109,605,654-110,086,128	480474	q22.1	LOH	2	1	100.0
chr5:130,146,514-130,560,692	414178	q23.3	LOH	2	0	100.0
chr6:15,255,688-15,743,928	488240	p22.3	LOH	2	0	100.0
chr6:48,524,782-49,353,062	828280	p12.3	LOH	0	0	100.0
chr6:97,645,281-98,180,734	535453	q16.1	LOH	3	0	100.0
chr6:116,177,802-117,079,925	902123	q22.1	LOH	16	0	100.0
chr6:128,530,979-129,060,850	529871	q22.33	LOH	1	0	100.0
chr7:77,104,498-77,606,655	502157	q11.23 - q21.11	LOH	5	0	100.0
chr7:98,820,525-99,465,188	644663	q22.1	LOH	21	0	100.0
chr7:104,620,677-105,143,060	522383	q22.3	LOH	5	0	100.0
chr7:123,012,750-123,448,271	435521	q31.32	LOH	7	0	100.0
chr8:66,456,396-67,089,473	633077	q13.1	LOH	7	0	100.0
chr8:145,085,554-145,567,655	482101	q24.3	LOH	22	5	100.0
chr10:32,701,399-33,354,991	653592	p11.22	LOH	3	0	100.0
chr10:103,471,966-103,879,746	407780	q24.32	LOH	8	0	100.0
chr11:14,341,632-14,895,559	553927	p15.2	LOH	4	0	100.0
chr11:84,843,246-85,386,644	543398	q14.1	LOH	4	0	100.0
chr11:111,390,064-111,983,755	593691	q23.1	LOH	17	0	100.0
chr12:33,444,058-35,800,000	2355942	p11.1 - q11	LOH	2	0	100.0
chr12:37,876,400-39,325,273	1448873	q11 - q12	LOH	2	0	100.0
chr12:50,686,641-51,147,777	461136	q13.12	LOH	3	0	100.0
chr14:55,308,648-55,883,645	574997	q22.2 - q22.3	LOH	10	1	100.0
chr14:81,065,439-81,747,472	682033	q31.1	LOH	5	0	100.0
chr15:43,256,833-43,905,857	649024	q15.2 - q15.3	LOH	16	0	100.0
chr15:56,785,467-57,254,721	469254	q21.3	LOH	3	0	100.0
chr15:64,312,554-64,970,632	658078	q22.31	LOH	9	0	100.0
chr15:67,463,337-68,238,646	775309	q22.33 - q23	LOH	9	0	100.0
chr15:72,104,485-73,332,708	1228223	q23 - q24.1	LOH	17	1	100.0
chr15:76,540,526-77,232,177	691651	q24.2 - q24.3	LOH	5	1	100.0
chr16:21,738,928-22,674,107	935179	p12.2	LOH	13	0	100.0
chr16:28,328,430-28,825,827	497397	p11.2	LOH	18	2	100.0
chr16:30,556,442-31,010,980	454538	p11.2	LOH	25	2	100.0
chr16:32,151,873-34,231,404	2079531	p11.2	LOH	16	0	100.0
chr17:27,890,925-29,303,662	1412737	q11.2	LOH	25	2	100.0
chr17:30,106,603-30,612,412	505809	q11.2	LOH	8	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	mi-RNAs	Frequency %
chr17:58,321,300-58,826,484	505184	q23.2	LOH	6	0	100.0
chr18:38,845,074-39,409,691	564617	q12.3	LOH	1	0	100.0
chr19:42,803,185-43,392,144	588959	q13.2	LOH	16	1	100.0
chr20:21,553,601-21,754,925	201324	p11.22	LOH	3	0	100.0
chrX:18,882,933-19,538,442	655509	p22.13 - p22.12	LOH	5	0	100.0
chrX:19,703,881-20,334,542	630661	p22.12	LOH	9	1	100.0
chrX:20,537,739-21,689,798	1152059	p22.12	LOH	2	0	100.0
chrX:36,799,171-37,772,276	973105	p21.1 - p11.4	LOH	7	0	100.0
chrX:49,104,417-50,534,869	1430452	p11.23 - p11.22	LOH	79	8	100.0
chrX:53,909,369-54,497,617	588248	p11.22	LOH	5	0	100.0
chrX:64,420,830-67,003,457	2582627	q11.2 - q12	LOH	9	1	100.0
chrX:70,483,328-71,301,116	817788	q13.1	LOH	16	0	100.0
chrX:71,844,251-73,542,576	1698325	q13.2	LOH	31	5	100.0
chrX:73,648,249-73,662,587	14338	q13.2	LOH	1	0	100.0
chrX:73,791,519-77,370,364	3578845	q13.2 - q21.1	LOH	18	2	100.0
chrX:84,167,819-84,961,903	794084	q21.1 - q21.2	LOH	6	0	100.0
chrX:98,465,617-99,154,899	689282	q22.1	LOH	1	0	100.0
chrX:99,253,470-99,765,160	511690	q22.1	LOH	1	0	100.0
chrX:110,110,735-111,200,966	1090231	q23	LOH	8	0	100.0
chrX:111,657,286-111,856,925	199639	q23	LOH	1	0	100.0
chrX:126,962,408-127,595,158	632750	q25	LOH	1	0	100.0
chrX:132,567,373-133,181,878	614505	q26.2	LOH	1	0	100.0
chrX:134,612,278-134,797,939	185661	q26.3	LOH	2	0	100.0
chrX:151,278,776-151,798,204	519428	q28	LOH	7	3	100.0
chrX:153,063,333-154,918,789	1855456	q28	LOH	96	8	100.0

2 Mb ROH (runs of homozygosity) analysis

CLL Family 1

Region	Region Length	Cytoband Location	Event	Genes	mi-RNAs	Frequency %
chr5:104,431,051-104,505,444	74393	q21.2 - q21.3	CN Loss	1	0	100.0
chrY:2,655,180-3,274,356	619176	p11.31 - p11.2	CN Loss	4	0	100.0
chrY:6,088,397-6,453,668	365271	p11.2	CN Loss	14	0	100.0
chrY:6,614,711-9,974,735	3360024	p11.2	CN Loss	41	0	100.0
chrY:13,504,884-19,567,718	6062834	q11.21 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0
chr4:48,353,699-50,400,000	2046301	p11 - q11	LOH	7	0	100.0
chr8:47,002,900-49,782,743	2779843	q11.1 - q11.21	LOH	10	0	100.0
chr9:40,714,011-44,887,313	4173302	p13.1 - p11.2	LOH	29	0	100.0
chr9:65,629,772-69,410,435	3780663	q12 - q21.11	LOH	19	3	100.0
chrX:53,158,285-55,189,679	2031394	p11.22 - p11.21	LOH	28	5	100.0
chrX:120,222,656-122,317,172	2094516	q24 - q25	LOH	0	1	100.0

CLL Family 2

Region	Region Length	Cytoband Location	Event	Genes	miR-NAs	Frequency %
chr12:31,240,839-31,407,801	166962	p11.21	Allelic Imbalance	1	0	100.0
chr12:0-256,127	256127	p13.33	CN Gain	4	0	100.0
chr12:3,302,395-3,340,081	37686	p13.32	CN Gain	1	0	100.0
chr12:9,219,050-9,272,773	53723	p13.31	CN Gain	2	0	100.0
chr12:17,525,916-17,639,517	113601	p12.3	CN Gain	0	0	100.0
chr12:19,414,992-20,922,051	1507059	p12.3 - p12.2	CN Gain	5	0	100.0
chr12:21,447,001-21,464,915	17914	p12.1	CN Gain	1	0	100.0
chr12:31,240,839-31,407,801	166962	p11.21	CN Gain	1	0	100.0
chr12:33,846,842-35,800,000	1953158	p11.1 - q11	CN Gain	1	0	100.0
chr12:52,960,073-53,010,650	50577	q13.13	CN Gain	4	0	100.0
chr12:54,385,273-54,687,878	302605	q13.13	CN Gain	19	3	100.0
chr12:59,459,881-62,137,864	2677983	q14.1	CN Gain	2	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr12:64,324,996-64,409,482	84486	q14.2	CN Gain	1	0	100.0
chr12:69,513,557-69,542,929	29372	q15	CN Gain	0	0	100.0
chr12:71,249,238-71,388,234	138996	q15	CN Gain	1	0	100.0
chr12:71,482,919-71,570,918	87999	q15 - q21.1	CN Gain	1	0	100.0
chr12:72,728,822-73,077,229	348407	q21.1	CN Gain	1	0	100.0
chr12:73,343,040-74,402,940	1059900	q21.1	CN Gain	1	0	100.0
chr12:74,850,958-75,015,653	164695	q21.1	CN Gain	1	0	100.0
chr12:76,636,821-76,941,675	304854	q21.2	CN Gain	2	0	100.0
chr12:77,215,437-77,398,066	182629	q21.2	CN Gain	2	0	100.0
chr12:77,722,882-78,269,390	546508	q21.2	CN Gain	1	0	100.0
chr12:78,508,794-81,089,543	2580749	q21.2 - q21.31	CN Gain	8	1	100.0
chr12:82,537,785-82,976,126	438341	q21.31	CN Gain	2	0	100.0
chr12:83,147,536-85,410,579	2263043	q21.31	CN Gain	3	0	100.0
chr12:85,920,682-87,533,262	1612580	q21.31 - q21.32	CN Gain	4	0	100.0
chr12:98,207,772-98,487,513	279741	q23.1	CN Gain	1	2	100.0
chr12:99,196,800-99,229,824	33024	q23.1	CN Gain	1	0	100.0
chr12:101,194,298-101,379,071	184773	q23.1	CN Gain	1	0	100.0
chr12:126,738,610-126,881,735	143125	q24.32	CN Gain	0	0	100.0
chr12:127,850,708-128,208,987	358279	q24.32	CN Gain	1	0	100.0
chr12:128,406,449-128,522,391	115942	q24.32	CN Gain	1	0	100.0
chr12:129,171,957-129,247,690	75733	q24.32	CN Gain	1	0	100.0
chr12:129,459,964-129,530,498	70534	q24.33	CN Gain	1	0	100.0
chr12:129,808,003-129,870,681	62678	q24.33	CN Gain	1	0	100.0
chr12:130,808,601-130,911,986	103385	q24.33	CN Gain	2	0	100.0
chr12:131,557,897-131,654,487	96590	q24.33	CN Gain	2	0	100.0
chrY:5,893,803-6,088,397	194594	p11.2	CN Gain	0	0	100.0
chr1:111,927,724-111,939,188	11464	p13.2	CN Loss	1	0	100.0
chr8:9,054,811-9,063,535	8724	p23.1	CN Loss	1	0	100.0
chr15:21,903,815-22,701,317	797502	q11.2	CN Loss	15	4	100.0
chr19:24,378,791-24,629,285	250494	p12 - p11	CN Loss	0	0	100.0
chrX:2,699,012-58,483,247	55784235	p22.33 - p11.1	CN Loss	460	34	100.0
chrX:61,719,290-154,918,789	93199499	q11.1 - q28	CN Loss	802	82	100.0
chrY:2,655,180-3,205,097	549917	p11.31 - p11.2	CN Loss	4	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chrY:6,088,397-10,077,460	3989063	p11.2	CN Loss	55	0	100.0
chrY:12,500,000-19,567,718	7067718	q11.1 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0
chrY:27,390,536-28,817,458	1426922	q11.23 - q12	CN Loss	7	0	100.0
chr1:146,982,244-150,273,115	3290871	q21.1 - q21.2	LOH	72	2	100.0
chr14:98,891,530-107,349,540	8458010	q32.2 - q32.33	LOH	195	70	100.0
chr3:145,338,487-149,232,963	3894476	q24 - q25.1	LOH	20	0	100.0
chr4:48,333,953-50,400,000	2066047	p11 - q11	LOH	7	0	100.0
chr5:162,737,230-174,289,546	11552316	q34 - q35.2	LOH	67	9	100.0
chr8:112,612,536-114,668,761	2056225	q23.3	LOH	2	1	100.0
chr9:40,714,011-44,887,313	4173302	p13.1 - p11.2	LOH	29	0	100.0
chr9:65,629,772-69,410,435	3780663	q12 - q21.11	LOH	19	3	100.0
chrX:2,699,012-58,483,247	55784235	p22.33 - p11.1	LOH	460	34	100.0
chrX:61,719,290-88,460,879	26741589	q11.1 - q21.31	LOH	151	14	100.0
chrX:92,364,640-154,918,789	62554149	q21.32 - q28	LOH	647	68	100.0
chrY:14,368,629-19,567,718	5199089	q11.21 - q11.221	LOH	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	LOH	55	0	100.0
chrY:7,464,715-10,077,460	2612745	p11.2	LOH	38	0	100.0

CLL Family 3

Region	Region Length	Cytoband Location	Event	Genes	miRNAs	Frequency %
chr2:117,777,567-117,942,955	165388	q14.1	CN Gain	0	0	100.0
chr4:64,131,761-64,152,984	21223	q13.1	CN Loss	0	0	100.0
chr13:50,565,275-50,977,946	412671	q14.2 - q14.3	CN Loss	8	3	100.0
chr13:69,250,062-69,279,507	29445	q21.33	CN Loss	0	0	100.0
chrY:2,655,180-2,915,938	260758	p11.31	CN Loss	4	0	100.0
chrY:6,088,397-6,453,668	365271	p11.2	CN Loss	14	0	100.0
chrY:6,614,711-9,974,735	3360024	p11.2	CN Loss	41	0	100.0
chrY:13,504,884-19,567,718	6062834	q11.21 - q11.221	CN Loss	12	0	100.0
chrY:20,812,848-25,201,107	4388259	q11.222 - q11.223	CN Loss	55	0	100.0

Region	Region Length	Cytoband Location	Event	Genes	mi-RNAs	Frequency %
chrY:27,390,536-28,817,458	1426922	q11.23 - q12	CN Loss	7	0	100.0
chr12:33,444,058-35,800,000	2355942	p11.1 - q11	LOH	2	0	100.0
chr16:32,151,873-34,231,404	2079531	p11.2	LOH	16	0	100.0
chrX:64,420,830-67,003,457	2582627	q11.2 - q12	LOH	9	1	100.0
chrX:73,648,249-73,662,587	14338	q13.2	LOH	1	0	100.0
chrX:73,791,519-77,370,364	3578845	q13.2 - q21.1	LOH	18	2	100.0

Chapter 6

The presence of CLL-associated stereotypic B cell receptors in the normal BCR repertoire from healthy individuals increases with age

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ABSTRACT

Background

Aging is known to induce immunosenescence, resulting in alterations in both the innate and adaptive immune system. Here we evaluated the effects of aging on B cell subsets in peripheral blood of 155 immunologically healthy individuals in four age categories (range 20-95y) via multi-parameter flow cytometry. Furthermore, we studied the naive and antigen-experienced B cell receptor (BCR) repertoire of different age groups and compared it to the clonal BCR repertoire of chronic lymphocytic leukemia (CLL), a disease typically presenting in elderly individuals.

Results

Total numbers and relative frequencies of B cells were found to decline upon aging, with reductions in transitional B cells, memory cell types, and plasma blasts in the 70+y group. The BCR repertoire of naive mature B cells and antigen-experienced B cells did not clearly alter until age 70y. Clear changes in IGHV gene usage were observed in naive mature B cells of 70+y individuals, with a transitional pattern in the 50-70y group. IGHV gene usage of naive mature B cells of the 50-70y, but not the 70+y, age group resembled that of both younger (50-70y) and older (70+y) CLL patients. Additionally, CLL-associated stereotypic BCR were found as part of the healthy control BCR repertoire, with an age-associated increase in frequency of several stereotypic BCR (particularly subsets #2 and #5).

Conclusion

Composition of the peripheral B cell compartment changes with ageing, with clear reductions in non-switched and CD27+IgG+ switched memory B cells and plasma blasts in especially the 70+y group. The BCR repertoire is relatively stable until 70y, whereafter differences in IGHV gene usage are seen. Upon ageing, an increasing trend in the occurrence of particular CLL-associated stereotypic BCR is observed.

Keywords: aging, B-lymphocyte, BCR repertoire, CLL, stereotypic BCR

BACKGROUND

Changes in the immune system related to aging generally lead to increased susceptibility to infections, poor responses to new and evolving pathogens, poor vaccination responses, and higher incidence of autoimmune disorders and malignancies [1, 2]. This decline in function of the immune system, also referred to as immunosenescence, is the result of alterations occurring in both innate and adaptive immunity [3].

Age-related changes in humoral immune responses have generally been ascribed to defects in the T cell compartment and a lack of T cell help for B cell function [3]. Nevertheless, mouse studies do provide evidence for changes in the B cell compartment itself during aging. Although total B cell numbers did not alter much, shifts in the distribution of functional subsets were apparent with old age. In fact, in old mice nearly 100% of splenic B cells exhibited an antigen-experienced phenotype [4] and circulating immunoglobulins (Ig) were predominantly derived from post-germinal center B cells, as deduced from the presence of somatic hyper mutations (SHM) [5].

In human, age-related alterations in peripheral blood (PB) B cell subset distribution have also been reported, with circulating CD19+ B cells declining in absolute numbers and frequencies [6, 7, 8, 9, 10]. In some studies numbers and percentages of CD27+ memory B cells were found to decline [7, 8], whereas others showed an increase of these cells [10, 11, 12]. Similarly, numbers and percentages of naive CD27-IgD+ B cells were found to decrease by some studies [9, 10, 12], whereas others reported an increase [7, 8]. These inconsistent results may be explained by different B cell subset definitions and/or by large inter-individual variations in the studied age groups [11, 13].

Changes in B cell subsets during aging will likely also impact on B cell receptor (BCR) repertoire diversity. Indeed, in several mouse models age-related changes in the naive BCR repertoire were reported [14]. In some elderly humans, Ig heavy chain (IGH) complementarity determining region 3 (HCDR3) spectratyping analysis of PB B cells revealed a significant loss of diversity, which was associated with poor health status and poor survival [13]. Conflicting data, however, were reported on SHM in IGHV genes of the memory B cell compartment upon aging, varying from increased mutation rates in IgG+ but not in IgM+ PB memory B cells, to increased mutation rates in IgM+ memory B cells but not in other tonsillar subsets [15, 16].

Introduction of next generation sequencing (NGS) technologies has opened new possibilities to analyze the aging BCR repertoire, particularly in the light of immune diseases that typically arise in elderly. One category of immune diseases with a higher change to develop in elderly humans are B cell malignancies, with chronic lymphocytic leukemia (CLL) being the most common type. Notably, in about one-third of CLL patients, quasi-identical (stereotypic) BCRs are observed, which are characterized by restricted IGHV, IGHD, and IGHJ

gene usage plus similarities in HCDR3 length and amino acid sequence [17]. One study reported on stereotypic BCRs within the normal IGHV1-69-IGHJ6 repertoire [18]. Little is known however about the overall existence of CLL-associated stereotypic BCRs in the normal BCR repertoire of different age groups. We hypothesize that these CLL-associated stereotypic BCR could be a reflection of changes in B cell subset distribution and the normal BCR repertoire upon aging.

