Modeling the B-cell receptor signaling on single cell level reveals a stable network circuit topology between nonmalignant B cells and chronic lymphocytic leukemia cells and between untreated cells and cells treated with kinase inhibitors

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Abstract

B-cell receptor (BCR) signaling is central for the pathomechanism of chronic lymphocytic leukemia (CLL), and inhibitors of BCR signaling have substantially improved treatment options. To model malignant and nonmalignant BCR signaling, we quantified five components of BCR signaling (ZAP70/SYK, BTK, PLCγ2, AKT, ERK1/2) in single cells from primary human leukemic cells and from nonmalignant tissue. We measured signaling activity in a time-resolved manner after stimulation with BCR crosslinking by anti-IgM and/or anti-CD19 and with or without inhibition of phosphatases with H2O2. The phosphorylation of BCR signaling components was increased in malignant cells compared to nonmalignant cells and in IGHV unmutated CLL cells compared to IGHV mutated CLL cells. Intriguingly, inhibition of phosphatases with H2O2 led to higher phosphorylation levels of BCR components in CLL cells with mutated IGHV compared to unmutated IGHV. We modeled the connectivity of the cascade components by correlating signal intensities across single cells. The network topology remained stable between malignant and nonmalignant cells. To additionally test for the impact of therapeutic compounds on the network topology, we challenged the BCR signaling cascade with inhibitors for BTK (ibrutinib), PI3K (idelalisib), LYN (dasatinib) and SYK (entospletinib). Idelalisib treatment resulted in similar effects in malignant and nonmalignant cells, whereas ibrutinib was mostly active on CLL cells. Idelalisib and ibrutinib had complementary effects on the BCR signaling cascade whose activity was further reduced upon dasatinib and...
entospletinib treatment. The characterization of the molecular circuitry of leukemic BCR signaling will allow a more refined targeting of this Achilles heel.

**KEYWORDS**
BCR signaling, CLL, ibrutinib, modeling

**What’s new?**
Targeting B-cell receptor signaling has become standard of care in treating chronic lymphocytic leukemia. Further adjustment of B-cell receptor inhibition is however crucial to tackle adverse events and treatment resistance. Here, the authors shed light on the B-cell receptor signaling cascade topology by comparing healthy and malignant cells and chronic lymphocytic leukemia patient subgroups. They use a quantitative characterization model including poststimulation signaling strength, time-resolved dynamics and network interconnections. The model can assess the rewiring capability of the signaling cascade and evaluate potential combination treatments, allowing a more refined targeting of this Achilles heel.

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**1 | INTRODUCTION**

The main characteristics of B cell chronic lymphocytic leukemia (CLL) are a combination of enhanced survival in the periphery and increased proliferation in secondary lymphoid tissue. Both processes are stimulated by B cell receptor (BCR) signaling which is central to the pathomechanism of CLL. In physiological B cell development, the BCR is somatically hypermutated. CLL patients whose BCR contains an unmutated immunoglobulin heavy chain variable region (U-CLL) suffer from a more severe disease phenotype than patients with mutated IGHV (M-CLL). U-CLL has been associated with an enhanced BCR activity.

In nonmalignant B cells, BCR antigen engagement promotes proliferation and B cell selection, leading to differentiation upon antigen binding. Antigen-specific Ig and an Ig-α/Ig-β heterodimer compose a BCR. Antigen binding leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs of the Ig-α/Ig-β tails inside the cytoplasm. Subsequent BCR oligomerization and microcluster growth initiate the signaling cascade via docking and protein phosphorylation of the Spleen tyrosine kinase (SYK). The activation of SYK, PLCγ2 and PI3K pathways leads to membrane localization of Protein kinase B (AKT) and Bruton’s tyrosine kinase (BTK) and AKT and BTK amplify the BCR signal toward the transcription factors NF-κB and NFAT. The activation of SYK, PLCγ2 and PI3K pathways leads to membrane localization of Protein kinase B (AKT) and Bruton’s tyrosine kinase (BTK) and AKT and BTK amplify the BCR signal toward the transcription factors NF-κB and NFAT and additionally activate the extracellular signal-related kinases 1 and 2 (ERK1/2) pathway. Gene expression profiling has revealed that the BCR pathway is the most prominently activated in CLL cells in lymphatic tissue, the site of CLL cell proliferation. Interestingly, gene expression profiling also associated poor prognosis of CLL patients with increased activity of genes downstream of the BCR. These observations have led to the development of new therapeutic strategies focusing on the inhibition of BCR-related kinases such as BTK, PI3K, LYN or SYK. Inhibiting these components of the BCR signaling network is a landmark improvement in the treatment response of CLL.

