Toll-like receptor 9 stimulation can induce IκBζ expression and IgM secretion in chronic lymphocytic leukemia cells

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ABSTRACT

Chronic lymphocytic leukemia cells strongly depend on external stimuli for their survival. Both antigen receptor and co-stimulatory receptors, including Toll-like receptors, can modulate viability and proliferation of leukemic cells. Toll-like receptor ligands, and particularly the TLR9 ligand CpG, mediate heterogeneous responses in patients’ samples reflecting the clinical course of the subjects. However, the molecular framework of the key signaling events underlying such heterogeneity is undefined. We focused our studies on a subset of chronic lymphocytic leukemia cases characterized by expression of CD38 and unmutated immunoglobulin genes, who respond to CpG with enhanced metabolic cell activity. We report that, while CpG induces NFKBIZ mRNA in all the samples analyzed, it induces the IκBζ protein in a selected group of cases, through an unanticipated post-transcriptional mechanism. Interestingly, IκBζ plays a causal role in sustaining CpG-induced cell viability and chemoresistance, and CpG stimulation can unleash immunoglobulin secretion by IκBζ-positive malignant cells. These results identify and characterize IκBζ as a marker and effector molecule of distinct key pathways in chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent adult leukemia, characterized by the proliferation and accumulation of mature clonal B-lymphocytes in the peripheral blood and lymphoid tissues. Despite significant progress in treatment modalities, CLL remains incurable, with a sizeable fraction of patients experiencing progressive refractory disease. CLL is actually a heterogeneous disorder where aggressive cases are characterized by distinct clinical and biological markers including the presence of unmutated immunoglobulin heavy chain variable region (IGHV) genes, and CD38 expression.1

CLL cells strongly depend on external stimuli for survival and proliferation.1 Distinct cell types can support leukemia development and progression through different cytokine receptors. Moreover, both antigen receptor and co-stimulatory receptors, including Toll-like receptors (TLR), are involved in the pathobiology of CLL.3,4 Interestingly, novel B-cell receptor signaling inhibitors that have recently been approved for clinical use in CLL (i.e. BTK and PI3Kα inhibitors) target pathways that regulate tumor-microenvironment interactions;5 a better understanding of all the molecules and pathways involved in these processes may, therefore, help to fully understand CLL biology and design more effective targeting strategies.

TLR are transmembrane proteins devoted to the recognition of and binding to molecular patterns that can be derived either from microbes or from endogenous proteins (e.g. danger signals).4 TLR are mainly expressed by monocytes and macrophages where they trigger an innate immune response; nevertheless, both normal and malig-
nant B cells also express functional TLR and their stimulation influences leukemic cell survival and proliferation.24

Heterogeneity of response to different TLR ligands is observed in different patients, reflecting their clinical course. In detail, distinct TLR ligands binding to TLR1/2, TLR2/6 and TLR9 induce co-stimulatory molecules in virtually all cases, but they can induce proliferation and chemoresistance only in a select subset of cases characterized by adverse prognostic markers (e.g., unmutated IGHV genes and CD38).3,20 It should be noted that the levels of expression of TLR9 do not explain differential responsiveness of the cells to CpG.27 Cytokines (e.g., tumor necrosis factor-α), anti-apoptotic molecules (e.g., BclX) and distinct miRNA are selectively induced in IGHV unmutated cases.11-13 However, the molecular framework of the key signaling molecules leading to such variability in response is poorly defined.

We focused on the atypical IkBκ protein because it is specifically induced by TLR in different leukocyte populations but its role has never been characterized in CLL or in normal human B cells. IkBκ [also known as Interleukin-1 inducible nuclear ankyrin-repeat protein (INAP) or Molécule possessing ankyrin-repeat induced by lipopolysaccharide (MAL)] is selectively induced by interleukin-1 and TLR ligands but not by other inflammatory cytokines such as tumor necrosis factor.14,15 IkBκ expression is low to undetectable in unstimulated immune cells, and is rapidly induced after TLR stimulation by transcriptional, post-transcriptional and translational mechanisms.20-23 IkBκ is an atypical IκB family member, in contrast to the classic IκB family members (but similar to other atypical members), it is a nuclear protein and a direct transcriptional regulator. IkBκ can act as a negative or positive modulator in combination with distinct transcription factors including p50 and p65 nuclear factor-κB (NF-κB) family members. In particular IkBκ controls the induction of secondary response genes including interleukin-6 and interleukin-10.10,13