In this study we determined absolute numbers and relative distribution of PB B cell subsets in healthy individuals of different age categories. Additionally, we used NGS to investigate the BCR repertoire of naive mature B cells and different types of antigen-experienced B cells in healthy individuals upon aging. Finally, we compared IGHV gene usage of the normal BCR repertoire of different age groups with that of CLL leukemic cells and evaluated the occurrence of CLL-associated stereotypic BCR in the aging normal BCR repertoire.

RESULTS

Alterations in peripheral blood B cell subpopulations are minor upon aging

To study B cell dynamics during aging, we performed extensive flow cytometric immunophenotyping of peripheral blood (PB) cells in a cohort of 155 immunologically healthy individuals of 20-95 years [<50 (n=47), 50-60 (n=31), 60-70 (n=45), and 70+ (n=32)].

To validate our cohort, we first evaluated age-related dynamics of the total white blood cells and lymphocyte subpopulations (**Figure 1; Figure S1**). White blood counts (WBC) remained stable across the age groups (**Figure 1A**). Although differences were not significant, there was a trend that the absolute numbers of lymphocytes was lower in the 50-60y and 60-70y groups than in the <50y group (**Figure S1**). Both absolute and relative numbers of naive CD8+ T cells significantly declined >50y of age, whereas absolute and relative numbers of CD8+ effector (TemRA) T cells clearly increased with increasing age and CD8+ effector memory (TemRO) T cells remained stable (data not shown). CD4+ T cell and NK cell numbers did not alter between the age groups (**Figure S1**). These data are in line with previously reported data on T and NK cells [19], thus supporting the validity of our cohort for evaluating B cell aging effects.

Next, we focused on the composition of the B cell compartment (see Methods, **Table S1**, **Figure S2**) in the different age groups. The total B cell numbers and relative frequencies of B cells (as fraction of total lymphocytes) slightly declined during aging, resulting in a significant difference between the <50y and 70+y groups (**Figure 1B-C**). More specifically, we observed a significant reduction in absolute numbers of transitional B cells in the 70+y group, as well as reductions in the non-switched and CD27+IgG+ switched memory B cell populations, and plasma blasts in the two oldest age categories (**Figure 1D-M**). For naive mature B cells and all other types of memory B cells no clear alterations in absolute numbers were noted upon

aging (**Figure 1D-M**). The overall PB B cell subset distribution displayed only minor shifts between different age groups (**Figure 1N**), which was mainly reflected by the significantly lower frequencies of non-switched memory B cells and plasma blasts upon increasing age (**Figure S3**).

We then looked into CD5+CD43+ chronically activated B cells, as these have been associated with CLL and CLL-like MBL that typically appear in elderly [20, 21, 22]. We noted a small but significant increase in relative frequencies of CD5+CD43+ B cells in the 70+y group

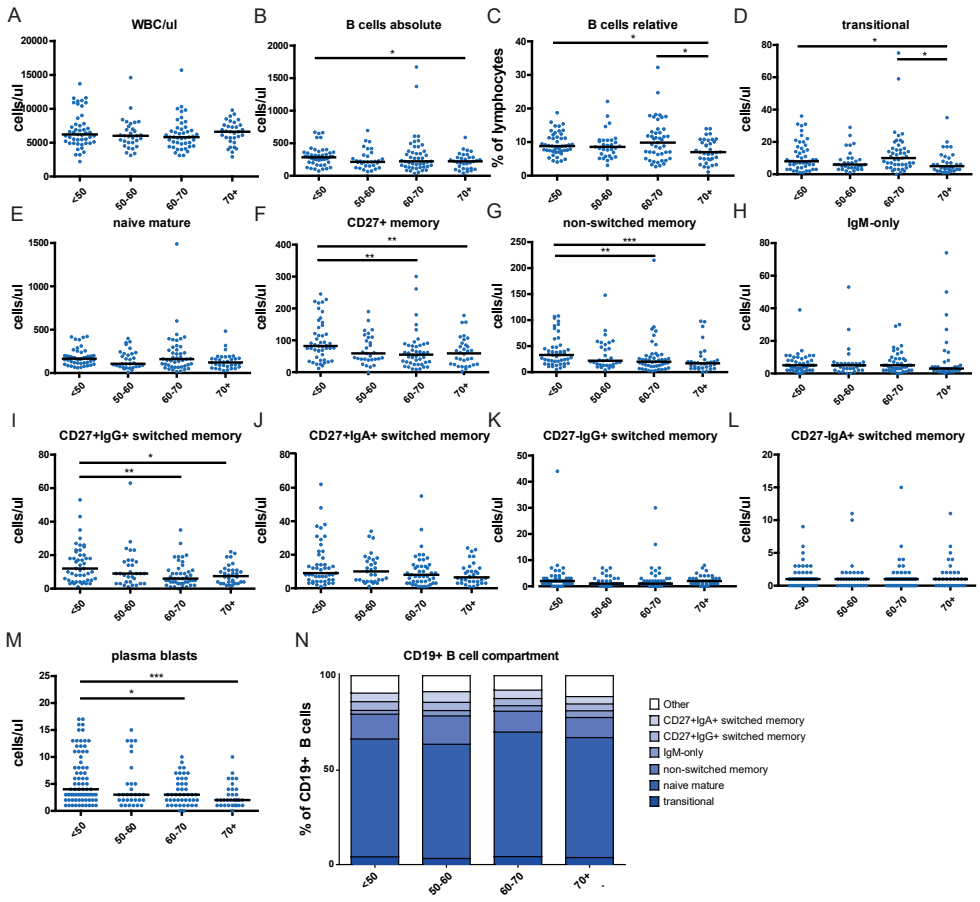

Figure 1. B cells and B cell subpopulations in peripheral blood upon aging.

A. WBC counts. B-C. Analysis of total cell numbers (**B**) and relative frequencies (**C**) of B cells. D-N. Total numbers of different B cell subpopulations (D-M), and relative distribution of B cell subpopulations (N). Data were evaluated for four age categories: <50 (n=47), 50-60 (n=31), 60-70 (n=45), and >70 (n=32). Statistical significance between age groups was determined using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

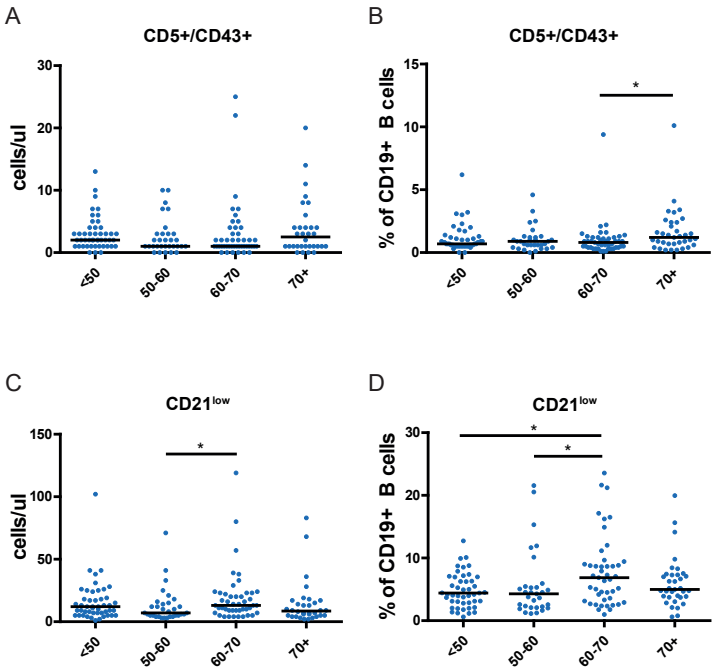

Figure 2. CD43+CD5+ B cells and CD21^{low} B cells in peripheral blood upon aging.

Analysis of total cell numbers (A,C) and relative frequencies (B,D) of CD5+CD43+ B cells and CD21^{low} B cells, respectively. Data were evaluated for four age categories: <50 (n=47), 50-60 (n=31), 60-70 (n=45), and >70 (n=32). Statistical significance between age groups was determined using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

(when compared with the 60-70y age group), together with a trend towards increased absolute numbers of these cells (**Figure 2A-B**). Next, we also evaluated CD21^{low} B cells, as high numbers of these cells have been associated with autoimmune disease [23]. Notably, a significant increase of CD21^{low} cell numbers and relative frequencies was seen between the 50-60y and 60-70y groups, which normalized again in the 70+y group (**Figures 2C-D**).

Taken together, our B cell subpopulation analysis mostly showed a decline in transitional B cells, non-switched and CD27+IgG+ switched memory B cells, and plasma blasts in elderly. The frequency of CD21^{low} B cells appeared to be increased in 60-70y group.

Composition of the BCR repertoire of naïve mature B cells is stable until 70y but shows changes in the 70+y group

Our next aim was to see which differences occur in the BCR repertoire of healthy donors during aging. To this end, we first sorted antigen-inexperienced naive mature B cells of healthy controls in the <50y, 50-70y, and 70+y groups (n=4-5 per age group) and analyzed

unique IGHV-IGHD-IGHJ sequences and their HCDR3 regions (**Table S2**). Despite slight variations in the mean HCDR3 lengths between the <50y (53.0 nucleotides), 50-70y (54.5 nucleotides), and 70+y (46.1 nucleotides) groups, the overall HCDR3 profiles showed no significant differences (**Figure 3A**).

When evaluating gene usage, differences in IGHV subgroup usage between the <50y and 50-70y groups appeared limited, but we did find a marked increase in IGHV1 and IGHV5 subgroup usage and a decrease in IGHV4 subgroup usage in the 70+y donors (**Figure 3B**). Upon further examination of IGHV gene usage, significant differences were mostly noted in the 70+y age group, with IGHV1-18, IGHV1-46, IGHV1-69, and IGHV5-51 gene usage being significantly higher, and IGHV4-34, IGHV4-39, and IGHV4-59 usage being significantly lower (**Figure 3C**), which could not be explained by small clonal proliferations. That said, the most commonly used IGHV gene in all three age groups appeared to be the IGHV3-23 gene, followed by IGHV3-21 (**Figure 3B**). We did not detect clear differences in IGHD and IGHJ gene usage between any of the three age groups.

Collectively, our data from healthy controls of different age groups suggest that the BCR repertoire of naive mature B cells is relatively stable until 70y. In contrast, in the 70+y group IGHV gene usage does differ, which most probably should be interpreted as an aging effect, since there were no other indications that can explain this difference.

Differences in BCR repertoire of memory B cell subpopulations are minor between <50y and 50-70y age groups

To evaluate age effects in the antigen-selected BCR repertoire, we then focused on non-switched, IgM-only, and CD27+IgG+ switched memory B cells, which are all antigen-experienced cells though arising via distinct activation routes. Some of the memory B cell populations are so small that they can only be sorted from buffy coats of healthy donors (n= 4-5 per age group); unfortunately blood donors are only allowed to give blood until 70y, so we could not the study the 70+y age group. Mean HCDR3 lengths did not differ significantly between the <50y and 50-70y age groups (**Figure S4**). IGHV, IGHD, and IGHJ gene usage and combined IGHV / IGHJ patterns of non-switched, IgM-only, and CD27+IgG+ memory B cells did not show significant differences either between these two age groups (**Figure S5**). Notably, when analyzing SHM frequencies for these memory B cell subpopulations, we did detect a higher mutation rate for non-switched and IgM-only memory B cells in the 50-70y group, whilst a small reduction in mutation frequency was seen in CD27+IgG+ switched memory B cells (**Figure S5**).

Collectively these data show that the BCR characteristics of memory B cell subpopulations do not differ statistically between the <50y and 50-70y groups.

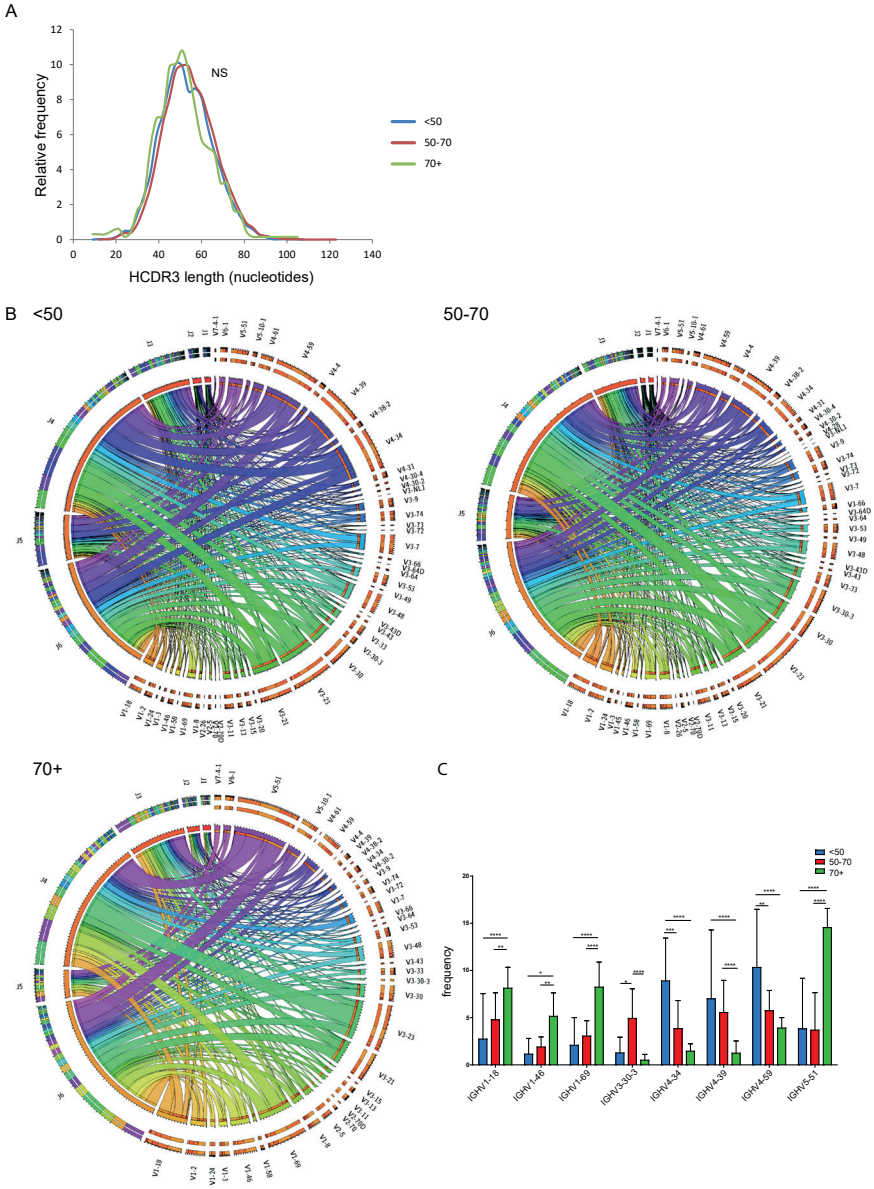

Figure 3. BCR repertoire analysis in naive mature B cells upon aging.

A. Comparison of HCDR3 lengths in <50, 50-70, and 70+y age categories. Statistical significance was determined using the Kolmogorov-Smirnov test. **B.** Circos plots of the combination of IGHV-IGHJ gene usage in <50, 50-70, and 70+y age categories. **C.** Differences in IGHV gene usage frequencies upon aging. Statistical significance between different age groups was determined using the two-way ANOVA test (with Bonferroni multiple comparisons correction); *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

IGHV gene usage in the clonal BCR repertoire of CLL patients is largely comparable to IGHV genes in naive mature B cells of 50-70y individuals

As the BCR is known to play an important role in disease onset and prognosis of CLL, which normally develops at elderly age (average 70+y), we then asked whether overall IGHV gene usage in CLL patients of different age groups would reflect the BCR repertoire of normal B cells of the same age groups. To this end we evaluated IGHV gene usage in different B cell subsets of healthy controls of different age groups and compared the profiles with Sanger sequencing-based data of a cohort of 920 CLL patients (**Figure 4**).

Overall IGHV gene usage profiles did not differ significantly between different types of normal B cell subpopulations, such as naive mature B cells, non-switched memory B cells, and IgM-only memory B cells, in the 50-70y age group ($p=0.99$-1 in all comparisons). Unfortunately, no such comparison was possible in 70+y individuals, due to the lack of available data from non-switched and IgM-only memory B cells for this age group. However, overall IGHV gene usage profiles in naive mature B cells of healthy individuals did clearly vary between the 50-70y and 70+y groups ($p=0.005$).

When we next compared overall IGHV gene usage profiles between CLL patients of the 50-70y and the 70+y groups we did not observe a significant difference ($p=0.995$). The IGHV profiles of naive mature B cells and CLLs in the 50-70y group appeared to look rather similar ($p=0.574$), albeit with a more dominant IGHV1-69 and IGHV4-34 usage in CLL. In contrast, naive mature B cell and CLL IGHV gene usage profiles in the 70+y groups were clearly different ($p<0.0001$), while the overall IGHV profile in 70+y CLL was in fact rather similar to that of naive mature B cells of the 50-70y age group ($p=0.110$). Furthermore, overall IGHV profiles in the 50-70y group appeared clearly different between CLL and non-switched memory B cells ($p=0.028$) or IgM-only memory B cells ($p=0.004$).

These data indicate that the overall IGHV gene usage profile in CLL patients, irrespective of the age of presentation, is similar to naive mature B cells of especially the 50-70y control group. The overall IGHV profile of the 70+y control group is different without obvious explanation as mentioned above.