Approximately 80% of patients with resistance against BCR inhibitors, treatment resistance is hypothesized to occur through rewiring and adaptation of the signaling network similar to the development of resistance in other malignancies, for example lung cancer under kinase inhibitor treatment.

Resistance mechanisms to the novel treatments lead to relapse in CLL patients, which is why understanding BCR signaling in CLL is especially important. Interestingly, patients with U-CLL do not differ from M-CLL patients in clinical prognosis under BTK or LYN inhibition. Approximately 80% of patients with resistance to BTK inhibition are associated with mutations in BTK or PLCγ2. For the remaining subpopulation of CLL patients with resistance against BCR inhibitors, treatment resistance is hypothesized to occur through rewiring and adaptation of the signaling network similar to the development of resistance in other malignancies, for example lung cancer under kinase inhibitor treatment.

For a more in-depth characterization of the BCR signaling cascade, we here provide insights into the dynamics, the heterogeneity and the signaling topology of BCR signaling in malignant CLL cells and in nonmalignant B cells. Our findings complement measurements of BCR signaling upon stimulation with a new model of the signaling network topology. Such network connectivity analysis contributes to patient stratification and thus ultimately to new combination treatment strategies by identification of central network nodes that could potentially be inhibited. We utilized single-cell phospho-specific flow cytometry (phospho-flow) to quantify signaling dynamics upon BCR stimulation. Protein phosphorylation is used as a marker for activation of the signaling network components. We demonstrate that the BCR signaling topology remains largely unchanged in CLL cells compared to nonmalignant B cells. In contrast, BCR-signaling strength and duration of signaling upon stimulation differ not only between CLL cells compared to CD19+ B cells of healthy donors but also between U-CLL and M-CLL cells. We furthermore analyzed the effect of the four inhibitors ibrutinib, idelalisib, entospletinib and dasatinib on BCR signaling. Using Bayesian network modeling of quantitative single-cell data, we found a novel interrelation between PLCγ2 and AKT that is stronger in U-CLL than in M-CLL. In addition, we found that inhibition of PLCγ2...
abrogates phosphorylation of AKT, suggesting a novel option for overcoming treatment resistance in CLL.

2 | MATERIALS AND METHODS

2.1 | Cell culture conditions and cell isolation

Peripheral blood was collected from patients with CLL of normal karyotype or deletion of chromosome 13q (Table S1). Buffy coats from healthy donors were obtained from the German Red Cross in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation in Biocoll (Biochrom, Berlin, Germany) separation solution. PBMCs of CLL patients were frozen without further manipulation in culture medium supplemented with 10% DMSO (Sigma-Aldrich, Munich, Germany). CD19⁺ B cells from Buffy coats were isolated via negative magnetic enrichment using EasySep Human B cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada) as described by the manufacturer. The purity of PBMCs and CD19⁺ sorted cells was determined by flow cytometry analysis using anti-CD5-FITC and anti-CD19-APC antibodies (BD Biosciences). Primary CLL cells were cultured in DMEM (Sigma-Aldrich), 10% FCS (Biochrom), 20% Human AB serum (MP Biomedicals, Eschwege, Germany) and 1% Penicillin/Streptomycin (1000 U/mL, Life Technologies, Darmstadt, Germany).

2.2 | Inhibitor treatments and stimulation

Prior to the stimulation experiments, a buffer exchange was performed for the biotin-coupled antibodies to remove sodium azide (NaN₃). For every experiment, a DMSO solution was transferred to Pierce protein formers for the biotin-coupled antibodies to remove sodium azide. Prior to the stimulation experiments, a buffer exchange was performed for the biotin-coupled antibodies to remove sodium azide.

For permeabilization, cells were resuspended in 100 μL 100% ice-cold methanol (Sigma) and fluorescent cell barcoding was applied as previously described. Briefly, cells in each well (≈one time point) were treated with a unique combination of amine-reactive fluorescent dyes, namely Pacific Blue (final concentrations of 0.001, 0.1 and 0.5 ng/μL) and Pacific Orange (final concentrations of 0.004 and 0.06 ng/μL) (Invitrogen). Based on their fluorescent signature, it was possible to distinguish 6 different cell populations. After 30 minutes incubation on ice, permeabilization and barcoding was stopped by the addition of 100 μL FACS buffer (5% FCS, 1% BSA, 0.05% NaN₃ in PBS) and the plates were centrifuged at 2000 rpm at 4°C. The cells were washed twice with FACS buffer and samples of six different time points of one condition could then be combined into one tube for further staining.