Mouse models lacking IkBκ demonstrated that this protein is a key regulator of both innate and adaptive immune responses, such as Th17 development, natural killer-cell-derived interferon-γ production, and interleukin-6 production in macrophages.14,21,22 In epithelial cells, a deficiency in IkBκ causes apoptosis, which induces Sjögren syndrome-like inflammation.25 In the context of B-cell types, it has been recently shown that IkBκ controls the proliferation of mouse B-lymphocytes and triggers a TLR-dependent but T-independent antibody response.25

We herein analyzed, for the first time, IkBκ expression, regulation and function in leukemic cells from CLL patients. Stimulation of TLR9 with the CpG ligand induced IkBκ in leukemic cells; this up-regulation was distinctly higher, as a result of a post-transcriptional mechanism, in a subgroup of CLL cases characterized by CD38 expression and unmutated IGHV genes. Moreover, we addressed the expression pattern and functional role of IkBκ in malignant cells. Our results provide novel insights into the pathobiology of CLL, and shed light onto the molecular pathways that mark and regulate distinct CLL cases.

Methods

Chronic lymphocytic leukemia samples

Leukemic lymphocytes were obtained from peripheral blood of CLL patients diagnosed according to the International Workshop on CLL/National Cancer Institute 2008 guidelines.26 All patients were either untreated or off therapy for at least 6 months before the study. The following parameters were analyzed for each patient: age, sex, disease stage at diagnosis, CD38 expression, and IGHV gene mutational status (Online Supplementary Table S1). All tissue samples were obtained with the approval of the institutional Ethics Committee of San Raffaele Scientific Institute (Milan, Italy), after informed consent.

Cell purification

CLL cells were negatively selected and purified using a B-cell enrichment kit (RosetteSep; StemCell Technologies) following the manufacturer’s instructions. Normal B cells from buffy coat and B cells from tonsils were purified by negative selection (EasySep; StemCell Technologies). The purity of all leukemic CD19+ CD5- and normal CD19 preparations was always >98% as checked by flow cytometry (FC500; Beckman Coulter). Buffy coats from anonymized healthy donors were obtained from ASST Rhodense Hospital (Rho, Italy), IRCCS AOU San Martino – IST (Genova, Italy) and “San Raffaele” Hospital (Milano, Italy). Tonsils from patients not affected by CLL were obtained from the “G. Gaslini” Hospital and “San Raffaele” Hospital.

Analysis of B-cell subpopulations

The percentage of anti-IκBκ-positive cells was determined in each B-cell subset; 4 h after CpG stimulation, by a multiparametric flow cytometry gating strategy described elsewhere.26,27 Briefly, cells were first stained for surface antigens with anti-IgD FITC, anti-CD38 PE-Cy5.7, anti-CD19 APC-H7, anti-CD5 Alexa-Fluor700, anti-CD24 PE-CF594 and anti-CD27 PE-CY5.5 monoclonal antibodies (BD Biosciences), then fixed, permeabilized and stained with anti-IgM PerCP-Cy5.5, anti-CD27 PE-CFS94 and anti-CD24 Alexa-Fluor647 monoclonal antibodies (BD Biosciences), then fixed, permeabilized and stained with anti-IκBκ (see below for details). Flow cytometry analyses were performed using FACSAria II DIVA 6 software (BD Biosciences). Data were processed with Prism (GraphPad Software Inc.).

Buffy-coat CD19+ B-cell subsets comprised naive B cells (IgD+IgM+ CD27-), memory (MEM) B cells (IgD-CD27-), CD5-positive (CD5+) B cells, CD38-positive (CD38+) B cells and CD38bright plasmablasts. MEM B cells comprised the following subsets: IgM memory (M-MEM) B cells (IgD-CD27+), switched memory B cells (S-MEM) (IgD-CD27+), and double-negative memory cells (IgD-CD27-) (DN-MEM).