CLL-associated stereotypic BCR are present in naive mature B cells and increase with age

In view of the occurrence of quasi-identical (stereotypic) BCR with similar IGHV / IGHD / IGHJ and HCDR3 features in CLL clones of different patients, we then investigated whether we could also detect stereotypic BCR in healthy individuals of different age groups. To identify CLL-related stereotypic BCRs in the normal repertoire of healthy controls of different age groups, a reference database of stereotypic BCR from CLL patients [24] was used for assigning individual IGH sequences from healthy controls. With this algorithm we could indeed identify CLL-associated stereotypic BCRs based on HCDR3 characteristics in the repertoire of naive

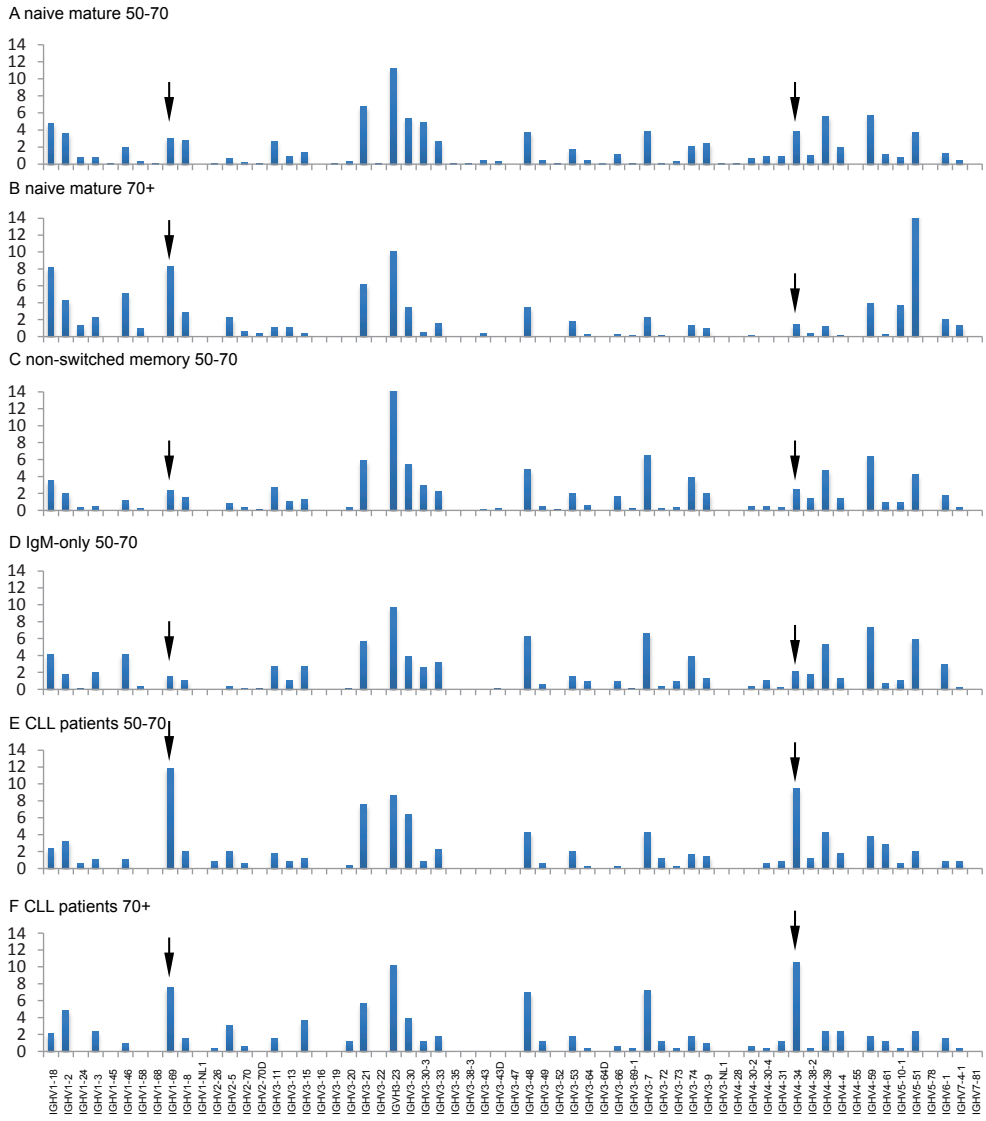

Figure 4. Comparison of IGHV gene usage in the normal BCR repertoire of healthy individuals and the clonal CLL repertoire of different age groups.

A-B. IGHV gene usage in naive mature B cells of 50-70y (A) and 70+y (B) healthy controls. **C-D.** IGHV gene usage in non-switched (**C**) and IgM-only B (**D**) cells of 50-70y healthy controls. **E-F.** IGHV gene usage in clones of CLL patients of 50-70y (C) and 70+y (**D**). Statistical analysis was determined using the Fisher's exact test; see text for details on significance levels.

mature B cells, and also of non-switched, IgM-only, and CD27+IgG+ switched memory B cells (**Figure 5**). The presence of stereotypic BCR receptors in naive mature B cells showed an increasing trend with age and was most apparent in the 70+y group (**Figure 5A**). In naive mature B cells, the most prominent stereotypic BCR belonged to CLL subsets #2, #5, and #64B (**Figure 5B**). In non-switched memory B cells the most frequently found stereotypic BCRs concerned CLL subsets #2 and #14 (**Figure 5C**). The CLL#14 BCR also appeared most prominent in IgM-only and CD27+IgG+ switched memory B cells, especially in the 50-70y category (**Figure 5D-E**).

From these findings we conclude that CLL-associated stereotypic BCRs are present in the normal BCR repertoire during life and that their frequency showed an increasing trend upon aging.

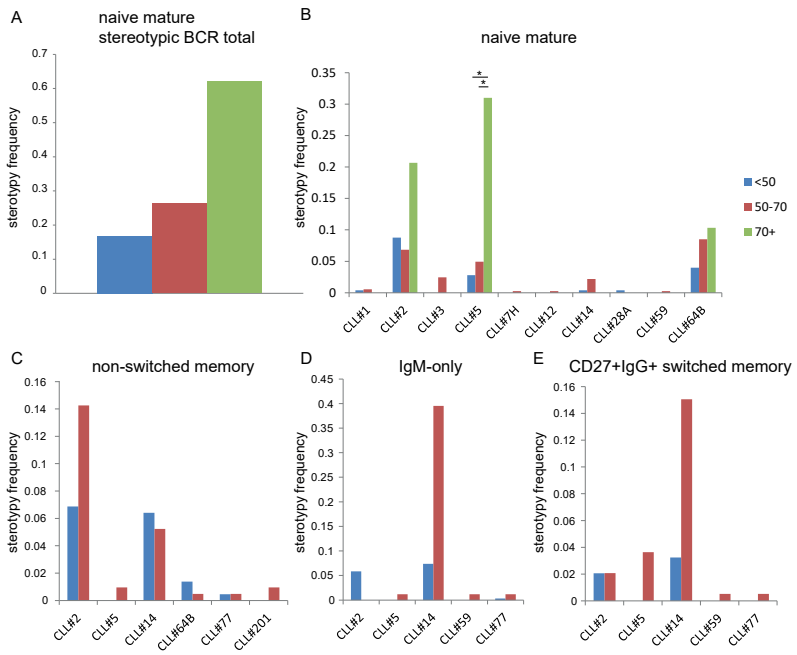

Figure 5. Stereotypic BCR in the normal BCR repertoire of healthy aging individuals.

A. Total frequency of stereotypic BCR in the normal BCR repertoire in naive mature B cells of healthy individuals. **B-E.** Frequency of stereotypic BCR assigned to CLL-associated stereotypic subsets in naive mature (**B**), non-switched (**C**), IgM-only (**D**), and CD27+IgG+ switched (**E**) memory B cells. Statistical significance between different age groups was determined using the two-way ANOVA test (with Bonferroni multiple comparisons correction); *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

DISCUSSION

Here we evaluated age-related changes in B cell subset composition as well as BCR repertoire with a focus on CLL-associated stereotypic BCR usage. In the most elderly (70+y) individuals we observed a decline in absolute numbers of transitional B cells, total memory B cells, and plasma blasts. The BCR repertoire of naive mature B cells and distinct memory B cell populations was found to be relatively stable until 70y. In naive mature B cells of the 70+y group differences were noted, especially with respect to IGHV gene usage. Notably, IGHV gene usage in the clonal BCR repertoire in CLL did not differ with the age of presentation of the CLL patients, and largely resembled naive mature B cells of the 50-70y group rather than the 70+y group. Finally, CLL-associated stereotypic BCR were found as part of the BCR repertoire of healthy individuals and their frequencies increased with age.

The observed decline in total B cell numbers in the 70+y group could result from a reduced output from the bone marrow [25, 26]. An alternative explanation could be the reduction in CD27+ antigen-experienced B cell subpopulations. Non-switched and CD27+IgG+ memory B cells together make up the majority of the CD27+ memory B cell compartment, and were previously described to decline upon aging [6]. The decline in non-switched memory B cells could underlie the higher vulnerability to diseases caused by encapsulated bacteria like *Streptococcus pneumoniae* in elderly [27]. CD27+IgG+ memory B cells are mainly, albeit not exclusively, formed in T cell-dependent immune responses and play a role in recall responses to previously encountered pathogens [28]. The reduction of plasma blasts upon aging is in line with earlier observations [29] and fits the lower immunoglobulin levels in the circulation as reported in elderly [30]. Together these data could, at least partially, explain the reduced effects of vaccination and immune responses against infections in elderly.

Chronically activated B cells express CD5 and CD43 [31, 32] and might trigger MBL onset. [33, 34]. MBL are found in healthy adult individuals, with an incidence that increases with age to roughly 10% of individuals >65y [35]. Based on their phenotypical association with MBL [20] and CLL [22], the increase of CD5+CD43+ B cells upon aging might thus correlate with the higher risk of MBL and CLL clones in elderly. Another B cell subset related with chronic activation concerns $CD21^{low}$ B cells, increased numbers of which can be found in patients showing chronic inflammation in the context of autoimmune disease [23]. As we excluded individuals with inflammatory and (auto)immune disease in our immunologically healthy cohort, unfortunately we could not link the higher number of $CD21^{low}$ B cells in the 60-70y group to overt autoimmune disease occurrence. Nevertheless, increased numbers of $CD21^{low}$ B cells in this age group might reflect an increased incidence of, yet undiagnosed, autoimmune diseases upon aging.

BCR repertoire changes were most apparent in naive mature B cells of the 70+y group. Since naive mature B cells are not affected by exogenous antigen, this is most likely the result

of changes in repertoire development and/or output from the bone marrow. Whilst HCDR3 length, IGHD, and IGHJ usage remained stable in all three age categories, IGHV gene usage did reveal alterations. Interestingly, IGHV4-34 usage, a gene often associated with autoimmunity, was found to be reduced upon aging in these healthy individuals. Upon aging we also noticed a combined increased usage of IGHV5-51 and IGHV1-69, in line with a previous report [36]. IGHV1-69 has been associated with broadly neutralizing antibodies against amongst others influenza, HIV, hepatitis C, and commensal bacteria antigens in the context of CLL [37].

Remarkably, IGHV gene usage in both the 50-70y and 70+y CLL patient groups most closely resembled IGHV gene usage in naive mature B cells of 50-70y, but not 70+y, healthy individuals. One could speculate that CLL clones, even the ones in elderly CLL patients (70+y), would have developed from B cells with a BCR repertoire of relatively younger age (<70y), but it might also reflect selection for IGHV specificities in the younger repertoire that could be predisposing for CLL development.

Stereotypic BCR, which are found in roughly one third of CLL clones, have previously been documented in RNA from a total lymphocyte pool of three healthy individuals (age 50, 69, and 69) [38, 39]. In this study we show that stereotypic BCR can be found in the normal BCR repertoire of both naive mature and antigen-experienced B cells. Interestingly, a frequently observed stereotypic BCR was the CLL#2 BCR (IGHV3-21 in combination with IGHJ6 with a short HCDR3 length of 9 amino acids), which is the most common stereotypic BCR seen in CLL patients and is associated with an aggressive form of CLL [17, 40]. Stereotypic CLL#5 BCR (IGHV1-69, IGHD3-10/3-3, IGHJ6, 20 amino acids HCDR3) as well as stereotypic CLL#64 BCR (IGHV3 subgroup genes, IGHD2 subgroup genes, IGHJ6, 21 amino acids HCDR3) [17] were relatively often detected in naive mature B cells of especially individuals 70+y. In antigen-experienced B cells, stereotypic CLL#14 BCR (IGHV4-4, no specific IGHD, IGHJ4, and short 10 amino acids HCDR3) was frequently found. The possibility to detect stereotypic BCRs with short HCDR3 lengths in antigen-experienced B cell subpopulations would be in line with our observation that on average the complete memory BCR repertoire shows selection for shorter HCDR3 lengths in comparison with naive B cells. Notably, other common CLL-associated stereotypic BCR, such as CLL#4, CLL#6, and CLL#8 BCR, could not be detected.

Even though the frequency of stereotypic BCR in healthy B cells shows a trend towards increase with age, stereotypic BCR can already be detected in cord blood (data not shown) and thus are to be considered as part of the normal BCR repertoire. Moreover, even though the increase of CLL-associated stereotypic BCR in the aging normal BCR repertoire might imply an increased predisposition for CLL development in elderly, it should be stressed that two thirds of CLL show heterogeneous BCR specificities that can also mediate derailment of B cells leading to CLL. Investigations into the presence of CLL-associated stereotypic BCR in healthy individuals should therefore be extended to larger datasets including more healthy individuals of all age groups, as well as to individuals suffering from chronic infection,

immunodeficiency, or autoimmune disease. Such studies would allow to define the true impact of CLL-associated stereotypic BCR in CLL development.

CONCLUSION

We analyzed the peripheral B cell compartment and BCR repertoire during human ageing. Composition of the peripheral B cell compartment changes with ageing, with clear reductions in non-switched and CD27+IgG+ switched memory B cells and plasma blasts in especially the 70+y group. The BCR repertoire is relatively stable until 70y age, whereafter differences in IGHV gene usage are seen. Upon ageing, an increase in the occurrence of particular CLL-associated stereotypic BCR is observed, potentially reflecting the occurrence of such BCR in CLL in elderly.

METHODS

Sample inclusion

For B cell subpopulation analysis, peripheral blood (PB) of immunologically healthy individuals was obtained from pre-surgery patients (Dept. Orthopedics, Erasmus MC) with the following *exclusion* criteria: (auto)immune or inflammatory diseases; malignancies; usage of anti-inflammatory or immunosuppressive drugs; surgery in the past 30 days; alcohol and drug abuse. To increase the number of subjects per age group, additional samples from the SENEX healthy aging cohort (Rheumatology and Clinical Immunology, UMCG, Groningen, Netherlands), and samples from co-workers from the department were included. Subjects (n=155) were divided into four age categories: <50 (n=47), 50-60 (n=31), 60-70 (n=45), and 70+ (n=32). For the BCR repertoire study, peripheral blood samples (n=5 per age group) were additionally obtained from Sanquin Blood bank (Amsterdam, The Netherlands). Diagnostic samples from CLL patients were collected upon informed consent and anonymized for further usage. Written consent was obtained in accordance with the Declaration of Helsinki after medical ethics committee approval (MEC 2011-409, 2016-202, and 2012375).

Immunophenotyping of B cell subpopulations

Folllowing white blood cell count (WBC) measurement on a Coulter®Ac.T diff analyzer (Beckman Coulter, Fullerton, CA, USA), flowcytometry was performed on whole blood [after red blood cell lysis with ammonium chloride] using an LSR Fortessa™ (BD Biosciences, San Jose, CA). Absolute cell counts of monocytes, natural killer (NK) cells, T cells, and B cells were calculated from WBC numbers using Infinicyt software (Cytognos, Salamanca, Spain).

Lymphocytes were first gated based on FSC / SSC characteristics, and B cells were defined by expression of the pan-B cell marker CD19. Further gating was performed for defined CD19+ B cell subpopulations, i.e. transitional B cells (CD38hi/CD27-), naive mature B cells (CD38-/CD27-/IgM+/IgD+), non-switched memory B cells (CD38-/CD27+/IgM+/IgD+), IgM-only B cells (CD38-/CD27+/IgM+/IgD-), switched memory B cells (CD38-/CD27+ or -/IgM-/IgD-IgG+ or IgA+ or IgE+), plasma blasts ($CD38^{hi}$/CD27+), CD5+/CD43+ B cells (CD38-/CD5+/CD43+), and $CD21^{low}$ B cells ($CD38^{dim}$/$CD21^{low}$) according to published data [28] (see also (**Table S1**), using 11-color flowcytometric stainings (**Table S3**) and FACS DIVA software (BD Biosciences) for analysis.

Sorting of B cell subpopulations and DNA isolation

PB mononuclear cells (PB-MNC) were isolated via Ficoll Paque gradient centrifugation. Subsequently, B cells were purified with human CD19 MicroBeads via AutoMACS (Myltenyi Biotech, Bergisch Gladbach, Germany). Next, several B cell subpopulations (naive mature, non-switched memory, IgM only, and CD27+/IgG+) were collected using a FACSAria cell sorter (BD Biosciences). Immediately after collection cells were lysed in RLT+ buffer (QIAGEN, Valencia, CA) complemented with β-mercapto-ethanol. Cells were used for DNA isolation with the DNA/RNA/miRNA Easy kit (QIAGEN) and/or stored in -80°C for later processing.

NGS-based BCR repertoire analysis of healthy individuals

IGHV-IGHD-IGHJ rearrangements were amplified from 100 ng DNA of sorted B cell subpopulations (from n=3-5 healthy controls per age group; see Table S3) using the BIOMED-2 IGH multiplex PCR with 6 IGHV primers and 1 IGHJ consensus primers that were extended with adapter sequences for NGS. PCR products were purified by gel extraction (QIAGEN) and subsequently by Agencourt AMpure XP beads (Beckman Coulter, Brea, CA). Concentrations were measured using Quant-iT Picogreen dsDNA assay (Invitrogen, Carlsbad, CA). PCR products were sequenced on a 454 GS junior (Roche, Branford, CT), using the GS Junior Titanium emPCR, sequencing, and PicoTiterPlate kits (Roche), and partly on a MiSeq (Illumina, San Diego, CA) platform. Cross-validation experiments using B cells from healthy individuals showed comparability of data from both platforms (unpublished; see Suppl. Information). Sequences were demultiplexed based on their multiplex identifier sequence and trimmed via the ImmunoGlobulin galaxy (IGGalaxy) pipeline [41]. FASTA files were uploaded in IMGT/High-V-QUEST (http://www.imgt.org/HighV-QUEST/login.action) and IMGT output files were further analyzed in the Antigen Receptor Galaxy pipeline, as described before [42].

For comparison purposes, a local Erasmus MC cohort of 920 CLL patients (mean 65y) was used, in which Sanger sequencing-based IGHV mutation status analysis was performed using the BIOMED-2 primers and protocol [43], and following ERIC interpretation guidelines [44].

IGHV-IGHJ circos plots were generated via the Circos Table Viewer (http://mkweb.bcgsc.ca/tableviewer/). CLL-associated stereotypic BCR in healthy control samples were defined using the ARResT/AssignSubsets tool (http://tools.bat.infspire.org/arrest/assignsubsets/).

Statistical analysis

Significant differences in relative and absolute numbers of lymphocyte subpopulations and IGH SHM levels between age groups were determined using the Mann-Whitney U test. Differences in HCDR3 lengths were evaluated using Kolmogorov-Smirnov statistics. Relative IGHV gene usage and relative frequencies of stereotyped BCR between different age groups were analyzed using a two-way ANOVA with multiple comparisons. Overall IGHV gene usage between B cell subpopulations and CLL clones was analyzed using the Fisher's exact test. A p-value <0.05 was considered significant. Statistics were performed in GraphPad Prism v5.0 (La Jolla, CA).

DECLARATIONS

Ethics approval and consent to participate

Ethical approval and written consent to participate was obtained from the medical ethics committee of Erasmus MC, Rotterdam (MEC 2011-409, 2016-202) and UMCG, Groningen (2012375).