A master-mix containing all conjugated phospho-specific antibodies (Anti PLC-γ2 (pY759) Alexa Fluor488, Anti ERK 1/2 (pT202/pY204) PerCP-CyTM5.5, Anti ZAP70 (pY319)/SYK (pY352) PE-CyTM7, Anti AKT (pS473) Alexa Fluor 647, Anti BTK (pY551)ITK (pY511) PE, all from BD Biosciences) and an anti CD20 (Anti-Human CD20 APC-H7) antibody for gating were added to the cells and incubated for 30 minutes at 4°C. In parallel, compensation beads for the antibodies (BD Biosciences) and the fluorescent cell barcode (FCB) dyes (Thermo Scientific) were stained. Samples and beads were washed twice with FACS buffer and measured at an LSR II flow cytometer (BD Biosciences).

2.3 | Fluorescent cell barcode labeling and staining with phoso-specific antibodies

Data were acquired at an LSR II flow cytometer (BD Biosciences) and compensated by using BD FACSDiva software. Further analysis and deconvolution of the barcode labeling populations were done with FlowJo software and scale values were exported for further analysis.

All statistical modeling and analysis approaches were done with R. Variables in the principal component analysis were shifted to be zero centered and scaled to have unit variance. For the linear mixed modeling, the R package lme4 (version 1.1-19) was used. The Bayesian network learning was performed with the help of the R package bnlearn (version 4.2). We employed a score-based learning method, where each candidate network was assigned a goodness-of-fit score given a certain data set. Networks were calculated as directed graphs assuming an equivalent correlation that does not necessarily reflect causal dependencies. We performed a greedy hill climbing search in...
the space of directed acyclic graphs. A greedy hill climbing searches for local maxima by repeating single network changes and scoring the respective best fit to the underlying data. For the score, we used the logarithm of the Bayesian Dirichlet equivalent (BDe) assuming Dirichlet priors for the posterior probability distributions. The continuous data were discretized into a fixed number of quantiles. We applied four schemes, consisting of 3, 6, 10 or 15 discrete levels. For model averaging, we employed a nonparametric frequentist bootstrap approach by learning one network from each bootstrap sample. To ensure that our model did not learn connections randomly introduced by the finite sample of the true distribution, we resampled 100 times and chose the size of each bootstrap sample with replacement being 50% of the original data set.

3 | RESULTS

3.1 | Single-cell flow cytometry allows simultaneous quantification of phosphorylation of components of BCR signaling in primary leukemic and nonmalignant B cells

Phospho-flow provides fast and efficient means to measure the activity of signaling cascades. We utilized phospho-flow to measure phosphorylation as a marker for activation of downstream components of the BCR signaling cascade. To examine whether intracellular signaling is altered in U-CLL, M-CLL and normal B cells, a panel of five phospho-specific antibodies against components of the BCR signaling pathway was established (ZAP70/SYK, BTK, PLCγ2, AKT, ERK1/2). Selection of the coupled fluorophores allowed simultaneous detection of phosphorylation of these five distinct intracellular signaling molecules within the same cell. For stimulation of BCR signaling, B cells are capable of recognizing and responding to soluble and surface-bound antigens. Sustained activation is achieved in response to membrane-bound or immobilized antigens while soluble epitopes cause only transient activation. To stimulate cells by crosslinking, CLL PBMCs or normal CD19+ B cells were incubated with biotin-coupled antibodies against CD19 and IgM, either individually or by coligation of both receptors that were subsequently crosslinked with streptavidin (Figure 1A). To further increase the throughput and enable simultaneous analysis of different time points, FCB labeling was established as previously described. In this technique, amine-reactive fluorophores like Pacific Blue and Pacific Orange are added in different concentrations during the permeabilization of the cells. Depending on the defined concentration, the signal intensity varies and each sample can be identified as a distinct population during subsequent combined flow cytometry quantification (Figure 1A). The lymphocyte population was identified as a smaller and less granulated population using side scatter over forward scatter. Within the CD20+ population, the phosphorylations of BCR signaling components were analyzed in the six FCB populations representing the different time points (Figure 1A,B). Signaling in cells is fast, very sensitive and is modulated by different factors that can influence experimental reproducibility. We therefore verified reproducibility and robustness of our experimental design in technical replicates (Figure S1).

In summary, the simultaneous measurement of the five phosphoproteins allowed to correlate the activity of specific BCR signaling components at single-cell level (Figure 1C). To quantify malignant and nonmalignant BCR signaling, we performed experiments with primary CLL PBMCs from 11 previously untreated low-risk CLL patients with normal karyotype or single deletion of chromosome 13q. We compared CLL cells to CD19+ B cells from healthy donors (Table S1). CLL patients were selected to have a high tumor load and the CD19+/CD5+ population in samples to be above 90%. In PBMC samples from healthy donors, B cells were isolated by negative enrichment to prevent activation of the cells or downregulation of CD19. Upon cell sorting, no change could be detected in the intracellular phosphorylation level of proteins involved in the BCR signaling cascade (data not shown).