The tonsillar CD19+ B-cell subset comprised naive B cells (IgD+IgM+CD38-CD27-), memory (MEM) B cells (IgD-CD27-), germinal center (GC) B cells (IgD+CD38+CD24-), plasmablasts (CD38+CD24+), transitional B cells (TRANS) (CD38-CD24+), activated B cells (IgD-CD38-). MEM B cells included three different memory B-cell subsets: IgM memory (M-MEM) (IgM+IgD-CD27+), switched memory (S-MEM) (IgM+IgD-CD27+), and double-negative memory (IgD-CD27-) (DN-MEM) subsets, which were analyzed separately.

Online Supplementary Figures S1 and S2 describe the gating strategy for peripheral blood and tonsillar cells, respectively.

Further details regarding the methods can be found in the Online Supplementary Files.

Results

CpG induces IkBκ protein in a heterogeneous manner in patients with chronic lymphocytic leukemia

IkBκ protein was undetectable in unstimulated CLL cells, but was markedly induced, in selected cases, after TLR9 stimulation with the CpG ligand; a representative case analyzed by flow cytometry before and after CpG
stimulation, and stained with anti-\(\text{I}\kappa\text{B} \zeta\) or isotype control antibody is shown in Figure 1A. It should be noted that no significant cell death was induced at this time point, thus excluding apoptosis as influencing \(\text{I}\kappa\text{B} \zeta\) staining (data not shown and Online Supplementary Figure S4).\(^{11}\)

Western blot analysis demonstrated that a protein with a molecular weight of approximately 85 kDa, corresponding to the longer isoform of \(\text{I}\kappa\text{B} \zeta\), is detected in some but not all CLL samples after CpG stimulation (Figure 1B). Interestingly, analysis of a total of 75 CLL cases revealed

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**Figure 1.** Heterogeneous \(\text{I}\kappa\text{B} \zeta\) expression in chronic lymphocytic leukemia cells treated with CpG correlates with adverse prognostic markers. (A) \(\text{I}\kappa\text{B} \zeta\) flow cytometry analysis of two representative patients; CLL cells were incubated with or without CpG for 24 h, and stained with anti-\(\text{I}\kappa\text{B} \zeta\)-PE or IgG G1 K iso control-PE. (B) Western blot analysis of four representative patients; CLL cells were incubated with or without CpG for 24 h. (C-D) Higher percentage of \(\text{I}\kappa\text{B} \zeta\)+ cells and higher \(\text{I}\kappa\text{B} \zeta\) mean fluorescence intensity (MFI) are observed in CD38-positive samples; \(n=16\) CD38+ and 56 CD38−. (E-F) Higher percentage of \(\text{I}\kappa\text{B} \zeta\)+ cells and higher \(\text{I}\kappa\text{B} \zeta\) MFI are observed in unmutated CLL cases; \(n=23\) unmutated and 40 mutated. The Mann-Whitney test was used to analyze all the data. ** \(P<0.01\); *** \(P<0.001\). (G) Receiver operating characteristic (ROC) curve analysis was performed with the percentage of \(\text{I}\kappa\text{B} \zeta\)+ cells and IGHV mutational status; \(n=23\) unmutated and 40 mutated. (H) Distribution of CLL samples based on the increase in percentage of \(\text{I}\kappa\text{B} \zeta\)+ cells (\(n=75\)); the calculated cut-off value is indicated.
that IκBζ expression is different in patients characterized by different prognostic markers including CD38 expression and IGHV mutational status. Higher proportions of IκBζ-positive cells and higher levels of expression of IκBζ protein (mean fluorescence intensity; MFI) were significantly associated with CD38 expression (Figure 1C,D) and the presence of unmutated IGHV genes (Figure 1E,F). No significant differ-ence of IκBζ expression was found in CLL patients as a function of age, sex or in patients with progressive versus stable disease (Online Supplementary Table S1).

We performed a receiver operating characteristic (ROC) analysis to discriminate IGHV-mutated versus -unmutated samples based on the levels of IκBζ; the ROC curve shown in Figure 1G demonstrated that the percentage of IκBζ-positive cells predicted IGHV mutated versus unmutated cases with an area under the curve (AUC) of 0.7804 (P=0.0002327).