Consent for publication

Written consent for publication was obtained from the medical ethics committee of Erasmus MC, Rotterdam (MEC 2011-409, 2016-202) and UMCG, Groningen (2012375).

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AFM and AWL drafted the study; AFM, MdJ, ILMWT, HIJ, MvdB, and WvIJ performed experiments and obtained data; AFM, JANV, WHA, EB, and AMHB were responsible for selecting cases and defining data; AFM, CT, ND, RS, RWH, JJMvD, and AWL analyzed and interpreted data; AFM, RWH, JJMvD, and AWL wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Grubeck-Loebenstein, B., Della Bella, S., Iorio, A.M., Michel, J.P., Pawelec, G. & Solana, R. Immunosenescence and vaccine failure in the elderly. *Aging Clin Exp Res* **21**, 201-209 (2009).
2. Ademokun, A., Wu, Y.C. & Dunn-Walters, D. The ageing B cell population: composition and function. *Biogerontology* **11**, 125-137 (2010).
3. Goronzy, J.J. & Weyand, C.M. Understanding immunosenescence to improve responses to vaccines. *Nat Immunol* **14**, 428-436 (2013).
4. Johnson, S.A., Rozzo, S.J. & Cambier, J.C. Aging-dependent exclusion of antigen-inexperienced cells from the peripheral B cell repertoire. *J Immunol* **168**, 5014-5023 (2002).
5. Williams, G.T., Jolly, C.J., Kohler, J. & Neuberger, M.S. The contribution of somatic hypermutation to the diversity of serum immunoglobulin: dramatic increase with age. *Immunity* **13**, 409-417 (2000).
6. Chong, Y., Ikematsu, H., Yamaji, K., Nishimura, M., Nabeshima, S., Kashiwagi, S. *et al.* CD27(+) (memory) B cell decrease and apoptosis-resistant CD27(-) (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances. *Int Immunol* **17**, 383-390 (2005).
7. Frasca, D., Landin, A.M., Lechner, S.C., Ryan, J.G., Schwartz, R., Riley, R.L. *et al.* Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol* **180**, 5283-5290 (2008).
8. Shi, Y., Yamazaki, T., Okubo, Y., Uehara, Y., Sugane, K. & Agematsu, K. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. *J Immunol* **175**, 3262-3267 (2005).
9. Veneri, D., Ortolani, R., Franchini, M., Tridente, G., Pizzolo, G. & Vella, A. Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. *Blood Transfus* **7**, 29-34 (2009).
10. Morbach, H., Eichhorn, E.M., Liese, J.G. & Girschick, H.J. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol* **162**, 271-279 (2010).

11. Colonna-Romano, G., Aquino, A., Bulati, M., Di Lorenzo, G., Listi, F., Vitello, S. *et al.* Memory B cell subpopulations in the aged. *Rejuvenation Res* **9**, 149-152 (2006).
12. van Gent, R., van Tilburg, C.M., Nibbelke, E.E., Otto, S.A., Gaiser, J.F., Janssens-Korpela, P.L. *et al.* Refined characterization and reference values of the pediatric T- and B-cell compartments. *Clin Immunol* **133**, 95-107 (2009).
13. Gibson, K.L., Wu, Y.C., Barnett, Y., Duggan, O., Vaughan, R., Kondeatis, E. *et al.* B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell* **8**, 18-25 (2009).
14. Cancro, M.P., Hao, Y., Scholz, J.L., Riley, R.L., Frasca, D., Dunn-Walters, D.K. *et al.* B cells and aging: molecules and mechanisms. *Trends Immunol* **30**, 313-318 (2009).
15. Chong, Y., Ikematsu, H., Yamaji, K., Nishimura, M., Kashiwagi, S. & Hayashi, J. Age-related accumulation of Ig V(H) gene somatic mutations in peripheral B cells from aged humans. *Clin Exp Immunol* **133**, 59-66 (2003).
16. Kolar, G.R., Mehta, D., Wilson, P.C. & Capra, J.D. Diversity of the Ig repertoire is maintained with age in spite of reduced germinal centre cells in human tonsil lymphoid tissue. *Scand J Immunol* **64**, 314-324 (2006).
17. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* **119**, 4467-4475 (2012).
18. Forconi, F., Potter, K.N., Wheatley, I., Darzentas, N., Sozzi, E., Stamatopoulos, K. *et al.* The normal IGHV1-69-derived B-cell repertoire contains stereotypic patterns characteristic of unmutated CLL. *Blood* **115**, 71-77 (2010).
19. Lin, Y., Kim, J., Metter, E.J., Nguyen, H., Truong, T., Lustig, A. *et al.* Changes in blood lymphocyte numbers with age in vivo and their association with the levels of cytokines/cytokine receptors. *Immun Ageing* **13**, 24 (2016).
20. Nieto, W.G., Teodosio, C., Lopez, A., Rodriguez-Caballero, A., Romero, A., Barcena, P. *et al.* Non-CLL-like monoclonal B-cell lymphocytosis in the general population: prevalence and phenotypic/genetic characteristics. *Cytometry B Clin Cytom* **78 Suppl 1**, S24-34 (2010).
21. Griffin, D.O., Holodick, N.E. & Rothstein, T.L. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* **208**, 67-80 (2011).
22. Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Durig, J. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* **209**, 2183-2198 (2012).
23. Thorarinsdottir, K., Camponeschi, A., Gjertsson, I. & Martensson, I.L. CD21 -/low B cells: A Snapshot of a Unique B Cell Subset in Health and Disease. *Scand J Immunol* **82**, 254-261 (2015).
24. Bystry, V., Agathangelidis, A., Bikos, V., Sutton, L.A., Baliakas, P., Hadzidimitriou, A. *et al.* ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukemia based on B cell receptor IG stereotypy. *Bioinformatics* **31**, 3844-3846 (2015).
25. Duggal, N.A., Upton, J., Phillips, A.C., Sapey, E. & Lord, J.M. An age-related numerical and functional deficit in CD19(+) CD24(hi) CD38(hi) B cells is associated with an increase in systemic autoimmunity. *Aging Cell* **12**, 873-881 (2013).
26. van der Geest, K.S., Lorencetti, P.G., Abdulahad, W.H., Horst, G., Huitema, M., Roozendaal, C. *et al.* Aging-dependent decline of IL-10 producing B cells coincides with production of antinuclear antibodies but not rheumatoid factors. *Exp Gerontol* **75**, 24-29 (2016).

27. Kruetzmann, S., Rosado, M.M., Weber, H., Germing, U., Tournilhac, O., Peter, H.H. *et al.* Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med* **197**, 939-945 (2003).
28. Berkowska, M.A., Driessen, G.J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A. *et al.* Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* **118**, 2150-2158 (2011).
29. Caraux, A., Klein, B., Paiva, B., Bret, C., Schmitz, A., Fuhler, G.M. *et al.* Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. *Haematologica* **95**, 1016-1020 (2010).
30. Lock, R.J. & Unsworth, D.J. Immunoglobulins and immunoglobulin subclasses in the elderly. *Ann Clin Biochem* **40**, 143-148 (2003).
31. Mageed, R.A., Garaud, S., Taher, T.E., Parikh, K., Pers, J.O., Jamin, C. *et al.* CD5 expression promotes multiple intracellular signaling pathways in B lymphocyte. *Autoimmun Rev* **11**, 795-798 (2012).
32. Gary-Gouy, H., Harriague, J., Bismuth, G., Platzer, C., Schmitt, C. & Dalloul, A.H. Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. *Blood* **100**, 4537-4543 (2002).
33. Perez-Andres, M., Grosserichter-Wagener, C., Teodosio, C., van Dongen, J.J., Orfao, A. & van Zelm, M.C. The nature of circulating CD27+CD43+ B cells. *J Exp Med* **208**, 2565-2566 (2011).
34. Covens, K., Verbinnen, B., Geukens, N., Meyts, I., Schuit, F., Van Lommel, L. *et al.* Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. *Blood* **121**, 5176-5183 (2013).
35. Shim, Y.K., Rachel, J.M., Ghia, P., Boren, J., Abbasi, F., Dagklis, A. *et al.* Monoclonal B-cell lymphocytosis in healthy blood donors: an unexpectedly common finding. *Blood* **123**, 1319-1326 (2014).
36. Martin, V., Wu, Y.C., Kipling, D. & Dunn-Walters, D.K. Age-related aspects of human IgM(+) B cell heterogeneity. *Ann N Y Acad Sci* **1362**, 153-163 (2015).
37. Hwang, K.K., Trama, A.M., Kozink, D.M., Chen, X., Wiehe, K., Cooper, A.J. *et al.* IGHV1-69 B cell chronic lymphocytic leukemia antibodies cross-react with HIV-1 and hepatitis C virus antigens as well as intestinal commensal bacteria. *PLoS One* **9**, e90725 (2014).
38. Henriques, A., Rodriguez-Caballero, A., Nieto, W.G., Langerak, A.W., Criado, I., Lecrevisse, Q. *et al.* Combined patterns of IGHV repertoire and cytogenetic/molecular alterations in monoclonal B lymphocytosis versus chronic lymphocytic leukemia. *PLoS One* **8**, e67751 (2013).
39. DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L. *et al.* Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* **64**, 252-271 (2014).
40. Baliakas, P., Agathangelidis, A., Hadzidimitriou, A., Sutton, L.A., Minga, E., Tsanousa, A. *et al.* Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. *Blood* **125**, 856-859 (2015).
41. Moorhouse, M.J., van Zessen, D., H, I.J., Hiltemann, S., Horsman, S., van der Spek, P.J. *et al.* ImmunoGlobulin galaxy (IGGalaxy) for simple determination and quantitation of immunoglobulin heavy chain rearrangements from NGS. *BMC Immunol* **15**, 59 (2014).
42. IJspeert, H., van Schouwenburg, P.A., van Zessen, D., Pico-Knijnenburg, I., Stubbs, A.P. & van der Burg, M. Antigen Receptor Galaxy: A User-Friendly, Web-Based Tool for Analysis and Visualization of T and B Cell Receptor Repertoire Data. *J Immunol* **198**, 4156-4165 (2017).

43. van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L. *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* **17**, 2257-2317 (2003).
44. Langerak, A.W., Davi, F., Ghia, P., Hadzidimitriou, A., Murray, F., Potter, K.N. *et al.* Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. *Leukemia* **25**, 979-984 (2011).

SUPPLEMENTARY FILES

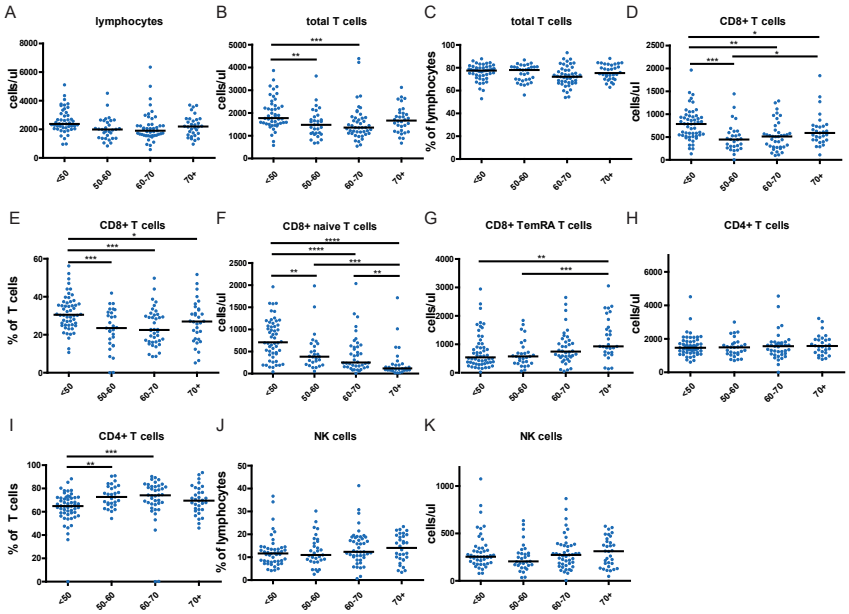


Figure S1. Frequencies and absolute numbers of T cell subsets and NK cells to validate the cohort for evaluating peripheral blood B cell subpopulations upon aging.

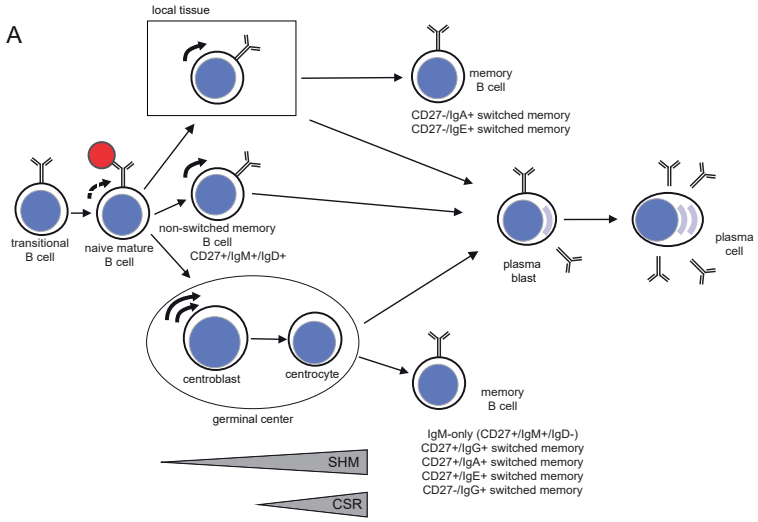


Figure S2. Scheme of different human B-cell subpopulations in peripheral blood.

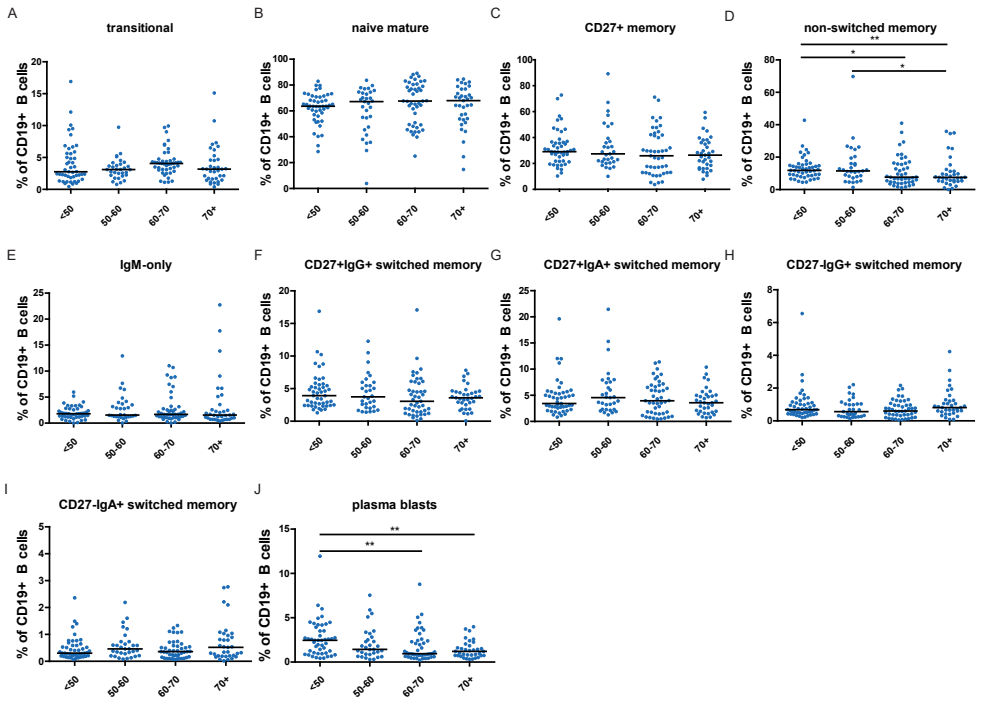


Figure S3. Relative frequencies of B cell subpopulations in peripheral blood upon aging.

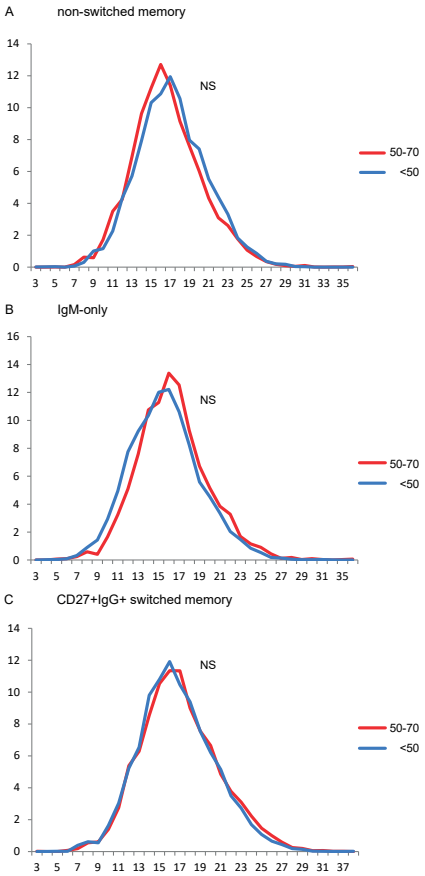


Figure S4. No difference in HCDR3 lengths of antigen-experienced B cells upon aging.