This phospho-flow approach was used to collect comprehensive and quantitative single-cell data that we modeled to unravel differences in the topology, heterogeneity and dynamics of the signaling machinery both between normal and malignant B cells and between M-CLL and U-CLL subgroups.

3.2 | BCR signaling cascade components display quantitative differences in phosphorylation upon stimulation between IGHV mutated and nonmutated CLL and between leukemic and nonmalignant cells

First, we quantified and compared the activity of the components of the BCR signaling cascade between CLL cells and nonmalignant B cells and between M-CLL and U-CLL cells. As described previously, B cells are generally capable of recognizing and responding to soluble and surface-bound antigens. However, responses in CLL cells are heterogeneous between patients, and some patients are even nonreactive to standard stimulations. BCR signaling is not only driven by kinase activation. The duration and strength of signaling response are also controlled by protein tyrosine phosphatases (PTPs). H2O2 is an oxidant that is naturally produced in B cells to regulate the activity of PTPs by selectively oxidizing essential cysteine residues of the active site in a reversible manner. Antibody-crosslinking alone leads to minimal responses while the addition of H2O2 within a specific concentration range can amplify and prolong the signal. We therefore stimulated CLL PBMCs and normal CD19+ B cells by crosslinking the modulators anti-IgM(μ), anti-CD19 or both. CD19 stimulation has previously been shown to reduce the amount of antigen required to stimulate the BCR and thereby promote a more effective immune response. The phosphorylation signal of BCR signaling components was amplified by concomitant blocking of PTPs with H2O2. Comparison of time-resolved average mean fluorescence intensity (MFI) values of phospho-flow for different components of the BCR signaling cascade and different stimulatory conditions allowed us to validate qualitative differences (Figure 2A). Within the group of healthy donor-derived B cells, the
sensitivity toward H₂O₂ was homogeneous and only weak activation with respect to phosphorylation of components of the BCR signaling cascade was seen (Figure 2). In contrast to this, CLL cells were more sensitive toward H₂O₂ than normal B cells with stronger effects in M-CLL cells compared to U-CLL cells. All five assessed components of the BCR-signaling cascade displayed measurable amounts of
phosphorylation (Figure S2). The degree of phosphorylation was positively correlated between the different signaling molecules (Figure 1C), confirming previous observations. The H$_2$O$_2$-mediated response of p-AKT was of similar strength both in normal and CLL cells independent of the reaction of other proteins (Figure 2A). This suggests that in our setup PTPs that act on AKT are not differently regulated in CLL and normal B cells as was hypothesized earlier. Activation of the BCR signaling after receptor crosslinking in addition to inhibition of PTPs by H$_2$O$_2$ was weaker in healthy donor B cells compared to CLL cells. At early time points, anti-IgM crosslinking augmented by H$_2$O$_2$ led to a rapid increase in phosphorylation of PLC$_{\gamma}2$, ZAP70/SYK, ERK 1/2 and BTK. AKT phosphorylation increased strongly upon incubation with H$_2$O$_2$ alone and displayed no differences in the overall signal intensity between the different B cells in all

**FIGURE 2** Signaling response is quantitatively different between M-CLL, U-CLL and nonmalignant B-cells. (A) Average group-specific kinetics of the median fluorescence intensity (MFI) for all p-proteins and different stimulatory conditions. Standard error (SE) is displayed as shaded ribbon around the mean of individual patients or healthy donors, respectively; (B) to evaluate the contribution of receptor engagement, data was normalized to the MFI after H$_2$O$_2$ stimulation only; (C) first two principle components (PC) of a PCA based on the MFI (normalized to unstimulated condition) as well as the coefficient of variation (CV) of the five assessed p-proteins before (0 minute) and 15 minutes after receptor crosslinking; (D) variables of the PCA show the relationship between all variables and their contribution to the first two PCs, positively correlated variables are grouped together, negatively correlated variables are positioned on opposite quadrants of the plot, the distance between variables and the center point represents their weight (loading) on the factor scores.
tested stimulation combinations. As H2O2 impacted very strongly on the phosphorylation of components of the BCR-signaling cascade in CLL cells, we normalized all stimulation conditions to the respective MFI after stimulation with H2O2 alone (Figure 2B). This normalization allowed to evaluate the individual contribution of the engagement of the BCR with anti-IgM and anti-CD19 without the influence of H2O2. Normalization revealed that the actual contribution of receptor engagements in normal B cells is not generally weaker compared to malignant B cells. Especially the phosphorylation of PLCy2 was prominently enhanced in U-CLL compared to M-CLL and healthy donor B cells (Figure 2B). The phosphorylation patterns of BCR signaling components in the three different sample groups M-CLL, U-CLL and normal B cells were distinctly different as assessed by principal component analysis (PCA). Interestingly, anti-IgM-stimulated M-CLL samples showed higher variability in the PCA (Figure 2C). Dimension reduction showed that phosphorylation patterns of AKT were distinct from phosphorylation patterns of the other signaling cascade components. The remaining four BCR signaling components were more correlated both before and 15 minutes after stimulation. Also, the dispersion of poststimulatory phosphorylation of both AKT and ZAP70/SYK was negatively correlated to the normalized, poststimulatory MFI of AKT (Figure 2D). Thus, quantification of receptor engagement and signaling strength confirmed quantitative differences between the B cell subgroups while H2O2 was identified as the main contributor to activation of BCR downstream components in this setup. In U-CLL, costimulation with anti-CD19 and anti-IgM crosslinking resulted in additive signal strength increase while anti-CD19 did not increase the signaling strength in M-CLL cells.