The Youden index (a measure of specificity and sensitivity) was calculated for each value of the curve, and the maximum index corresponded to a cut-off of 29.75% IκBζ-positive cells. Using this cut-off value, we detected a higher frequency of IκBζ-positive samples among cases that were CD38-positive, IGHV unmutated, and higher Rai stage (Online Supplementary Figure S3).

We thus applied 29.75% IκBζ-positive cells as the cut-off value for the subsequent biological analyses comparing IκBζ-positive to IκBζ-negative samples.

**Metabolic activation driven by TLR9 stimulation is mediated by IκBζ**

*In vitro* TLR9 stimulation is known to upregulate costimulatory molecules in virtually all CLL cases after 24 h; in contrast, it induces metabolic cell activation, cell viability and proliferation only in those cases characterized by poor prognostic markers including disease progression, CD38 expression, unmutated IGHV genes and unfavorable cytogenetic aberrations. To study the specific signaling pathways that are involved in the TLR-mediated activation program, we cultured patient-derived leukemic CLL cells in the presence of the TLR9 ligand CpG.
ODN2006 for 24 h, and analyzed the expression of \( \text{iK}B_\zeta \). In parallel, after an additional 24 h, we measured metabolic activation by a specific assay that quantifies the ATP present as an indirect indicator of cell viability.\(^{11}\) As expected, CpG induced metabolic activation in a group of patients, herein referred to as “ATP+” but had no effect on the other group of patients (“ATP-”)(Online Supplementary Figure S4A). Strikingly, TLR9 stimulation induced higher levels of \( \text{iK}B_\zeta \) in the group of “ATP+” samples (Figure 2A,B).

Since we previously reported that CpG can induce chemoresistance to fludarabine \(^{\text{i}}\) in vitro in a proportion of cases,\(^{11}\) we investigated whether the observed rapid \( \text{iK}B_\zeta \) induction also mediated this effect. We stimulated the cells with or without CpG for 24 h, and analyzed \( \text{iK}B_\zeta \) expression (Figure 2C,D); subsequently, we treated the cells with fludarabine for another 24 h, and measured metabolic activation at the end of the treatment (Online Supplementary Figure S4B). Both the percentage of \( \text{iK}B_\zeta \) -positive cells and the mean expression of \( \text{iK}B_\zeta \) were significantly higher in the group of samples in which CpG induced fludarabine resistance (Figure 2C,D).

To test the functional role of \( \text{iK}B_\zeta \), CLL cells from “ATP+” cases were electroporated with \( \text{iK}B_\zeta \) specific or control siRNA to inhibit \( \text{iK}B_\zeta \) expression, as demonstrated by western blot analysis (Figure 2E); cellular metabolic activity was then analyzed 48 h after CpG treatment, in the absence or presence of 5 \( \mu \)M fludarabine. Upon \( \text{iK}B_\zeta \) silencing before CpG stimulation, a significant decrease in metabolic activation cells was evident in both fludarabine-treated and untreated cells (Figure 2F and G, respectively).

These data implicate \( \text{iK}B_\zeta \) in the mechanism whereby...
CpG induces metabolic cell activation, and modulates the sensitivity of CLL cells to fludarabine.

**TLR9 stimulation differentially induces \( \kappa B \zeta \) protein through a post-transcriptional mechanism.**

To better characterize the molecular mechanism underlying differential induction of \( \kappa B \zeta \) among different patients’ samples, we measured \( NFKBIZ \) mRNA levels at different time points in cells from two subgroups of patients (the previously characterized “ATP+” and “ATP-” groups). \( NFKBIZ \) mRNA was up-regulated by CpG in all patients’ samples at all time points (Figure 3A; “ATP+” cases: n=15 at 2 h; n=18 at 4 h; n=4 at 24 h; “ATP-” cases: n=9 at 2 h; n=9 at 4 h; n=4 at 24 h); the amount of mRNA detected peaked 2 h after CpG stimulation, remained high at 4 h, and declined in a similar manner in both groups after 24 h (Figure 3A).