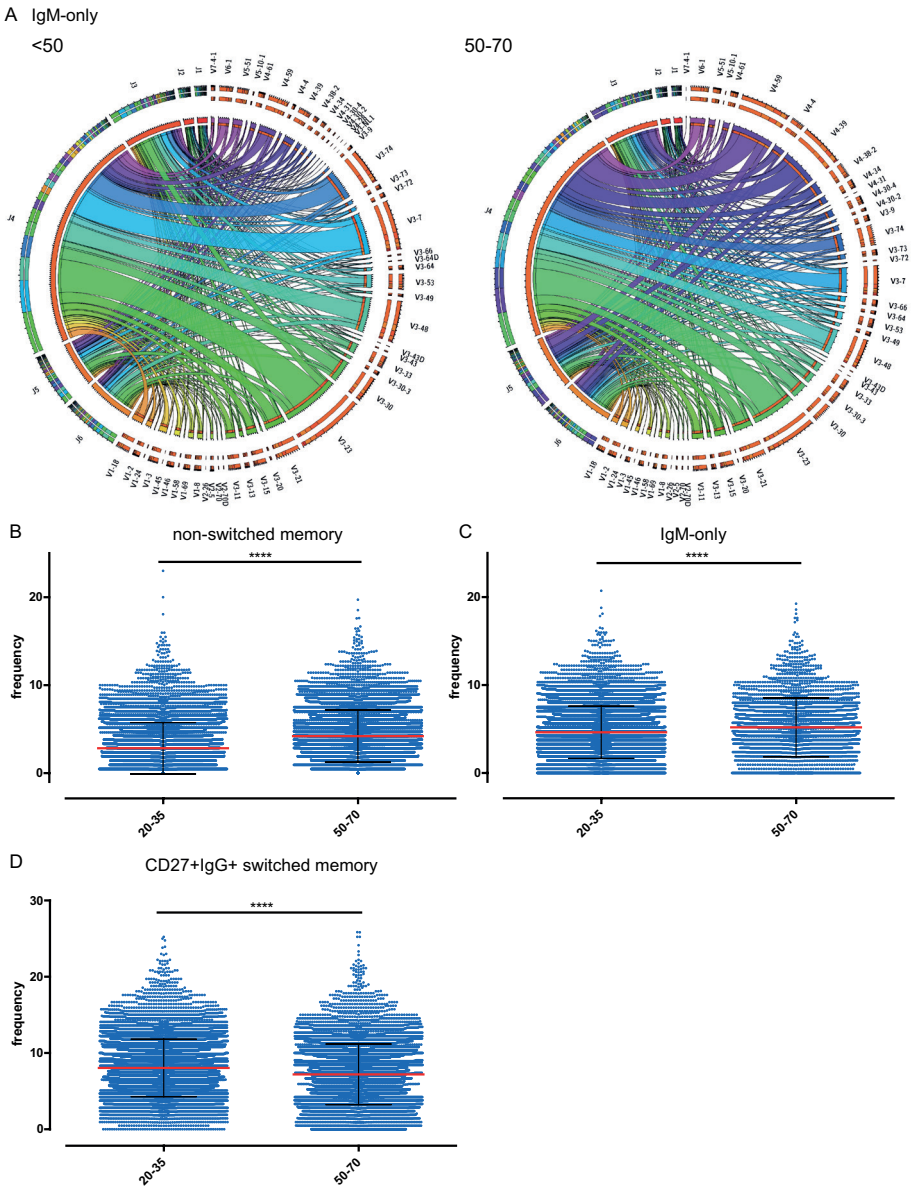


Figure S5. Minor differences in BCR repertoire of antigen-experienced B cell subpopulations in different age groups.

SUPPLEMENTARY TABLES

Table S1. Definition of human B cell subpopulations

B cell subpopulation	Markers
transitional B cells	CD19+/CD38hi/CD27-
naive mature B cells	CD19+/CD38-/CD27-/IgM+/IgD+
non-switched memory B cells	CD19+/CD38-/CD27+/IgM+/IgD+
IgM-only B cells	CD19+/CD38-/CD27+/IgM+/IgD-
CD27+ or – IgG+ switched memory B cells	CD19+/CD38-/CD27+ or -/IgM-/IgD-/IgG+
CD27+ or – IgA+ switched memory B cells	CD19+/CD38-/CD27+ or -/IgM-/IgD-/IgA+
CD27+ or – IgE+ switched memory B cells	CD19+/CD38-/CD27+ or -/IgM-/IgD-/IgE+
plasma blasts	CD19+/CD38^{hi}/CD27+
CD5+/CD43+ B cells	CD19+/CD38-/CD5+/CD43+
CD21^{low} B cells	CD19+/CD38^{dim}/CD21^{low}

Table S2. Overview of productive and unique IGH sequences in NGS analysis

B cell subpopulation	Age group	Productive sequences	Unique sequences
naïve mature B cells	<50 (n=5)	20507	14257
	50-70 (n=5)	31211	20367
	>70 (n=4)	657	648
non-switched memory B cells	<50 (n=5)	17793	9495
	50-70 (n=4)	16012	10973
IgM-only B cells	<50 (n=5)	26519	10112
	50-70 (n=3)	7248	3200
switched memory B cells (CD27+IgG+)	<50 (n=5)	27606	9329
	50-70 (n=4)	15030	8994

Table S3. Composition of different antibody panels for staining B cell subpopulations

Panel 1		**Panel 2**	
Marker	Fluorochrome	Marker	Fluorochrome
CD27	BV421	**CD27**	BV421
IgM	BV510	**IgM**	BV510
CD20	BV605	**CD20**	BV605
CD21	BV711	**CD21**	BV711
IgE	FITC	CD43	FITC
IgA	FITC		
IgG	PE	CD11b	PE
IgA	PE		
IgD	PE-CF594	**IgD**	PE-CF594
CD79b	PerCP	CD11c	PerCP
CD19	PE-Cy7	**CD19**	PE-Cy7
CD23	APC	CD5	APC
CD38	APC-H7	**CD38**	APC-H7

To allow combining data from the two panels. a backbone of markers (indicated in bold) was used.

SUPPLEMENTARY INFORMATION

Cross-validation between different NGS platforms was performed using DNA from 8 controls that were used for paired analysis of IGH DNA and RNA/cDNA libraries on 454 (Roche. Branford. CT) and MiSeq (Illumina. San Diego. CA) platforms and data analysis using the ImmunoGlobulin galaxy (IGGalaxy) pipeline (Moorhouse et al.. 2014).

Comparison of productive and unique productive sequencess

Donor		All	Productive		Unique Productive	
NWK16	454	4363	2849	65%	1539	35%
NWK214	454	7640	3845	50%	1951	26%
NWK276	454	8183	6486	79%	4286	52%
NWK279	454	11111	8517	77%	4716	42%
NWK31	454	3322	1226	37%	928	28%
NWK43	454	7868	4994	63%	2669	34%
NWK53	454	32403	24107	74%	3449	11%
NWK54	454	4547	2966	65%	1528	34%
Mean	454			64%		33%
NWK16	ILLUMINA	29043	21964	76%	9858	34%
NWK214	ILLUMINA	30202	20884	69%	10790	36%
NWK276	ILLUMINA	41469	28165	68%	16173	39%
NWK279	ILLUMINA	36737	24792	67%	13725	37%
NWK31	ILLUMINA	44931	33886	75%	19209	43%
NWK43	ILLUMINA	41800	19032	46%	13834	33%
NWK53	ILLUMINA	44482	35421	80%	11212	25%
NWK54	ILLUMINA	30746	19682	64%	9498	31%
Mean	ILLUMINA			68%		35%

Comparison of junctional region characteristics

Donor		Total,Del	Total,N	Total,P	CDR3,Length
NWK16	454	18.9	11	0.6	15
NWK214	454	20.2	11.9	0.5	14
NWK276	454	17.2	12.5	0.6	16
NWK279	454	17.7	12.8	0.5	16
NWK31	454	19.8	10.9	0.5	14
NWK43	454	21.1	11	0.4	14
NWK53	454	20.8	12.1	0.5	14
NWK54	454	18.7	11.4	0.4	14
Mean	454	18.8	11.2	0.5	14.5
NWK16	ILLUMINA	19.5	12	0.5	15
NWK214	ILLUMINA	20.9	12.4	0.4	14
NWK276	ILLUMINA	17.4	11.7	0.6	16
NWK279	ILLUMINA	17.7	12.3	0.6	16
NWK31	ILLUMINA	18.9	11.7	0.5	15
NWK43	ILLUMINA	19.7	11	0.5	14
NWK53	ILLUMINA	21.5	12.8	0.4	14
NWK54	ILLUMINA	19	12.5	0.4	14
Mean	ILLUMINA	19.25	12.25	0.45	14.5

Comparison of V subgroup usage

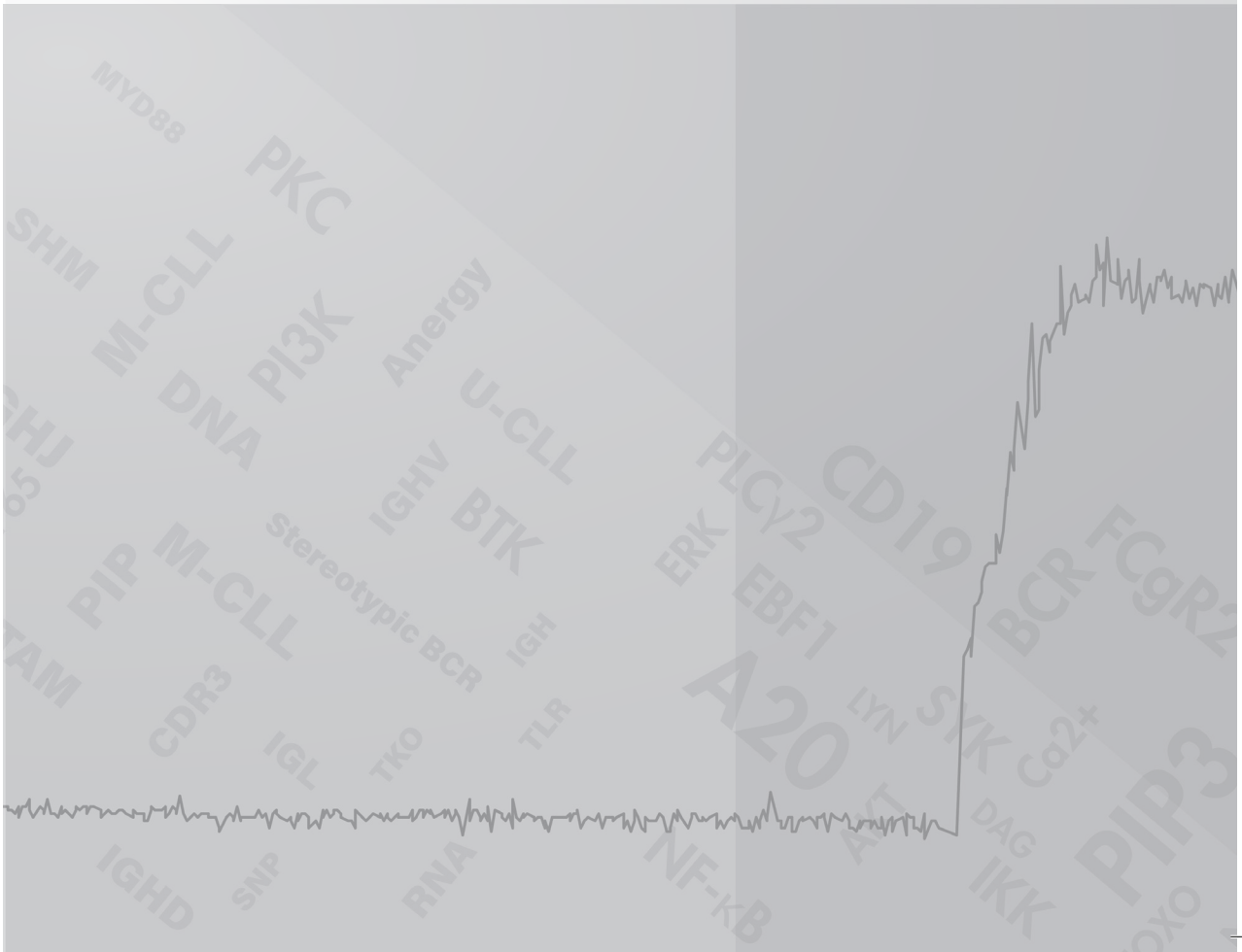
Donor		IGHV1	IGHV2	IGHV3	IGHV4	IGHV5	IGHV6	IGHV7
NWK16	454	8.77	0.06	65.95	19.75	5.26	0.19	
NWK214	454	8.25	0.46	40.80	47.87	1.90	0.72	
NWK276	454	7.44	1.61	55.27	32.13	2.24	1.31	
NWK279	454	8.69	1.38	54.52	32.46	2.04	0.91	
NWK31	454	14.44	1.62	51.08	30.39	1.94	0.54	
NWK43	454	4.57	1.09	61.22	26.71	3.11	3.18	0.11
NWK53	454	7.36	0.58	66.98	20.50	3.89	0.70	
NWK54	454	3.93	0.20	69.31	21.34	4.65	0.46	0.13
Mean	454	7.93	0.87	58.14	28.89	3.13	1.00	0.12
NWK16	ILLUMINA	19.32	0.87	53.04	22.95	3.62	0.19	
NWK214	ILLUMINA	0.38	0.05	66.29	30.92	1.42	0.94	0.01
NWK276	ILLUMINA	39.49	1.79	40.75	8.64	8.68	0.64	0.01
NWK279	ILLUMINA	44.92	1.70	39.81	6.87	5.68	0.98	0.03
NWK31	ILLUMINA	15.20	2.34	40.05	26.12	14.35	1.38	0.56
NWK43	ILLUMINA	10.16	2.11	36.29	35.39	12.06	3.43	0.56
NWK53	ILLUMINA	16.33	1.31	40.90	36.59	4.17	0.67	0.02
NWK54	ILLUMINA	0.64	0.03	48.96	46.11	3.33	0.93	
Mean	ILLUMINA	18.30	1.28	45.76	26.70	6.66	1.14	0.20

Comparison of J gene usage

Donor		IGHJ1	IGHJ2	IGHJ3	IGHJ4	IGHJ5	IGHJ6
NWK16	454	1.30	2.86	17.48	45.35	8.77	24.24
NWK214	454	2.72	3.74	9.33	52.49	15.89	15.84
NWK276	454	1.33	2.75	11.78	43.89	11.06	29.19
NWK279	454	1.95	2.84	12.77	43.83	12.13	26.48
NWK31	454	5.50	4.42	8.19	48.38	14.87	18.64
NWK43	454	3.33	4.50	12.51	50.88	12.89	15.89
NWK53	454	2.17	4.03	9.51	53.93	13.31	17.05
NWK54	454	2.29	2.88	12.30	54.25	11.26	17.02
Mean	454	2.57	3.50	11.73	49.13	12.52	20.54
NWK16	ILLUMINA	1.13	3.95	15.56	41.72	11.09	26.56
NWK214	ILLUMINA	2.80	4.08	9.84	53.20	14.09	16.00
NWK276	ILLUMINA	1.16	1.28	10.33	37.28	11.21	38.75
NWK279	ILLUMINA	1.88	1.54	14.70	35.72	10.14	36.02
NWK31	ILLUMINA	2.76	3.00	12.04	49.73	13.00	19.46
NWK43	ILLUMINA	2.76	3.79	12.28	51.63	14.03	15.50
NWK53	ILLUMINA	1.96	3.29	10.60	49.31	15.61	19.23
NWK54	ILLUMINA	2.10	2.07	12.16	54.32	13.22	16.13
Mean	ILLUMINA	2.07	2.88	12.19	46.61	12.80	23.46

Chapter 7

General discussion and summary



GENERAL DISCUSSION

In this thesis, we studied the role of the B cell receptor (BCR) in chronic lymphocytic leukemia (CLL), which is the most common type of leukemia in elderly in the Western world. Although the cause of CLL is still not fully clear, the BCR was found to be important in the development of the disease. It has been known for a long time that CLL can be separated into mutated (M-CLL) and unmutated (U-CLL) subgroups based on the somatic hypermutation (SHM) status of the BCR[1, 2]. An important role of the BCR is further supported by the restricted usage of immunoglobulin (Ig) heavy chain variable, diversity, and joining gene (IGHV, IGHD, and IGHJ respectively), with a restricted heavy chain determining region 3 (HCDR3) length and composition, in combination with a restricted light chain usage, in subsets of CLL patients. These so-called stereotypic BCR, which are found in one-third of CLL cases, suggest antigenic selection for certain BCRs in the development of CLL[3]. Most U-CLLs have been found to be polyreactive, recognizing self- and non-self-antigens such as DNA, insulin, apoptotic cells, LPS, oxidized LDL, and cytoskeletal proteins myosin and vimentin, with low affinity binding[4, 5, 6, 7, 8]. For M-CLLs more specific, high-affinity antigen binding is expected, but for only a few stereotypic M-CLL receptors the specific antigen has actually been identified. Hoogeboom et al. described a stereotypic receptor consisting of the IGHV3-7 containing IGH with an extremely short HCDR3 length of 5 or 6 amino acids, in combination with an IGKV2-24-containing light chain, which recognizes the fungal antigen pustulan (β-1,6-glucan)[9].

To get more insight in the role of the BCR in CLL pathogenesis, in this thesis we explored signaling pathways downstream of the BCR and focused on characteristics of the BCR itself, especially also in families with CLL patients. Furthermore, as CLL is a disease of the elderly, in parallel we evaluated changes in B cell subset distribution and BCR repertoire of healthy individuals upon aging.

BCR signaling

The crucial role of the BCR downstream signaling cascade in CLL development is thoroughly outlined in our review on signaling pathways in CLL (Chapter 2)[10]. CLL cells show increased basal Ca^{2+} signaling compared to normal B cells, as we have described in Chapter 3. We detected an association between the IGHV SHM status of the CLL cases and basal Ca^{2+} signaling levels, being higher in M-CLL compared with U-CLL[11]. In another CLL cohort (described in Chapter 4), we could detect higher basal Ca^{2+} signaling for CLL in comparison with normal B cells, but we could not validate the association with the SHM status of the CLL cases. One possible explanation for this difference could be that the samples used for the cohort in Chapter 3 were taken from our CLL biobank and these stored, frozen samples were thawed for the Ca^{2+} signaling experiments. In contrast, for the studies described in Chapter 4,

fresh CLL samples were used. Nevertheless, when we performed experiments to compare Ca^{2+} signaling in samples from CLL patients both fresh and after freezing and thawing, we noticed no major differences in Ca^{2+} signaling (data not shown). Thawed samples might contain more apoptotic cells, which are known to have higher cytosolic Ca^{2+} levels, but we excluded these cells as much as possible from the analysis by stringent gating strategies. Another explanation for the difference between the two cohorts could be the heterogeneity between CLL patients, which could impact on the mean levels of Ca^{2+} signaling of the included M-CLL and U-CLL samples in the two relatively small cohorts. That notwithstanding, we detected in both CLL cohorts (Chapters 3 and 4) that U-CLL are in general more responsive to anti-IgM stimulation than M-CLL. For IgD stimulation we did not notice any dissimilarities between U-CLL and M-CLL[11]. Differences in signaling capacity between U-CLL and M-CLL after BCR stimulation were also reported previously, whereby U-CLL were more capable to respond to IgM stimulation than M-CLL, which was explained by the latter being more anergic[12].

In Chapter 4 we analyzed the phosphorylation status of various signaling molecules from the BCR signaling pathway (**Figure 1**) to further zoom in on BCR responsive and non-responsive CLL cases. We found a higher basal phosphorylation level of the signaling molecule PLCγ2 in the former as compared with the latter. For the signaling molecules SYK and AKT we did not find such differences. After anti-IgM-mediated BCR stimulation, responsive CLL cases showed an increase in phosphorylation of PLCγ2 and AKT (**Figure 1**). In addition, CLL cells from anti-IgM responsive CLL cases appeared to have higher expression levels of IgM, IgD, CD19, CD38, and CD43, and thus a more activated phenotype than unresponsive CLL cases. Furthermore, we detected a strong correlation between IgM and CD21 expression and the capability of CLL cells to respond to IgM stimulation. CD21 is an important component of the BCR co-receptor complex and enhances signaling capacity[13]. Downregulation of CD21 is associated with anergy[14] and high numbers of CD21 low B cells are often found in patients with autoimmune diseases[15] and in healthy individuals with increasing age (Chapter 6).