3.3 Inhibition of LYN and SYK synergizes with inhibition of BTK

We next investigated the influence of clinically relevant kinase inhibitors on the activity and network circuit topology of BCR signaling. The PI3K inhibitor idelalisib and the BTK inhibitor ibrutinib have recently significantly advanced the therapy of CLL.20,22 As expected, incubation with ibrutinib or idelalisib caused a clear reduction of phosphorylation levels in both CLL subgroups. Ibrutinib treatment led to reduced phosphorylation of SYK, BTK, PLCy2 and ERK1/2 but not AKT whereas idelalisib was observed to reduce phosphorylation levels of AKT only (Figure 3A). To extract the particular contribution of each inhibitor to phosphorylation of the BCR signaling components under specific stimulation conditions, we applied a piecewise linear mixed effects regression model and an integral-based method for assessing the strength of different fixed effects. In the regression model, each observation was described by a vector of fixed effects. These effects represented major influences of interest that is, receptor engagement and inhibitor effects, a vector of random effects modeling the unknown variability across individual subjects and experimental conditions, and a random measurement error. A piecewise temporal trend approximation was introduced by splitting the x-axis into two segments with a breakpoint at 15 minutes. The model was fitted against a multidimensional data set as in Figure 1A to obtain optimal parameter values for the different fixed and random effects. After fitting, the integral area under curve (AUC) was calculated for each condition and in a subsequent step, the integral change (ΔAUC) was derived as a measure of strength for each fixed effect (Figure 3B). All three sample groups showed similar contributions of anti-IgM stimulation to the BCR signaling activity. The effect on SYK phosphorylation appeared to be stronger in normal B cells compared to CLL cells. Ibrutinib significantly reduced BCR signaling activity for both CLL groups but had almost no effect in normal B cells. Again, the influence of ibrutinib on AKT phosphorylation was negligible. In contrast, idelalisib treatment did not reveal clear differences between the CLL and normal B cells and had a negative effect solely on AKT phosphorylation (Figure 3C). A complementarity of molecular effects could warrant the combination of inhibitors. Combination therapies of BCR inhibitors have been widely discussed to prevent or overcome resistance.49,50 Thus, we included dasatinib, a c-ABL/BCR-ABL and SRC kinase inhibitor41 with a strong inhibitory capacity toward LYN,52 an upstream activator of SYK.53 We also included entospletinib, a SYK inhibitor.54 Treating cells with dasatinib reduced all five measured BCR component activation levels while entospletinib reduced four of the BCR signaling components to a lower degree and had no effect on phosphorylation levels of SYK when applied alone. Dasatinib in combination with ibrutinib showed additive effects in reducing the phosphorylation of SYK, BTK, PLCy2 and ERK while the reduction of AKT phosphorylation went beyond the expected additive effect. Also, entospletinib had additive effects to BTK inhibition by ibrutinib (Figure 3D). In summary, all tested BCR inhibitors reduced the BCR-mediated activation of downstream signaling components, and the combination of compounds could further reduce this activation. Also by inhibiting LYN or SYK, the activation of several downstream pathways could be reduced while targeting BTK or PI3K had effects only on a subset of downstream components. Our model allows us to differentiate the positive and negative effects of individual treatment components and thus, we were able to extract the impact of BCR inhibitors in a complex stimulation condition.