In order to understand whether different splicing isoforms are differentially induced by TLR stimulation in CLL samples, we performed a real-time polymerase chain reaction analysis with primers specifically recognizing the long isoform of \( \kappa B \zeta \).
isoform A, in “ATP+” and “ATP−” patients, 4 h after TLR stimulation. Both groups of patients expressed this IκBζ isoform; counter-intuitively, “ATP−” patients’ samples (n=4) showed even higher mRNA levels of the long isoform A, as compared to “ATP+” samples (n=4) (Online Supplementary Figure S5). Altogether, these data rule out differential mRNA expression of the long isoform as a probable cause of the differential induction of IκBζ protein by CpG in CLL cells.

Strikingly, time course analyses of protein expression by western blot revealed that IκBζ was expressed soon after 4 h of CpG stimulation, with a peak at 6 h in IκBζ-positive samples; IκBζ protein remained detectable after 24 h of TLR stimulation in IκBζ-positive samples (Figure 3B for 1 representative case). In contrast, IκBζ protein was barely detectable if present in IκBζ-negative samples (Figure 3B for 1 representative sample). Densitometric analysis of the western blots for all the six samples studied is shown in Online Supplementary Figure S6.

We further analyzed IκBζ expression by immunofluorescence microscopy, which revealed IκBζ mainly in the nucleus, in punctate structures of IκBζ-positive cases (Figure 3C); low to undetectable immunofluorescence for IκBζ was observed in IκBζ-negative cases before and after CpG treatment (Figure 3C).

To dissect the signaling pathways involved in this specific IκBζ upregulation, we treated CpG-stimulated CLL cells with an IRAK1/4 inhibitor that blocks the activity of the TLR-signaling complex including IRAK kinases; drug treatment significantly blunted IκBζ protein expression (Figure 3D), while no significant cell death was induced at this time point (data not shown).

In conclusion, IκBζ protein is specifically upregulated after TLR9 ligation by an IRAK-dependent post-transcriptional mechanism in a distinct group of CLL samples.

TLR9 stimulation promotes IgM secretion in IκBζ-positive chronic lymphocytic leukemia

It was recently shown that stimulation of CLL cells with a mix of CpG and cytokines can induce immunoglobulin secretion, at least in a proportion of cases.31-33 We hypothesized that IκBζ could mark and/or be involved in CpG-induced immunoglobulin production.

To test this hypothesis, we first analyzed the expression of surface and intracellular IgM by flow cytometry, upon incubation of CLL cells in the presence or absence of CpG; we calculated both the percentage of IgM-positive cells and MFI of IgM expression. Surface IgM was similar in the two conditions (Figure 4A,B). Conversely, we found significant induction of intracellular IgM levels (MFI but not percentage of positive cells) after 72 h of CpG stimulation (Figure 4B). There was no difference between IκBζ-positive and IκBζ-negative patients in terms of intracellular IgM induction (Figure 4C). In contrast, when we quantified IgM secreted into the culture medium, we detected

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Figure 5. CpG induces autophagy is required for IgM secretion. (A) Immunoblot analysis of converted LC3 (LC3-II) following treatment with CpG for the indicated times. (B) Autophagic fluxes were assessed as the rate of lysosomal digestion of LC3-II (quantified as the difference of LC3-II band intensity in the presence or absence of NH4Cl normalized by β-actin) by lysosomal inhibition with 50 mM NH4Cl for 1 h. One out of four experiments from one representative IκBζ+ CLL patient is shown. (C-D) IgM expression analysis was performed by flow cytometry (C) and its secretion was quantified by ELISA (D) on eight IκBζ+ CLL samples (mean and SEM are indicated). CLL cells were stimulated with CpG for 24 h and treated with 50 nM of bafilomycin for an additional 48 h. A Wilcoxon matched pairs test was performed and ** indicates a P<0.001.

A

B

C

D

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significantly higher levels in the group of IκBζ-positive patients (Figure 4D).

Next, we analyzed the expression of Activation-induced (cytidine) deaminase (AICDA or AID) and Blimp-1, both representing key regulators of antibody response. AID mRNA transcripts were significantly induced in the group of IκBζ-positive samples (Figure 4E). Moreover, a trend of increased Blimp-1 protein expression was observed 48 h after CpG stimulation (Figure 4F shows 2 representative IκBζ-positive samples); a densitometric analysis of different cases is reported in Online Supplementary Figure S6. As expected, at the same time point low but detectable levels of IκBζ protein were revealed (Figure 4F).