Evaluation of gene expression via RNA sequencing of CLL cells of six BCR responsive and six BCR non-responsive CLL cases, showed differential expression of several NF-κB-related genes between these CLLs (Chapter 4). Interestingly, in unresponsive CLL cases transcripts of several regulators of NF-κB signaling, such as inhibitor of NF-κB (IκB) genes *NFKBIB*, *NFKBID* and *NFKBIE*, and NF-κB components *NFKB1*, *NFKB2*, and *BCL3* were found to be upregulated. RQ-PCR confirmed higher expression levels of *NFKB1*, *NFKB2*, *NFKBIB*, and *NFKBIE* in anti-IgM unresponsive CLL samples. Stimulation of known responsive and unresponsive CLL samples with anti-IgM, led to a clear upregulation in the expression of *NFKB2*, NF-κB component *REL*, *NFKBID*, *NFKBIE*, and NF-κB regulator *TNFAIP3* (A20) in responsive CLL compared with unresponsive CLL cases (**Figure 1**).

These differences in response to BCR stimulation seem to reflect differences in the anergic state of the CLL cases. Anergy in B cells is defined by loss of the capacity to respond

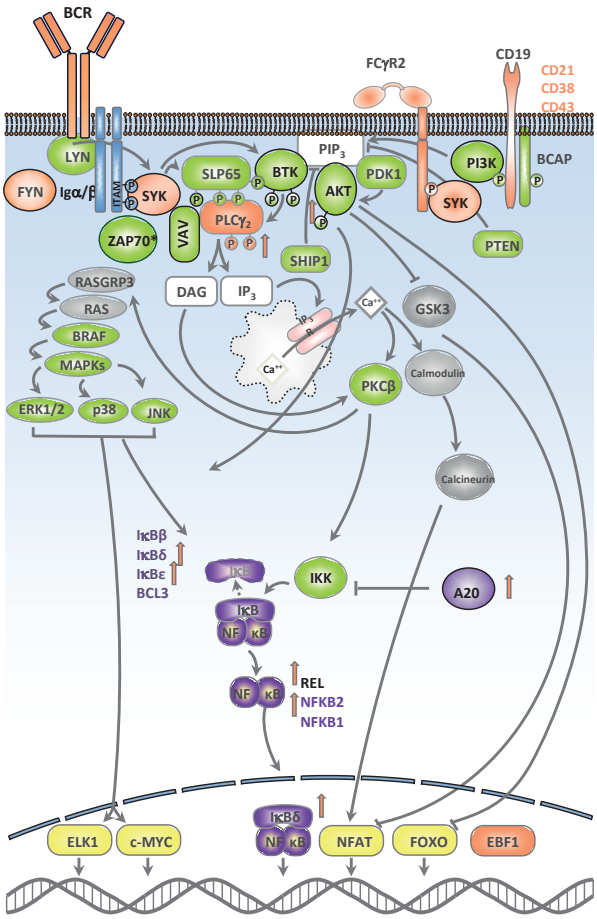


Figure 1. Differences between BCR signaling in α-IgM responsive CLL versus α-IgM unresponsive CLL.

Schematic overview of BCR signaling pathway in α-IgM responsive CLL versus α-IgM unresponsive CLL. Upon antigen binding to the BCR, signaling is initiated by LYN-mediated phosphorylation of ITAMs in the cytoplasmic tail of Igα/β and CD19, recruiting SYK and PI3K, respectively. PI3K converts PIP2 into PIP3 to enable membrane recruitment of BTK. SYK-induced phosphorylation of SLP65 promotes a docking platform for BTK and PLCγ2, and formation of the BCR micro-signalosome, resulting into phosphorylation of PLCγ2 by BTK. This induces an influx of Ca^{2+} in the cytoplasm, finally resulting in activation of the transcription factors NF-κB, NFAT, FOXO, and ELK1/c-Myc[10].

α-IgM unresponsive CLL have higher protein expression levels of IgM, IgD, CD19, CD21, CD38, and CD43, and in addition higher basal P-PLCγ2 (indicated in orange). Also, *EBF1*, *FCGR2A*, *SYK* and *FYN* RNA expression levels are increased (orange). Furthermore, unresponsive CLL have higher RNA expression levels of NF-κB-related genes (*NFKBIB*, *NFKBID* and *NFKBIE)*, and NF-κB components (*NFKB1*, *NFKB2*, and *BCL3*), and in addition *TNFAIP3* encoding A20 (indicated in purple). Upon α-IgM stimulation of responsive CLL-cases P-PLCγ2 and P-AKT levels increase, relative to unresponsive cases, while *NFKB2*, *REL*, *NFKBID*, *NFKBIE* and *A20* RNA expression levels are upregulated (indicated with orange arrows).

to auto-antigenic stimulation via the BCR. As a result, the anergic state prevents the B cell to go into apoptosis. Anergic B cells internalize especially their IgM BCR and show increased cytosolic Ca^{2+} levels[16, 17]. In addition, the anergic cells fail to mobilize Ca^{2+} [17], and do not proliferate upon stimulation[18]. Several phosphatases and kinases play a role in the maintenance of this anergic state. LYN seems to function as a driver for the inhibition of PI3K signaling via the inositol phosphatases SHIP and PTEN, which convert PIP3 into PIP2[18] (**Figure 1**). In addition, LYN was found to recruit SHP-1 that dephosphorylates signaling molecules and thereby acts as a regulator. This eventually leads to activation of the ERK and Ca^{2+}-calcineurin-NFAT signaling pathways, without activation of JNK and NF-κB[19, 20]. Also, anergic CLL cells exhibit constitutive activation of ERK1/2 and NFAT2[21]. Recently, it was shown that NFAT2 is critical in maintaining the anergic phenotype of CLL, whereas loss of NFAT2 expression plays a role in CLL transformation (Richter's syndrome)[22]. Loss of IκBε, leading to NF-κB activation and translocation to the nucleus is associated with a more aggressive type of CLL[23]. In addition, upon IgM stimulation U-CLL have in general higher phosphorylation levels of NF-κB and ERK than M-CLL[24]. It thus seems likely that unresponsive CLL with a more anergic phenotype are controlled by Ca^{2+}-calcineurin-NFAT signaling. Moreover, higher basal levels of NF-κB regulatory components, and especially of NF-κB inhibitory proteins, could make these CLL cells unresponsive and independent of BCR stimulation. In responsive CLL, which are less anergic than unresponsive CLL, though more than their normal B cell counterparts, NF-κB signaling is required. Based on these findings it would be tempting to speculate that if unresponsive CLL shift from control via NFAT signaling towards NF-κB signaling and become more responsive, this goes hand in hand with disease progression.

BCR characteristics

Apart from BCR signaling, we also investigated characteristics of the BCR of CLL cases in more detail. In Chapter 5, we studied the BCR characteristics of CLL cells in three families with multiple siblings who developed CLL. Whereas in CLL family 1 the four siblings were recognized as M-CLL cases, the family 2 siblings could be characterized as U-CLL cases. Interestingly, family 3 was identified as a stereotypic subset #2(-like) CLL family, in which all siblings expressed the specific CLL#2 λ light chain. One of the family 3 siblings appeared to carry the specific CLL#2 BCR, and another was found to have a CLL#169 BCR, which is known to be very alike to CLL#2. Three completely different groups of CLL, are represented by these families, being M-CLL (family 1), U-CLL (family 2) and CLL#2-like CLL (family 3), with member 3C being a true CLL#2 case and member 3B a closely related CLL#169 case.

To study the reactivity of the BCRs of the M-CLL familial CLL cases, we attempted to clone and express the BCR of the various siblings of family 1. For CLL family member 1A we detected by use of ELISA that the recombinant BCR (IgM), which consisted of the IGHV1-3, IGHD3-3, and IGHJ3 genes (SHM status 92.9%) in combination with IGKV1-9, IGKJ5 light chain

genes, bound to LPS, but did not show affinity for insulin, actin, or fungal antigen pustulan (**Figure 2**). LPS binding by this CLL-BCR could be specific, since this BCR has undergone SHM, although it cannot be entirely excluded that this BCR is polyreactive when more antigens would have been tested. Unfortunately, as we were not able to test antigen binding of the

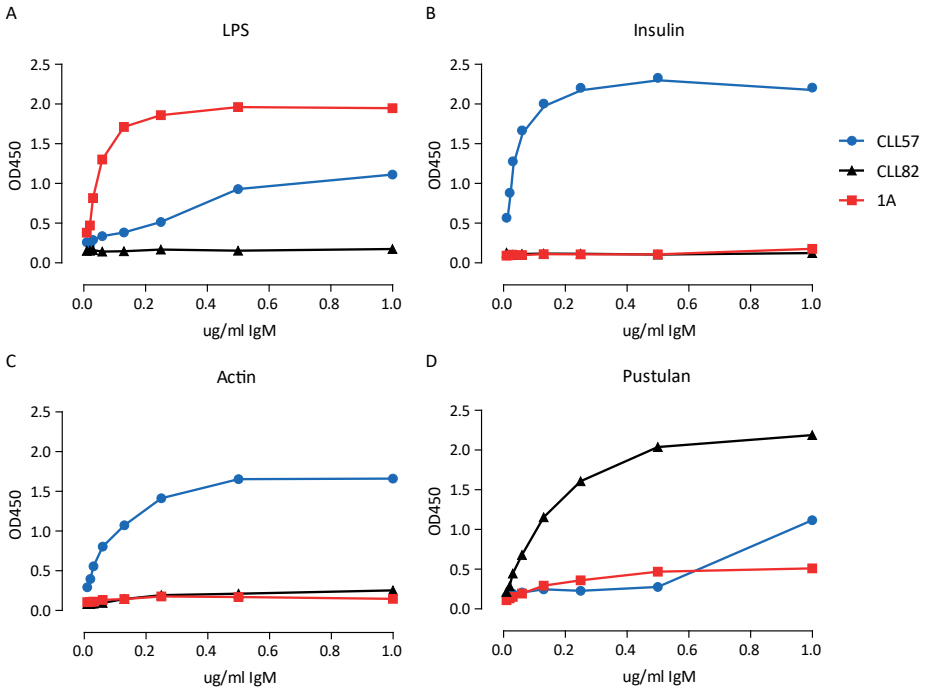


Figure 2. Evaluation of the binding of recombinant IgM from CLL family member 1A towards different molecules.
By use of ELISA the binding of recombinant IgM of familial case 1A towards different molecules such as LPS (**A**), insulin (**B**) actin (**C**) and the fungal antigen pustulan (**D**), as indicated, were analyzed.
On the x-axis the titrated concentrations of the soluble recombinant pentameric IgM from familial CLL sibling 1A consisting of IGHV1-3/IGHD3-3/IGHJ3 with a HCDR3 length of 19 amino acids combined with IGKV1-9/IGKJ5, and two controls. I.e.; CLL57 IgM consisting of IGHV3-30-3/IGHD3-3/IGHJ6 and a HCDR3 length of 25 amino acids in combination with IGLV1-51/IGLJ3 is used as a positive control since it is known to be polyreactive, whereas CLL82 IgM, which consists of IGHV3-7/IGHD2-21/IGHJ5 with a HCDR3 length of 5 amino acids combined with IGKV2-24/IGKJ2 and which specifically recognizes pustulan (β-1,6-glucan) was used as a negative control[9]. An antibody was considered to be reactive when the absorbance of the titration exceeds the absorbance of the blank mean times three (3x Optical density 450 (OD450)) as is shown on the y-axis.
Method used for production of soluble IgM was described by Bende et al[37]. ELISA plates were coated overnight with actin (Sigma-Aldrich) (3 ug/ml), insulin (Sigma-Aldrich) (3 ug/ml), LPS (E. Coli O55:B5, Sigma-Aldrich) (3 ug/ml) or pustulan (10 ug/ml) (Invivogen). This was followed by overnight incubation with titrated concentrations of the soluble recombinant pentameric IgM as described before[9]. Absorbance was measured at 450 nm.

BCR of the other family 1 members due to the multiple rearrangements of their heavy and light chains[25], we cannot address if recognition of a common antigen acts as a driving factor in this CLL family. Irrespective of the involvement of common antigens, it seems likely that the familial CLL occurrence is also impacted by a common genetic predisposition in the three families. Our SNP data seem to indicate a shared path of CLL clonal evolution, as we detected similarities in known CLL-related chromosomal alterations within our CLL families. Currently, genetic predisposition is the topic of further study, exploiting whole genome sequencing on paired CLL cell / non-malignant T cell genomic DNA samples of the various siblings.

Additionally, we tested the reactivity of several CLL-derived BCR from the CLL cohort as described in Chapter 3 (see **Table 1**) by expressing their heavy and light chain as IgM in an *in vitro* model system. We took advantage of the triple knock-out (TKO) cell system, which is derived from a signaling-competent mouse pre-B cell line lacking expression of endogenous pre-BCRs due to inactivation of *RAG2* and λ*5* genes[26, 27]. The λ*5* gene encodes one of the components of the surrogate light chain, which forms the pre-BCR together with the Ig H chain[27]. Upon evaluating antigen recognition through flow cytometry, we found that five out of six tested U-CLL recognized one or more auto-antigens, in contrast to only one out of four M-CLL. The antigens which bound to the CLL-derived BCR were LPS, LDL, oxidized-LDL, liposomes, and double-stranded DNA (**Figure 3A**). Unfortunately, we were not able to analyze the BCR response upon stimulation with these antigens. Altogether, our results are in line with other reports showing that autoantigen recognition is more common in U-CLL than in M-CLL.

In the *E*μ-*TCL1* transgenic CLL mouse model, it was found that viral infections induced expansion of virus-specific B cells, but did not alter leukemogenesis directly nor resulted

Table 1. Characteristics of CLL cases

Sample	SHM status*)	IGHV	IGHD	IGHJ	CDR3 AA length	CDR3 AA
2010-135	97.36	IGHV1-2*04	IGHD3-9*01	IGHJ3*02	28	ARSSRPGSRRGLYDILTNYRGEGDAFDI
2011-308	97.76	IGHV3-21*01	IGHD1-14*01	IGHJ4*02	12	VRTTQDVTLFDS
2006-207	96.82	IGHV4-34*01	IGHD3-22*01	IGHJ6*02	20	VRGYPEDTTTRRYYYYGMEV
2009-115	92.73	IGHV4-34*01	IGHD3-16*01	IGHJ6*02	20	VRGfPYDYTTRRYYYYGMDV
2008-022	100	IGHV4-34*01	IGHD3-3*01	IGHJ3*02	19	ARGPYYDFWSGYSRRAFDI
2011-304	100	IGHV3-23*01	IGHD6-13*01	IGHJ6*02	20	AKPLYSSSWYLNYYYYGMDV
2011-467	100	IGHV3-30*01	IGHD3-3*01	IGHJ6*02	27	ARASDFWSGYYKYEDPYGYYYYYGMDV
2006-225	100	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	21	ARGGGYDYVWGSYRPNDAFDI
2006-284	100	IGHV1-69*01	IGHD3-16*02	IGHJ3*02	21	ARGGGYDYIWGSYRSNDAFDI
2009-335	100	IGHV1-69*06	IGHD3-16*02	IGHJ3*02	21	ARGGDYDYIWGSYRPNDAFDI

*) Values represent % identity with germline DNA sequence

in earlier onset or progression of CLL[28]. When comparing sera from young non-leukemic *E*μ-*TCL1* mice with that of aged CLL-bearing *E*μ-*TCL1* mice, the former did not show autoreactivity, while the latter did recognize a broad range of autoantigens, mostly directed via light chain usage[28]. In line with these findings, it was shown in the *IgH.TE*μ CLL mouse model that the unmutated CLL-BCR repertoire is dependent on B cell-T cell interaction. This was explored *in vivo* using crosses with mice deficient for activation-induced cytidine deaminase (AID) expression in B cells or CD40L expression in T cells, as well as antigenic stimulation pressure[29]. Hereby, It was also found that the age of onset was not influenced by high expression levels of AID or CD40L[29]. These observations in mouse CLL models further underline the importance of the BCR including the role of (auto)antigens, as well as T-cell derived signals in disease onset[28, 29]. However, it should be further investigated whether this is comparable with CLL pathogenesis in human.

Next to the antigen reactivity concept, over the last years another concept has emerged in which the CLL BCR itself can act as an antigen, thus resulting in autonomous stimulation. Dühren-Von Minden et al. found that CLL-derived BCR recognized an internal epitope within framework 2 of the BCR, which causes cell-autonomous Ca^{2+} signaling[26]. This cell-autonomous signaling is induced by CLL BCRs expressed as IgM in the above described TKO cell system. When expressing the IGH and IGL genes from CLL cases described in Chapter 3 in TKO cells, we could indeed confirm autonomous signaling when these CLL BCRs were expressed as IgM isotypes. However, no autonomous Ca^{2+} signaling could be induced when they were expressed as IgD isotypes (**Figure 3B**). IgM can respond to monovalent and polyvalent antigens, while IgD requires polyvalent antigens for stimulation. This is found to be dependent on the specific hinge region in IgD, because after abrogation of the hinge region IgD can be stimulated by monovalent antigens[30]. Additionally, it was recently published that IgM-BCR can be autoreactive, while the same antigen shows less binding to and signaling induction in the corresponding IgG-BCR in TKO cells. Furthermore, it was also found that auto-antigen binding to soluble IgG, does not automatically correspond with autoantigen binding when this is expressed as IgM-BCR or IgG-BCR[31]. Taken together, these experiments corroborate the idea of cell-autonomous signaling in CLL, but also indicate the importance of the IGH constant region structure for this phenomenon and as a result perhaps for development of CLL.

The importance of the IGH constant region structure has been further shown in the publication by Minici et al, who investigated crystal structures of the stereotypic subsets CLL#2 and CLL#4[32]. The CLL#4 BCR consists of IGHV4-34, and IGHJ6*02 with a HCDR3 length of 20 amino acids, combined with an IGKV2-30/IGKJ2 light chain, and is expressed as IgG. For CLL#4 BCRs the class switch to IgG appeared to be required to induce autonomous signaling. The authors provided evidence that this is caused by the positively charged residue Lys214H, of which the side chain in the IgG CH1 domain forms hydrogen bonds that stabilize

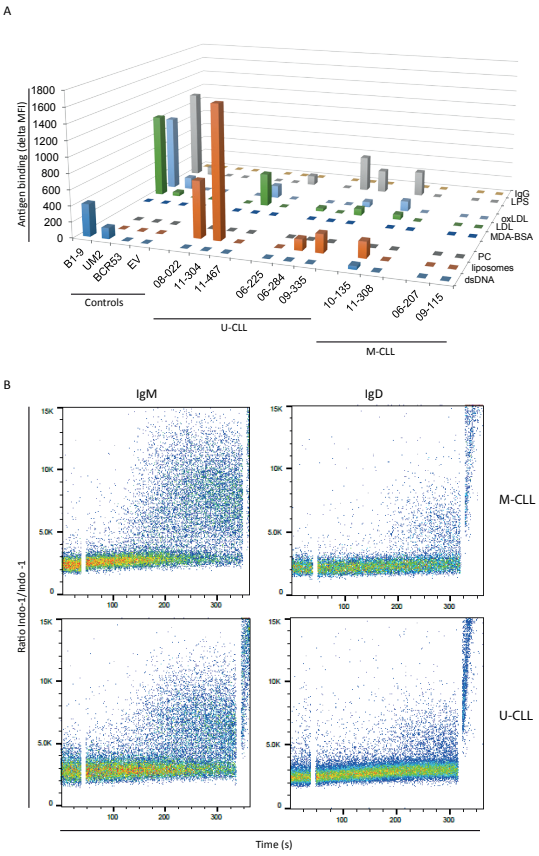


Figure 3. Analysis of the binding capacity and Ca2+ influx induction of CLL-derived BCR in TKO cells.