3.4 Network interdependencies can be modeled by correlation of phosphorylation levels of BCR signaling cascade components in single cells

After characterization of quantitative dynamics of BCR-signaling in CLL-cells and healthy B cells, we used the quantitative single cell data to model signaling networks. Multiple Bayesian networks were derived from single-cell data by using a bootstrap approach to find the best fitting Bayesian network for each resampled data set. A Bayesian network is a probabilistic directed acyclic graph model describing dependencies among variables. Each node corresponds to one phosphorylated protein. A connection represented by an arc between two nodes indicates that these components are conditionally dependent on each other. Learning Bayesian networks can reveal significant relationships between protein phosphorylation levels. These may imply a functional and/or physical interaction while neither direction nor causality are assessed. To remove unlikely connections, the
FIGURE 3  Piecewise linear mixed effects modeling of stimulatory and inhibitory responses shows complementary effect of therapeutic inhibitors. (A) Average group-specific kinetics of the median fluorescence intensity fold change (MFI/unstimulated MFI) for different stimulatory conditions and p-proteins after pretreatment with 1 μM ibrutinib and/or idelalisib; (B) piecewise linear mixed effects regression model and an integral-based method for assessing the strength of different fixed effects; in the regression model, each observation is described by a vector of fixed effects representing major influences of interest, a vector of random effects modeling the unknown variability across individual subjects and experimental conditions, and a random measurement error; a piecewise temporal trend approximation is introduced by splitting the x-axis into two segments with a breakpoint at 15 minutes, colored points and lines represent data and fits of individual patients taking the random effects into account; black stars display the mean of individual patients data, additive fixed effects are shown by the solid black line. After fitting, the integral (area under curve, AUC) is calculated for each condition and in a subsequent step the integral change (ΔAUC) as a measure of strength for each fixed effect is derived; (C) strength of different stimulatory (+anti-IgM, +anti-CD19) and inhibitory (+ibrutinib, +idelalisib) effects on fluorescence signal intensities; results are based on the data depicted in panel A; M-CLL, U-CLL and normal B cells data are fitted separately; diamonds with error ticks are showing the mean and SD of ΔAUC for the indicated effect calculated from the data; (D) effects of pretreatment with 1 μM dasatinib, entospletinib and both in combination with ibrutinib.
average arc strength between nodes was derived from the number of occurrences of the respective connection in the set of best scoring networks. The arc strength depicts a confidence measure for a dependency between the respective nodes with a value between 0 and 1. This was done for all possible connections. For all individuals, including patients and healthy individuals, an individual consensus network was derived. With this analysis, we derived insights into conditional, temporal and group-specific differences by averaging among networks derived from all data sets. Each interconnection represents conditionally not independent components (Figure 4A). The overall BCR signaling network topology was found to be very similar between M-CLL and U-CLL and even between leukemic and non-malignant B cells. Overall, the phosphorylation of PLCy2 was strongly interrelated with all other signaling components. Of similar strong

![Bayesian network inference reveals robustness of network topology between M-CLL, U-CLL and nonmalignant B-cells.](image)

(A) Bayesian network learning and averaging approach: After discretizing the continuous single cell data, an optimal network was derived from each of R bootstrap samples; the Bayesian network learning strategy uses the BDe scoring function and a greedy hill-climbing algorithm to find the network model that represents the resampled data best; an average arc strength for each connection between nodes is derived from the number of occurrences of the respective connection in the set of R best scoring networks; further averaging among networks derived from different data sets can be applied for identifying conditional, temporal and group-specific differences; (B) interdependency of phosphorylation of BCR signaling components as displayed by Bayesian network topology showed a high level of similarity between nonmalignant B cells and both IGHV mutated and unmutated CLL; the networks shown are derived from data obtained after anti-IgM stimulation; besides the different subjects means within each sample group, the arc strength was further averaged over all poststimulatory time points and four different data discretization schemes with 3, 6, 10 and 15 discrete levels, respectively; (C) average arc strength over time for each potential interconnection of network nodes; the arc strength was averaged over four different data discretization schemes with 3, 6, 10 and 15 discrete levels, respectively; shaded ribbons depict the SE of the mean among all subjects within each sample group.
interrelation was the phosphorylation of BTK and ERK1/2. In CLL cells, there was a strong interrelation between BTK and SYK phosphorylation which was not prominent in normal B cells (Figure 4B). The network analysis was also performed in a time-resolved manner. Throughout all measured time points, AKT phosphorylation was mostly independent of the other signaling components except PLCγ2 phosphorylation. Of note, BTK and PLCγ2 showed opposite arc strengths with the downstream target ERK in CLL-cells and normal B cells (Figure 4B,C). Together, these data indicate that the overall network circuit topology of BCR signaling is not significantly different between CLL subgroups or between CLL and normal B cells but the temporary signal resolution is partially distinct between these subgroups, especially regarding the downstream effector ERK.