**Autophagy flux induced by CpG is required for IgM secretion**

We analyzed autophagy, a selective lysosomal recycling strategy recently implicated in the differentiation of antibody-secreting cells, and in immunoglobulin secretion induced by TLR stimulation. We treated CLL cells with or without CpG, and analyzed the expression of LC3 protein, whose short-lived faster-migrating lipidated form (LC3-II, 14 kDa) is an established biochemical marker of autophagosomes. CpG induced autophagy in CLL cells, as indicated by increased steady-state abundance of LC3-II, which further accumulated under short treatment with the lysosomal inhibitor NH4Cl, attesting to increased autophagic flux (Figure 5A,B). Moreover, when bafilomycin-A1, an inhibitor of autophagosome-lysosome fusion, was co-administered to CLL cells stimulated with CpG, IgM accumulated intracellularly (Figure 5C) at the expense of secretion (Figure 5D).

Overall, these data reveal that TLR9 stimulation unleashes IgM secretion selectively in IκBζ-positive cases through an autophagy-dependent mechanism.

**IκBζ expression and regulation in normal and leukemic B cells**

Having defined a novel molecular mechanism sustaining IκBζ overexpression in selected CLL cases, we compared the levels of this protein among different CLL cases and normal B-cell populations. We thus analyzed the expression of IκBζ in human purified B lymphocytes freshly isolated from buffy coats and tonsils by flow cytometry upon 24 h stimulation with CpG. As shown in Figure 6A,B, IκBζ was induced by TLR9 in both types of samples. Interestingly, CLL cells from “ATP+” cases showed higher proportions of IκBζ-positive cells and higher IκBζ MFI compared to circulating and tonsillar B cells, respectively (Figure 6C,D). Of note, 4 h after TLR9 stimulation, both circulating and tonsillar B cells expressed high levels of IκBζ protein which decreased rapidly over time, especially in the buffy-coat cells (Online Supplementary Figure S7). This further suggests that IκBζ-
negative CLL resemble normal B lymphocytes while Κζ-positive CLL show overexpression of this protein.

To address whether Κζ-positive cells resembled a specific sub-population of normal B cells, we analyzed Κζ expression by flow cytometry in different B-cell populations in peripheral blood and tonsil. Naïve, memory, germinal center, transitional, activated, and plasmablast B-cell subsets were detected by specific surface markers (see Methods section and Online Supplementary Figures S1 and S2).

Concomitant Κζ immunolabeling demonstrated high percentages of Κζ-positive cells among naïve and memory B lymphocytes in buffy coats (Figure 7A; naïve=58.4±9%; memory=31.2±8.7% mean and SD). In particular, a high percentage of peripheral blood B cells expressing CD38, excluding plasmablasts (CD38\(^{+}\)IgM\(^{-}\)), were Κζ-positive (58.5±14.5% mean and SD) whereas very few if any CD5\(^{+}\) B cells expressed Κζ (Figure 7A). It is of note that CD38\(^{+}\)IgM\(^{-}\) B cells from both buffy coats and tonsils stimulated by CpG were mainly plasmablasts (CD138 negative, not shown). Among memory B cells, switch memory and IgM memory expressed the highest levels of Κζ (Figure 7B).

Finally, when we analyzed tonsil samples we observed an enrichment of Κζ-positive cells among naïve and memory subsets (naïve=50.3±9.2%, memory=56.5±11.7%) (Figure 7C). Among memory B cells, in the tonsils, Κζ was expressed more by switch memory than by IgM memory and double-negative memory cells (Figure 7D).

**Discussion**

The aim of our project was to identify the mechanism that accounts for the differential response of CLL cells to TLR9 in regulating metabolic cell activation and immunoglobulin secretion.