TKO cells were transduced to express a selection of CLL-derived BCR (see table 1 for details) as IgM and IgD as described previously[26, 27].

A. Flow cytometric analysis of antigen binding capacity of CLL-derived BCR in TKO cells.

TKO cells expressing IgM were incubated with biotinylated antigens for antigen-binding analysis; IgG, LPS, oxidized-LDL, LDL, Malondialdehyde (MDA)-BSA, phosphorylcholine-conjugated BSA (PC-BSA), Liposomes, or double stranded DNA (from calf thymus), as indicated. Antigen binding was detected by APC-streptadavidin and measured by flow cytometry on a LSR-Fortessa. CLL-derived BCR and controls are plotted on the x-axis. Antigen binding is shown by mean fluorescence intensity with background subtraction of negative control (delta MFI) on the y-axis from 3 separate analysis.

B. Flow cytometric analysis of Ca^{2+} influx in TKO cells expressing CLL-derived BCR as IgM and IgD.

TKO cells were incubated with Indo-1 for Ca^{2+} influx analysis. Autonomous signaling was detected after addition of tamoxifen, which allows the cells to signal by release of SLP65, for IgM CLL-BCR and not for IgD CLL-BCR, both in M-CLL (upper panels) and U-CLL (lower panels). The shown Ca^{2+} influx assay plots are representative for all tested CLL-derived BCR. Time of measurement in seconds is plotted on the x-axis, Ratio of median fluorescence intensity of Indo1/Indo-1 is plotted on thy y-axis.

self-interaction, and which is not conserved in CH1 domains of IgM and IgE. Furthermore, substitution of tyrosine into histidine on position 31L of the IGKV2-30 gene, as caused by SHM, proved to be important for the stabilization of the heavy chain-light chain pairing site and thereby self-interaction in this CLL#4 BCR[32]. In addition, Catera et al, showed that CLL#4 BCR recognize naïve and memory B cells via an aspartic acid at position 66 of FR3 in the rearranged IGKV2-30 light chain gene, which is acquired by SHM[33]. Altering the DNA leading to an amino acid change at this position resulted in loss of binding to B cells. What the exact target is on the naïve and memory B cells needs to be further determined[33]. Furthermore, BCR-BCR interaction cannot only be induced by the constant domain or variable regions of the heavy chain, but also via the light chain. This has been shown for CLL#2 receptors which were found to contain an Arg110L residue because of a specific G to C nucleotide substitution induced by SHM[32]. This substituted amino acid was identified in CLL#2 receptors from a cohort of 17 CLL patients[32]. Of note, the primer pairs that we used for our analysis did not allow for the detection of this substitution in our subset #2(-like) CLL family 3 cases which use CLL#2 BCR light chains (Chapter 5). Hence this would require further investigation.

In summary, these findings in two independent stereotypic CLL-BCR show the importance of the BCR structure in self-recognition, which could also impact on the 'selection' of these BCR and provide an explanation why these BCR are stereotypic. Chronic activation through self-recognition may thus be a key step in the development of stereotypic CLL.

Since CLL is generally considered a disease of elderly, we also investigated the BCR repertoire in aging healthy individuals. Our BCR repertoire analysis (Chapter 6) showed that are no gross alterations in the repertoire of naïve mature B cells between 20 and 70 years of age. After 70y we noted a shift in IGHV gene usage with significantly higher IGHV1-18, IGHV1-46, IGHV1-69, and IGHV5-51 gene usage, and significantly lower IGHV4-34, IGHV4-39, and IGHV4-59 gene usage. When we compared IGHV gene usage of clones from CLL patients with the BCR repertoire in naïve mature B cells of different age groups, we observed that the IGHV gene usage in CLL clones is very similar with that of the 50-70y healthy group and not the 70+y group. Comparison of the clonal BCR repertoire of CLL patients with other B cell subpopulations did not show much resemblance. In addition, we also investigated the presence of stereotypic BCR in the BCR repertoire of healthy control B cell subpopulations. Interestingly, we could show that CLL-associated stereotypic BCR are present in the BCR repertoire in B cell subpopulations of healthy individuals, and that their presence increases with age. This might suggest that part of specific IGHV gene usage or CLL-associated stereotypic BCR usage can be explained by age-dependent narrowing or skewing in the BCR repertoire. In naïve mature B cells, stereotypic BCR belonging to CLL#2, CLL#5, and CLL#64B were most frequently detected. In non-switched memory B cells the mostly found stereotypic BCRs concerned CLL#2 and CLL#14, the latter also being most prominent in IgM-only and CD27+IgG+ memory B cells. Altogether, we observed that stereotypic CLL-associated BCR are

part of the normal BCR repertoire. The increase of their presence upon aging might reflect a predisposition towards the development of monoclonal B cell lymphocytosis (MBL) or CLL clones in elderly, although further research is required.

In addition to the BCR repertoire studies in elderly, we further looked into the distribution of B cell subpopulations (Chapter 6). Although B cell subset distribution did not alter dramatically with increase of age, our B cell subpopulations analyzed in peripheral blood did show a decline in both absolute numbers and proportions of transitional B cells, total memory B cells and plasma blasts in elderly. These changes were accompanied with signs of relatively increased chronically activated $CD5^{+}CD43^{+}$ B cell and $CD21^{low}$ B cell populations. Chronically activated B cells have been associated with MBL and CLL[34, 35], while high numbers of $CD21^{low}$ B cells have been associated with autoimmune disease[36]. Increased numbers of CD5+CD43+ B cells upon aging could relate to a higher risk of MBL and CLL development in elderly. Higher counts of $CD21^{low}$ B cells in our screened elderly population could point to undiagnosed autoimmune phenomena.

SUMMARY OF MAIN FINDINGS AND FUTURE PERSPECTIVES

In summary, in this thesis we studied the B cell populations and BCR repertoire of healthy individuals upon aging. We detected a decline in both absolute numbers and proportions of transitional B cells, total memory B cells, and plasma blasts in elderly, and noticed signs of increased proportions of chronically activated CD5+CD43+ B cells and CD21low B cells. Furthermore, we observed that CLL-associated stereotypic BCR are present in the normal BCR repertoire from healthy individuals and their usage appeared to increase with age.

Both immunogenetic and chromosomal similarities were detected within the three CLL families in a disease that is known to be quite heterogeneous. Immunogenetically, these families represent three known CLL subgroups being M-CLL (family 1), U-CLL (family 2), and CLL#2-like CLL (family 3). Common chromosomal aberrations having prognostic value in CLL, were found to be present in these families. Deletion of 11q14.2-11q14.3 was found in family 1 and family 3 members, while trisomy 12 was detected in family 2 members.

Finally, when evaluating basal Ca^{2+} levels we noted clear differences in BCR signaling in CLL patients compared with B cells of healthy individuals, especially upon BCR stimulation. We identified a relationship between the basal Ca^{2+} level and SHM status, with the Ca^{2+} level being higher in M-CLL. Furthermore, we observed striking differences in expression levels of several BCR signaling molecules as well as NF-κB-related genes between IgM-responsive and IgM-unresponsive CLL cases.

In future research the role of molecular processes including BCR signaling and the more downstream NF-κB signaling should be linked to analysis of clonal evolution of CLL cells.

Current research on clonal evolution is mainly focused on the associated genetic alterations. We anticipate that combining studies into clonal evolution and BCR downstream signaling can shed new light on how CLL progresses and can lead to novel diagnostic tools. This will pave the way for personalized medicine in CLL, taking advantage of innovative targeted therapies that are currently available or that will be further developed.

REFERENCES

1. Hamblin, T.J., Davis, Z., Gardiner, A., Oscier, D.G. & Stevenson, F.K. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 94, 1848-1854 (1999).
2. Damle, R.N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L. *et al.* Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 94, 1840-1847 (1999).
3. Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J. *et al.* Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood* 119, 4467-4475 (2012).
4. Herve, M., Xu, K., Ng, Y.S., Wardemann, H., Albesiano, E., Messmer, B.T. *et al.* Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* 115, 1636-1643 (2005).
5. Catera, R., Silverman, G.J., Hatzi, K., Seiler, T., Didier, S., Zhang, L. *et al.* Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol Med* 14, 665-674 (2008).
6. Chu, C.C., Catera, R., Hatzi, K., Yan, X.J., Zhang, L., Wang, X.B. *et al.* Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood* 112, 5122-5129 (2008).
7. Lanemo Myhrinder, A., Hellqvist, E., Sidorova, E., Soderberg, A., Baxendale, H., Dahle, C. *et al.* A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood* 111, 3838-3848 (2008).
8. Binder, M., Lechenne, B., Ummanni, R., Scharf, C., Balabanov, S., Trusch, M. *et al.* Stereotypical chronic lymphocytic leukemia B-cell receptors recognize survival promoting antigens on stromal cells. *PLoS One* 5, e15992 (2010).
9. Hoogeboom, R., van Kessel, K.P., Hochstenbach, F., Wormhoudt, T.A., Reinten, R.J., Wagner, K. *et al.* A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med* 210, 59-70 (2013).
10. Muggen, A.F., Singh, S.P., Hendriks, R.W. & Langerak, A.W. Targeting Signaling Pathways in Chronic Lymphocytic Leukemia. *Curr Cancer Drug Targets* 16, 669-688 (2016).
11. Muggen, A.F., Pillai, S.Y., Kil, L.P., van Zelm, M.C., van Dongen, J.J., Hendriks, R.W. *et al.* Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. *Leukemia* 29, 321-328 (2015).
12. Mockridge, C.I., Potter, K.N., Wheatley, I., Neville, L.A., Packham, G. & Stevenson, F.K. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood* 109, 4424-4431 (2007).

13. Cherukuri, A., Cheng, P.C., Sohn, H.W. & Pierce, S.K. The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. *Immunity* 14, 169-179 (2001).
14. Terrier, B., Joly, F., Vazquez, T., Benech, P., Rosenzwajg, M., Carpentier, W. *et al.* Expansion of functionally anergic CD21-/low marginal zone-like B cell clones in hepatitis C virus infection-related autoimmunity. *J Immunol* 187, 6550-6563 (2011).
15. Isnardi, I., Ng, Y.S., Menard, L., Meyers, G., Saadoun, D., Srdanovic, I. *et al.* Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood* 115, 5026-5036 (2010).
16. Blery, M., Tze, L., Miosge, L.A., Jun, J.E. & Goodnow, C.C. Essential role of membrane cholesterol in accelerated BCR internalization and uncoupling from NF-kappa B in B cell clonal anergy. *J Exp Med* 203, 1773-1783 (2006).
17. Gauld, S.B., Benschop, R.J., Merrell, K.T. & Cambier, J.C. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* 6, 1160-1167 (2005).
18. Getahun, A., Beavers, N.A., Larson, S.R., Shlomchik, M.J. & Cambier, J.C. Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells. *J Exp Med* 213, 751-769 (2016).
19. Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtree, G.R. *et al.* Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity* 6, 419-428 (1997).
20. Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. & Healy, J.I. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature* 386, 855-858 (1997).
21. Muzio, M., Apollonio, B., Scielzo, C., Frenquelli, M., Vandoni, I., Boussiotis, V. *et al.* Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood* 112, 188-195 (2008).
22. Marklin, M., Heitmann, J.S., Fuchs, A.R., Truckenmuller, F.M., Gutknecht, M., Bugl, S. *et al.* NFAT2 is a critical regulator of the anergic phenotype in chronic lymphocytic leukaemia. *Nat Commun* 8, 755 (2017).
23. Mansouri, L., Sutton, L.A., Ljungstrom, V., Bondza, S., Arngarden, L., Bhoi, S. *et al.* Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. *J Exp Med* 212, 833-843 (2015).
24. Cesano, A., Perbellini, O., Evensen, E., Chu, C.C., Cioffi, F., Ptacek, J. *et al.* Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica* 98, 626-634 (2013).
25. Fernhout, F., Dinkelaar, R.B., Hagemeijer, A., Groeneveld, K., van Kammen, E. & van Dongen, J.J. Four aged siblings with B cell chronic lymphocytic leukemia. *Leukemia* 11, 2060-2065 (1997).
26. Duhren-von Minden, M., Ubelhart, R., Schneider, D., Wossning, T., Bach, M.P., Buchner, M. *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 489, 309-312 (2012).
27. Meixlsperger, S., Kohler, F., Wossning, T., Reppel, M., Muschen, M. & Jumaa, H. Conventional light chains inhibit the autonomous signaling capacity of the B cell receptor. *Immunity* 26, 323-333 (2007).
28. Jimenez de Oya, N., De Giovanni, M., Fioravanti, J., Ubelhart, R., Di Lucia, P., Fiocchi, A. *et al.* Pathogen-specific B-cell receptors drive chronic lymphocytic leukemia by light-chain-dependent cross-reaction with autoantigens. *EMBO Mol Med* 9, 1482-1490 (2017).

29. Pal Singh, S., de Bruijn, M.J.W., de Almeida, M.P., Meijers, R.W.J., Nitschke, L., Langerak, A.W. *et al.* Identification of Distinct Unmutated Chronic Lymphocytic Leukemia Subsets in Mice Based on Their T Cell Dependency. *Front Immunol* 9, 1996 (2018).
30. Ubelhart, R., Hug, E., Bach, M.P., Wossning, T., Duhren-von Minden, M., Horn, A.H. *et al.* Responsiveness of B cells is regulated by the hinge region of IgD. *Nat Immunol* 16, 534-543 (2015).
31. Iype, J., Datta, M., Khadour, A., Ubelhart, R., Nicolo, A., Rollenske, T. *et al.* Differences in Self-Recognition between Secreted Antibody and Membrane-Bound B Cell Antigen Receptor. *J Immunol* 202, 1417-1427 (2019).
32. Minici, C., Gounari, M., Ubelhart, R., Scarfo, L., Duhren-von Minden, M., Schneider, D. *et al.* Distinct homotypic B-cell receptor interactions shape the outcome of chronic lymphocytic leukaemia. *Nat Commun* 8, 15746 (2017).
33. Catera, R., Liu, Y., Gao, C., Yan, X.J., Magli, A., Allen, S.L. *et al.* Binding of CLL subset 4 B-cell receptor immunoglobulins to viable human memory B lymphocytes requires a distinctive IGKV somatic mutation. *Mol Med* 23, 1-12 (2017).
34. Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Durig, J. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med* 209, 2183-2198 (2012).
35. Nieto, W.G., Teodosio, C., Lopez, A., Rodriguez-Caballero, A., Romero, A., Barcena, P. *et al.* Non-CLL-like monoclonal B-cell lymphocytosis in the general population: prevalence and phenotypic/genetic characteristics. *Cytometry B Clin Cytom* 78 Suppl 1, S24-34 (2010).
36. Thorarinsdottir, K., Camponeschi, A., Gjertsson, I. & Martensson, I.L. CD21 -/low B cells: A Snapshot of a Unique B Cell Subset in Health and Disease. *Scand J Immunol* 82, 254-261 (2015).
37. Bende, R.J., Aarts, W.M., Pals, S.T. & van Noesel, C.J. Immunoglobulin diversification in B cell malignancies: internal splicing of heavy chain variable region as a by-product of somatic hypermutation. *Leukemia* 16, 636-644 (2002).

Addendum

Abbreviations

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

List of publications

LIST OF ABBREVIATIONS

AKT	protein kinase B
BCR	B cell receptor
BTK	Bruton's tyrosine kinase
CDR3	complementarity determining regions
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CSR	class switch recombination
DAG	diacylglycerol
DLBCL	diffuse large B cell lymphoma
EBF1	Early B-Cell Factor 1
ERK	extracellular signal-regulated kinase
FCγR2	Fc receptor, IgG, low affinity II
FOXO	forkhead transcription factors
FR	framework
GC	germinal center
Ig	Immunoglobulin
IGH	immunoglobulin heavy chain
IGHD	immunoglobulin heavy chain diversity region
IGHJ	immunoglobulin heavy chain joining region
IGHV	Immunoglobulin heavy chain variable region
IGL	immunoglobulin light chain
IκB	inhibitor of nuclear factor kappa B
IKK	IκB kinase
ITAM	immunoreceptor tyrosine-based activation motif
LPS	Lipopolysaccharide
LYN	Lck/Yes novel tyrosine kinase
M-CLL	mutated chronic lymphocytic leukemia
MCL	mantle cell lymphoma
MYD88	myeloid differentiation primary response 88
MZ	marginal zone
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NHL	non-Hodgkin lymphoma
NGS	next generation sequencing
ORR	overall response rate
OS	Overall survival

PFS	progression-free survival
PI3K	phosphatidyl-inositol-3-kinase
PIP	phosphatidyl-inositol phosphate
PIP2	phosphatidyl-inositol 4,5-biphosphate
PIP3	phosphatidyl-inositol 1,3,5-triphosphate
PKC	protein kinase C
PLCγ	phospholipase C γ
SHM	somatic hypermutation
SLL	small lymphocytic lymphoma
SLP65	SH-2 domain leukocyte protein of 65 KD
SNP	single nucleotide polymorphism
SYK	spleen tyrosine kinase
TKO	triple knock-out
TLR	toll-like receptor
U-CLL	unmutated chronic lymphocytic leukemia

NEDERLANDSE SAMENVATTING (VOOR NIET INGEWIJDEN)

Alle levende organismen, inclusief de mens, worden blootgesteld aan ziekteverwekkers zoals virussen, bacteriën, schimmels en parasieten gedurende hun leven. Daarom is een goed werkend afweersysteem nodig om ons te beschermen tegen ziekten of zelfs overlijden veroorzaakt door infecties. Het immuunsysteem bestaat uit cellen die behoren tot het aangeboren afweersysteem, dat een eerste verdedigingslinie vormt, en uit cellen die behoren tot het verworven afweersysteem, dat een heel specifieke reactie op een ziekteverwekker oproept. Een speciaal soort witte bloedcellen zijn de B en T cellen, ook wel lymfocyten genoemd. Zij maken deel uit van de verworven afweersysteem en brengen heel specifieke receptoren tot expressie, welke gericht zijn tegen eiwitten of andere moleculen (antigenen) van ziekteverwekkers. Bij de eerder genoemde B cel worden deze specifieke receptoren B-celreceptoren (BCR) genoemd. B cellen hebben verder als heel specifieke eigenschap dat ze zich nadat ze een antigeen herkend hebben, gaan delen, zich verder ontwikkelen en in staat zijn om deze BCR ook als antistof (ook wel immuunglobuline (Ig) genoemd) uit te kunnen scheiden. Er zijn 5 verschillende Ig klassen; IgM, IgD, IgG, IgA en IgE. Om alle mogelijke ziekteverwekkende antigenen te kunnen herkennen is een breed repertoire van BCR moleculen nodig. Dit is mogelijk door middel van knip- en plakprocessen (herschikkingen) in het immuunglobuline DNA waarbij “variable” (V), “diversity” (D), en/of “joining” (J) genen aan elkaar gekoppeld worden. Dit vormt samen uiteindelijk een unieke BCR. Omdat alle B cellen een unieke BCR tot expressie brengen zorgt dit voor een heel breed BCR repertoire en kunnen er onnoemelijk veel antigenen herkend worden.