3.5 | Challenging BCR signaling networks with novel therapeutic inhibitors shows similar topology between IGHV mutated and nonmutated CLL and even between leukemic and nonmalignant B cells

As there can be hundreds to thousands of contributors to signaling dynamics, we sought to determine the interference of kinase inhibition on the signaling network circuit topology. We modeled the average Bayesian network connectivity based on the temporally resolved phosphorylation data. The phosphorylation levels were measured in both kinase inhibitor-treated and untreated cells under the previously described stimulation protocol. The arc strength of all analyzed patient samples was averaged over four data discretization schemes with 3, 6, 10 and 15 discrete levels, respectively. While Bayesian networks do not provide insight into the direction of signal flow, our temporally resolved phosphorylation measurements could provide a deeper understanding of the dynamics and causal dependencies between individual nodes of the BCR signaling pathway. Interestingly, the inhibition of BTK by ibrutinib caused a reduction in phosphorylation levels of SYK, BTK, PLCγ2 and ERK in CLL cells (Figure 3A,C). By normalizing the effect of ibrutinib with the stimulation effects, the change of the overall network topology was extracted. The connectivity between PLCγ2 and SYK as well as PLCγ2 and AKT was decreased in unstimulated cells after treatment with ibrutinib, especially in U-CLL. B cells from healthy donors and U-CLL cells exhibited additionally an increase in connectivity between BTK and SYK upon ibrutinib treatment at later stimulation time points. (Figure 5A). The inhibition of PI3K by idelalisib showed no major changes in the overall network topology (Figure 5B). Taken together, these data suggest that BTK and PI3K are central network nodes in

![Therapeutic inhibitors do not change the overall network topology of BCR signaling.](image-url)
the BCR signaling pathway that cannot be circumvented when inhibited in treatment-naïve CLL or healthy B cells (Figure 6).

4 | DISCUSSION

To sustain proliferative signals is one of the hallmarks of cancer. Signal transduction consists of a series of molecular events that process an external stimulus into a cellular response. This processing can be achieved by serial phosphorylation of effectors through protein kinases. At the end of this chain of events, transcription factors translate the signal into changes in target gene expression. In nonmalignant B cells, the BCR signaling cascade is the main player in regulating differentiation, survival, proliferation and antibody production. Activated BCR signals through PI3K and AKT activation. Although in CLL initial BCR signaling pathway mutations are rare, there is clear evidence that the BCR and its downstream signaling are essential for the pathomechanism of CLL and often constitutively active. Especially in the light of recent clinical breakthroughs by therapeutic inhibition of the BCR signaling cascade, it remains key to understand the dynamics and interconnections within this central signaling pathway to guide therapeutic strategies and overcome resistance to BCR inhibitors. By modeling the temporally resolved interconnection between the key signaling molecules of the BCR signaling pathway, we could show the strong interrelation between SYK, PLCγ2, BTK and ERK (Figures 3 and 4).

At the same time, our data emphasize the independent signaling properties of the AKT pathway. We also show that signaling changes that occur over time are not binary but a very dynamic process that we could model in our network circuitry. We stimulated normal and CLL cells with crosslinked anti-IgM and anti-CD19 antibodies and/or subsequent H2O2 treatment and resolved individual contributions of the respective stimulating agents (Figures 1 and 2). While 30 minutes of stimulation with both anti-IgM and anti-CD19 resulted in minor
increases of phosphorylation levels of SYK, BTK, PLCy2, ERK and AKT, H2O2 treatment alone or in addition to the receptor stimulation led to a several-fold increase in the mean fluorescence intensity of all five measured phospho-antibodies (Figure 2). This difference in signaling strength can be partly explained by the inactivation of phosphatases, especially protein tyrosine phosphatases by H2O2.47

We observed a stronger BCR response in M-CLL cells compared to U-CLL and normal B cells, confirming previous data.48 This stands in contrast to U-CLL generally responding stronger to single anti-IgM stimulation19,30 while M-CLL patients are generally showing more indolent clinical characteristics.4 Also, higher sensitivity to H2O2 treatment had been linked to indolent behavior. The reversible inhibition of phosphatases by H2O2 indicates the role the phosphatases play in the control of BCR signaling.43,48 Interestingly, our data suggest that B cells from healthy donors respond less to receptor stimulation and simultaneous H2O2 treatment compared to both U-CLL and M-CLL, in contrast to a previous study that found normal B cells to respond at an intermediate level.25 While we observed these differences for SYK, BTK, PLCy2 and ERK phosphorylation levels, measuring AKT phosphorylation between the subgroups did not reveal larger differences (Figures 2 and 6). This suggests that AKT activity is not differentially controlled by protein phosphatases between CLL subgroups and normal B cells at these early time points compared to later time points that likely aggregate secondary and tertiary effects.34

Furthermore, the principal component analysis revealed distinct BCR activity patterns between the three subgroups with U-CLL as intermediate between normal B cells and M-CLL cells (Figure 2C). Again, the MFI of AKT phosphorylation differed from the other factors in its direction and contribution to the PCA. The heterogeneity of AKT phosphorylation before and after stimulation contributed largely to the explained difference between samples, sample subgroups and stimulation conditions. This is of note as the response to AKT inhibition has been associated with treatment outcomes in U-CLL patients30 and p53/ATM dysfunction.61 These associations have been made on bulk patient samples. The single-cell quantification that we present here allowed us to detect two types of intrasample heterogeneity, a gradual (SYK, BTK, AKT, ERK) and a bimodal (PLCy2) distribution of phosphorylation (Figure 1). As the population with no PLCy2 phosphorylation almost completely corresponded to no or low phosphorylation of all other components except AKT, it could be speculated that the activation of PLCy2 is threshold-dependent and amplifies the signal in an all-or-nothing manner. PLCy2 phosphorylation showed the strongest correlation to all other components’ phosphorylation (Figure 4).

Interestingly, anti-IgM stimulation alone was shown to cause a simultaneous increase in BTK and PLCy2 phosphorylation but did not reveal any correlation to SYK activation.30 Mutations in BTK and PLCy2 have been shown to be central in the development of resistance against ibrutinib.27 Inhibiting BCR signaling activity with ibrutinib has introduced a new era in the treatment of CLL. Upon full stimulation of the BCR signaling cascade, we found the most significant increases in phosphorylation of BTK and PLCy2 in U-CLL cases (Figure 2B), which is suggestive taking into consideration that therapeutically, U-CLL benefit from BCR inhibition with ibrutinib20,23 similarly as M-CLL.

Because of its relevance, we were very interested in whether specific inhibition of single BCR pathway components would lead to the rewiring of the remaining components in the signaling network in primary CLL cells. One hallmark of signaling networks as compared to linear signaling cascades is a more robust connectivity. Thus, a deleterious event impacting one or even a few nodes can usually be compensated.31 This characteristic of complex signaling networks has led to the development of treatment strategies that include the simultaneous inhibition of several nodes, for example, in glioblastoma.32 Nevertheless, network connectivity can also be vulnerable to the loss of central, highly connected proteins.52

To investigate the BCR signaling circuitry in CLL and identify an Achilles heel in its connectivity, we modeled the BCR signaling network in primary CLL cells using a Bayesian network learning and averaging approach. With the derived model, we could show the impact of inhibition of BTK, PI3K, SYK and LYN on the overall signaling strength, dynamics and network connectivity. All four investigated inhibitors reduced the phosphorylation levels of one or more signaling components after stimulation of the BCR signaling cascade, and strikingly the general network connectivity remained largely unchanged, underlining the robustness of the network as described for other signaling cascades mentioned above (Figures 3, 5 and 6). With our network analysis approach, second generation BTK inhibitors could be evaluated for suitability in combination treatment approaches both as a preclinical and a correlative program. This BCR signaling quantification would also be applicable for studying long-term effects as well as for evaluating future therapeutic targets in signaling cascades.

AUTHOR CONTRIBUTIONS
Christine Wolf, Carsten Maus and Michael R. O. Persicke performed research, analyzed data and wrote the manuscript; Katharina Filarsky, Eugen Tausch and Christof Schneider performed research; Hartmut Döhner and Stephan Stilgenbauer provided vital reagents and wrote the manuscript; Peter Lichter planned experiments and wrote the manuscript; Daniel Mertens and Thomas Höfer planned and analyzed experiments and wrote the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

ACKNOWLEDGMENTS
We would like to acknowledge Sibylle Ohl and Karin Müller for excellent technical support, and Marc Seifert and Martina Seifert for helpful discussions. We would also like to thank the patients and blood donors for generous donation of primary material. This work was supported by the Else Kröner-Fresenius-Stiftung (2012_A146), the Virtual Helmholtz Institute (VH-VI-404), the DFG (SFB1074 projects B1/B2), the BMBF-Network “CancerEpiSys” (0316049C) and the Deutsche José Carreras Leukaemie Stiftung (DJCLS R 11/01). Open Access funding enabled and organized by Projekt DEAL.
CONFLICT OF INTEREST

Eugen Tausch declares potential conflict of interest: Research support by Roche, Abbvie and Gilead and honoraria, advisory board, speakers bureau with Roche, Abbvie, Beigene and AstraZeneca. All other authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The single-cell phospho-flow cytometric data are available at FlowRepository (http://flowrepository.org) with the Repository ID FR-FCM-Z5ZN. Other data that support the findings of our study are available from the corresponding author upon request.

ETHICS STATEMENT

Informed written consent was obtained (Ethics Committee, University Ulm, approval no. 459/19).

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