Wanneer een antigeen bindt aan de BCR wordt de B cel geactiveerd doordat diverse signaalmoleculen achtereenvolgens aangezet worden. Dit wordt BCR signalering genoemd (zie hoofdstuk 2, figuur 1). Een deel van deze signaalmoleculen zijn kinases die elkaar aanzetten door op een ander signaalmolecuul een fosfaat-groep te zetten (fosforylering). Dit zorgt voor het vrijkomen van calcium (Ca^{2+}), wat uiteindelijk zorgt dat ook andere eiwitten zoals transcriptiefactoren worden geactiveerd. BCR signalering leidt tot verdere ontwikkeling (differentiatie) van de B cel en celdeling (proliferatie). Tijdens de B cel differentiatie kan een enzym mutaties in het DNA van het antigeen-herkennende domein van de BCR aanbrengen. Dit proces wordt somatische hypermutatie (SHM) genoemd en kan de bindingssterkte (affiniteit) voor het antigeen verhogen. Daarnaast kan verandering van immunoglobuline klasse van het constante deel van de BCR (class switch recombination, CSR), de affiniteit voor een antigeen ook versterken. Op basis van verschillende kenmerken tijdens de differentiatiestappen kunnen B cellen gegroepeerd worden.

Chronische lymfatische leukemie (CLL), het meest voorkomende type leukemie bij ouderen, is een monoklonale tumor van B cellen die allen dezelfde BCR hebben. CLL is een

heel heterogene ziekte, wat betekent dat het ziekteverloop voor veel patiënten heel mild is en zij weinig klachten hebben, maar andere patiënten hebben een agressievere vorm. Kenmerken van de BCR zijn een belangrijke indicatie voor de prognose van CLL patiënten. Wanneer er SHM van de BCR heeft plaatsgevonden, wordt dit gemuteerde CLL genoemd (M-CLL). Dit heeft een gunstige prognose ten opzichte van ongemuteerde CLL (U-CLL), welke vorm agressiever is. Verder is het opvallend dat bij een-derde van alle CLL patiënten de CLL cellen een BCR hebben die heel erg lijkt op die van CLL cellen in andere patiënten. Dit worden stereotype BCR genoemd. CLL patiënten met dezelfde stereotype BCR kunnen ingedeeld worden in groepen of stereotype subsets en dit kan ook prognostische waarde hebben. Subset CLL#2 is geassocieerd met een agressieve vorm van CLL en subset CLL#4 heeft over het algemeen een gunstige prognose.

In dit proefschrift hebben we de rol van de BCR signalering onderzocht in CLL cellen van verschillende groepen CLL patiënten. We hebben onderzocht in hoeverre BCR signalering verschilt van normale B cellen en welke verschillen er zijn tussen CLL cellen van verschillende patiënten na stimulatie van de BCR. Verder hebben we in 3 families met meerdere CLL patiënten de karakteristieken van de BCR onderzocht en daarnaast gekeken naar genetische afwijkingen in deze families. Omdat CLL een ziekte is die vooral bij ouderen voorkomt, hebben we tenslotte ook de B cel differentiatie en BCR repertoire bij veroudering bestudeerd.

In **hoofdstuk 2** geven we een overzicht van de literatuur over belangrijke immuun-gerelateerde signaleringspaden die een rol spelen in het ontstaan en de verdere groei van CLL en welke tegenwoordig een doelwit zijn voor therapie. Dit zijn BCR signalering, chemokine receptor signalering en Toll-like receptor (TLR) signalering. Ook bespreken we hier de medicatie die heel specifiek signaleringsmoleculen blokkeren en de effecten daarvan op CLL cellen.

In **hoofdstuk 3** hebben we de verschillen in BCR signalering tussen B cellen van 14 gezonde personen en CLL cellen van 68 patiënten bestudeerd aan de hand van de hoeveelheid intracellulair Ca^{2+}. We vonden dat intracellulair Ca^{2+} hoger was in CLL cellen dan in normale B cellen. Wanneer we deze resultaten vergeleken tussen verschillende subpopulaties van CLL, M-CLL en U-CLL, zagen we dat M-CLL hogere basale intracellulaire Ca^{2+} niveaus had dan U-CLL. Wanneer we de BCR stimuleerden met een antilichaam zagen we dat CLL cellen een verminderde respons lieten zien ten opzichte van normale B cellen, en dat U-CLL iets meer respons vertoonde bij stimulatie van de IgM-BCR dan M-CLL. Hieruit concludeerden wij dat M-CLL minder gevoelig is voor BCR stimulatie dan U-CLL.

In **hoofdstuk 4** hebben we verder gekeken naar verschillen tussen CLL cellen van 52 patiënten die wel of niet reageren op stimulatie van de BCR door de vrijgifte van Ca^{2+}, en welke factoren in de cel een rol spelen bij deze verschillen. Wanneer we keken naar de fosforylering van verschillende signaleringsmoleculen zagen we dat CLL cellen die reageerden op IgM stimulatie (responsive CLL, n=24) meer fosforylering van PLCγ2 en AKT lieten zien dan

cellen die niet reageerden (unresponsive CLL, n=28). Deze responsive CLL bleken ook een hogere expressie van IgM, IgD, CD19, CD38 en CD43 te hebben dan unresponsive CLL. Onze RNA sequencing resultaten lieten zien dat verschillende NF-κB gerelateerde genen hoger tot expressie kwamen in unresponsive CLL. Dit waren voornamelijk genen die een rol spelen bij het remmen van NF-κB signalering, zo genoemde NF-κB inhibitors. Na stimulering van de IgM-BCR zagen we in responsive CLL een toename van de expressie van NF-κB inhibitors, maar ook van NF-κB subunit REL, terwijl in de unresponsive CLL er geen verschil was vergeleken met de basale (ongestimuleerde) niveaus. Hiermee toonden we aan dat NF-κB signalering heel belangrijk is voor CLL cellen en hun mogelijkheid om te reageren op BCR stimulatie.

In **hoofdstuk 5** hebben we binnen drie families met twee of meer CLL patiënten de karakteristieken van de BCR bestudeerd. Familie 1 bestond uit vier familieleden die allen een door SHM gemuteerde BCR vertoonden en dus M-CLL patiënt zijn, terwijl de twee broers van familie 2 beiden een ongemuteerde BCR hadden en dus tot de U-CLL groep behoren. Opmerkelijk was dat de vier patiënten van familie 3 allemaal een vrijwel identieke lichte keten hadden, waarvan bekend is dat deze behoort tot de stereotype CLL subset CLL#2. Een van deze vier patiënten had ook de kenmerkende CLL#2 zware keten, en is daarmee een prototype CLL#2, terwijl een ander familielid een BCR liet zien die sterk lijkt op een CLL#169. Daarnaast hebben we ook gekeken naar diverse genetische afwijkingen die bekend zijn voor CLL en prognostische waarde hebben. Hier zagen we ook overeenkomsten. Binnen familie 1 en familie 3 hadden alle familieleden een chromosoom 13q14 deletie en binnen familie 2 lieten beide familieleden een trisomie 12 zien. Van andere kleinere genetische veranderingen die eerder al geassocieerd zijn met CLL, vonden we enkele terugkerend binnen een familie. Of dit laatste komt door een genetische predispositie of een overeenkomstig patroon van klonale evolutie zal verder onderzocht moeten worden.

In **hoofdstuk 6** hebben we het effect van veroudering op B cel differentiatie en het BCR repertoire bestudeerd. Met behulp van flow cytometrie hebben we de B cel subsets in het bloed van 155 gezonde individuen, onderverdeeld in 4 leeftijdsgroepen, geanalyseerd. We vonden een afname van B cellen (totaal en relatief) bij veroudering, met een verlaging van transitionele B cellen, memory (geheugen) B cellen (met name non-switched en IgG+ memory cellen), en plasmablasten bij individuen boven 70 jaar. Verder hebben we het BCR repertoire van naïeve cellen en antigeen-gestimuleerde B cellen in verschillende leeftijdsgroepen bestudeerd en dit vergeleken met het klonale BCR repertoire van 920 CLL patiënten. We vonden weinig verschillen in BCR repertoire van naïeve mature B cellen en antigeen-gestimuleerde B cellen onder 70 jaar. Duidelijke verschillen in BCR repertoire, vooral voor IGHV-gengebruik vonden we in naïeve mature B cellen in de 70+ leeftijdsgroep. Het IGHV-gengebruik van CLL patiënten (zowel van de 50-70 en de boven 70 groep) leek het meest op dat van naïeve mature B cellen van de 50-70 leeftijdsgroep, maar niet van

de naïeve mature B cellen van de 70+ groep. Daarnaast vonden we dat CLL-geassocieerde stereotype BCR deel uitmaken van het normale BCR repertoire van gezonde individuen en dat hun aandeel toeneemt bij hogere leeftijd.

Tenslotte bespreken we onze resultaten uit alle hoofdstukken in de General Discussion (hoofdstuk 7) en komen tot conclusies en verdere richtingen voor het onderzoek naar de BCR bij CLL.

CURRICULUM VITAE

PERSONALIA

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WORK EXPERIENCE

Current position	
Since 2017(Aug)	**Clinical Research Associate** Novartis - Amsterdam
2016 (Nov)-2017 (Aug)	**Remote site monitor/in-house CRA** PPD – Bennekom
2011 (Sep)-2020 (Jan)	**PhD thesis with title 'Chronic lymphocytic leukemia: the B cell receptor and beyond'** Erasmus MC – Rotterdam
2014 (Sep-Dec)	**Research visit/stay abroad** Max Planck Institute of Immunobiology and Epigenetics – Freiburg am Breisgau (Germany)
2015 (July)	**Research visit/stay abroad** Institute of Immunology, University Hospital Ulm, Ulm, (Germany)
2009 (Jan-Dec)	**Internship Immuno-(epi)genetica, subject 'effect of inhibitors of chromatin modifying enzymes on monocyte differentiation'** Leids Universitair Medisch Centrum – Leiden
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EDUCATION

2011 (Sep)-2020 (Jan)	**PhD study Immunology** Erasmus MC - Rotterdam
2007-2010	**MSc. Oncology** Vrije Universiteit - Amsterdam
2004-2007	**BSc. Biomedical Sciences** Universiteit Utrecht
2001-2002	**Propedeuse HBO Voeding & Diëtetiek** Hanzehogeschool - Groningen
2000-2001	**VWO** Deltion College - Zwolle
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PERSONAL AWARDS

2014 **Erasmus Trustfonds, Travel grant** for working visit to Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

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2015 **EMBO Short Term Fellowship**, for working visit to Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

2015 **NVVI (Dutch Society of Immunology), Travel grant** for visit to the European Congress of Immunology, Vienna, Austria

PHD PORTFOLIO
SUMMARY OF PHD TRAINING AND TEACHING

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Immunology

Research school:	Postgraduate Molecular Medicine
PhD period:	September 2011 – August 2019
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PhD training

In depth courses

2011	Biomedical Research Techniques
2011	Medische Immunologie
2012	Molecular Immunology
2012	Basic and Translational Oncology
2013	English Biomedical Writing and Communication
2013	NIBI Management voor promovendi en postdocs
2014	Career Orientation for Scientists
2014	Biobusiness Summercourse
2015	SPSS

Seminars and workshops

2011-2015	Seminars and minisymposia at the department of Immunology
2011-2014	Journal club at the department of Immunology
2012	Regulatory B cell Symposium at Leids Universitair Medisch Centrum
2013	IgCLL workshop, Brno, Czech Republic
2013	Symposium Mucosal Immunology at Erasmus MC
2014	Seminars at the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

(Inter)national conferences

2012 Dutch Hematology Congress, Arnhem, The Netherlands
2012 Annual Meeting/Winter school of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands – *Poster presentation*
2013 Dutch Hematology Congress, Arnhem, The Netherlands – *Oral presentation*
2013 15th International Congress of Immunology (ICI), Milano, Italy – *Oral presentation*
2013 IWCLL meeting, Köln, Germany – *Poster presentation*
2014 Dutch Hematology Congress, Arnhem, The Netherlands
2014 B Cell Forum Meeting, Krickenbeck, Germany – *Oral presentation*
2014 European Hematology Congress (EHA), Milano, Italy – *Poster presentation*
2014 Annual Meeting/Winter school of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands – *Poster presentation*
2015 B cell Forum Meeting, Hitzacker, Germany – *Oral presentation*
2015 5th European Congress of Immunology (ECI), Vienna, Austria – *Poster presentation*
2016 B cell Forum Meeting, Zeist, Nederland – *Poster presentation*

Grants and awards

2014 Erasmus Trustfonds, Travel grant for working visit to Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany
2014 NVVI (Dutch Society of Immunology), Travel grant for working visit to Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany
2015 EMBO Short Term Fellowship, for working visit to Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany
2015 NVVI (Dutch Society of Immunology), Travel grant for visit European Congress of Immunology, Vienna, Austria

Teaching

2012-2015 Supervising 1st and 2nd year Medical students' histology and immunology
2012-2013 Supervising BSc internship 'HLO-student'
2014-2015 Supervising MSc internship 'Master Infection and Immunity student'
2015 Supervising BSc internship 'HLO-student'

LIST OF PUBLICATIONS

Muggen AF, de Jong M, Wolvers-Tettero ILM, Kallemeijn MJ, Teodósio C, Darzentas N, Stadhouders R, IJspeert H, van der Burg M, van IJcken WF, Verhaar JAN, Abdulahad WH, Brouwer E, Boots AMH, Hendriks RW, van Dongen JJM, Langerak AW. The presence of CLL-associated stereotypic B cell receptors in the normal BCR repertoire from healthy individuals increases with age. Immun Ageing. 2019 Aug 28;16:22.

Meijers RWJ*, **Muggen AF***, Leon LG, de Bie M, van Dongen JJM, Hendriks RW, Langerak AW. Responsiveness of Chronic Lymphocytic Leukemia cells to B cell receptor stimulation is associated with low expression of regulatory molecules of the Nuclear Factor-κB pathway. Haematologica. 2019 May 16. (Epub ahead of print) (* contributed equally)

Singh SP, Pillai SY, de Bruijn MJW, Stadhouders R, Corneth OBJ, van den Ham HJ, **Muggen AF**, van IJcken W, Slinger E, Kuil A, Spaargaren M, Kater AP, Langerak AW, Hendriks RW. Cell lines generated from a chronic lymphocytic leukemia mouse model exhibit constitutive Btk and Akt signaling. Oncotarget. 2017 May 26;8(42):71981-71995.

Nelde A, Walz JS, Kowalewski DJ, Schuster H, Wolz OO, Peper JK, Cardona Gloria Y, Langerak AW, **Muggen AF**, Claus R, Bonzheim I, Fend F, Salih HR, Kanz L, Rammensee HG, Stevanović S, Weber AN. HLA class I-restricted MYD88 L265P-derived peptides as specific targets for lymphoma immunotherapy. Oncoimmunology. 2016 Dec 23;6(3):e1219825.

Muggen AF, Singh SP, Hendriks RW, Langerak AW. Targeting Signaling Pathways in Chronic Lymphocytic Leukemia. Curr Cancer Drug Targets. 2016;16(8):669-688. Review.

Sutton LA, Young E, Baliakas P, Hadzidimitriou A, Moysiadis T, Plevova K, Rossi D, Kminkova J, Stalika E, Pedersen LB, Malcikova J, Agathangelidis A, Davis Z, Mansouri L, Scarfò L, Boudjoghra M, Navarro A, **Muggen AF**, Yan XJ, Nguyen-Khac F, Larrayoz M, Panagiotidis P, Chiorazzi N, Niemann CU, Belessi C, Campo E, Strefford JC, Langerak AW, Oscier D, Gaidano G, Pospisilova S, Davi F, Ghia P, Stamatopoulos K, Rosenquist R; ERIC, the European Research Initiative on CLL. Different spectra of recurrent gene mutations in subsets of chronic lymphocytic leukemia harboring stereotyped B-cell receptors. Haematologica. 2016 Aug;101(8):959-67.

Mansouri L, Sutton LA, Ljungström V, Bondza S, Arngården L, Bhoi S, Larsson J, Cortese D, Kalushkova A, Plevova K, Young E, Gunnarsson R, Falk-Sörqvist E, Lönn P, **Muggen AF**, Yan XJ, Sander B, Enblad G, Smedby KE, Juliusson G, Belessi C, Rung J, Chiorazzi N, Strefford JC, Langerak AW, Pospisilova S, Davi F, Hellström M, Jernberg-Wiklund H, Ghia P, Söderberg O, Stamatopoulos K, Nilsson M, Rosenquist R. Functional loss of IκBε leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia. J Exp Med. 2015 Jun 1;212(6):833-43.

Wierda RJ, Goedhart M, van Eggermond MC, **Muggen AF**, Miggelbrink XM, Geutskens SB, van Zwet E, Haasnoot GW, van den Elsen PJ. A role for KMT1c in monocyte to dendritic cell differentiation: Epigenetic regulation of monocyte differentiation. Hum Immunol. 2015 Jun;76(6):431-7.

Sutton LA, Ljungström V, Mansouri L, Young E, Cortese D, Navrkalova V, Malcikova J, **Muggen AF**, Trbusek M, Panagiotidis P, Davi F, Belessi C, Langerak AW, Ghia P, Pospisilova S, Stamatopoulos K, Rosenquist R. Targeted next-generation sequencing in chronic lymphocytic leukemia: a high-throughput yet tailored approach will facilitate implementation in a clinical setting. Haematologica. 2015 Mar;100(3):370-6.

Muggen AF, Pillai SY, Kil LP, van Zelm MC, van Dongen JJ, Hendriks RW, Langerak AW. Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia. Leukemia. 2015 Feb;29(2):321-8.