We focused on the atypical Κζ nuclear protein because it is specifically induced by TLR in different leukocyte populations; however, its role has never been characterized in CLL. We observed that CpG stimulation strongly induced Κζ protein in a selected group of CLL samples characterized by CD38 expression and unmutated IGHV genes. Moreover, we observed that Κζ was specifically induced in cells that showed metabolic cell activation. By knocking down Κζ protein before TLR9 stimulation, we proved that it was essential for CpG-induced cell survival, spontaneous or following treatment with fludarabine. Interestingly, recent findings suggest that Κζ is either mutated or upregulated in an aggressive type of lymphoma in which it controls cell survival. Along this line, our results suggest that Κζ controls an oncogenic pathway relevant in mature B-cell neoplasia, and its dysregulation can occur via genetic mechanisms (mutations) or following microenvironmental stimulation (e.g. through TLR).

Because Κζ is also an essential transcriptional regulator during T-independent antibody responses, we focused on the control of immunoglobulin production in CLL. Leukemic cells are mature clonal B-lymphocytes characterized by the expression of intracellular and surface IgM and/or IgD, although other Ig classes can rarely be expressed. These cells are somehow blocked throughout terminal differentiation. However, different cytokines together with the TLR9 ligand CpG were recently shown to induce immunoglobulin secretion, at least in a group of CLL cases. Nevertheless, the molecular mechanisms accounting for this regulation are poorly understood. Here we studied whether the observed upregulation of Κζ in select CLL cases could also explain this dichotomy of response. Indeed, we observed that Κζ is a hallmark of IgM-secreting CLL cells. Wagner et al. recently described a signaling framework that links TLR9 activation, in ZAP70-positive CLL, with protection from apoptosis mediated by IgM secretion. Our results support and extend this observation, and suggest that TLR9-induced IgM secretion and metabolic cell activation in Κζ-positive cases may play a key role in tuning CLL cell survival.

We also observed that CpG stimulation induces IgM secretion through an autophagy-dependent pathway, and that Κζ is overexpressed in CLL as compared to normal B cells. TLR9 signaling is known to induce autophagy in different cell types. Hence, while the observed induction of autophagy by CpG stimulation was expected, it was surprising to observe that IgM secretion – but not production – following TLR9 engagement was abolished by lysosomal inhibition in CLL cells. The underlying selective autophagic control on TLR-dependent immunoglobulin secretion warrants investigation. Being operative specifically in Κζ-positive CLL cells, such a mechanism may unfold relevant prognostic markers and therapeutic targets.

A previous publication reported a differential response of CLL cells to CpG (as well as interleukin-21) in terms of Blimp-1 induction and immunoglobulin secretion, while similar levels of Κζ \(\eta\) and Κζ mRNA expression were observed (no protein analysis of Κζ was performed). Such a differential response was attributed to specific epigenetic regulation marked by H3K4 trimethylation and to the presence or absence of “poised” Blimp-1 promoter. It should be noted that Κζ is a key regulator of the transcriptional activity of inflammatory cytokine genes during so-called “secondary response”. Indeed, it can bind to “poised” gene promoters to facilitate the recruitment of the transcription pre-initiation complex and H3K4 trimethylation. Moreover, in mouse models, Κζ mediates LPS-induced Blimp-1 induction in splenic B cells.

Based on these observations and on our results, it is tempting to speculate that differently induced Κζ protein may directly regulate Blimp-1 promoter activity in CLL cells.

The overexpression of Κζ in a selected group of CLL cases may represent an abnormal characteristic of the malignant clone, or may reflect the phenotype of a putative cell of origin. The cellular origin of CLL is still debated, one study demonstrated that CLL cells are strongly related to memory B cells in terms of gene expression profile while another study showed a significant similarity between CLL cells and naïve and mature CD5\(^{-}\) B cells, suggesting a heterogeneous origin of CLL cells from CD5\(^{-}\) B-cell subpopulations (CD27\(^{-}\) or CD27\(^{+}\)). MicroRNAome analysis of CLL in comparison to normal B-cell subsets highlighted similarities of CLL cells to antigen-experienced and marginal zone–like B cells but also naïve B cells. When we analyzed Κζ induction in different B-cell populations from buffy coat and tonsil we observed an enrichment of Κζ positivity in both naïve and memory B-cells, which is in agreement with some aforementioned results supporting the hypothesis that CLL could resemble a subset of normal B cells intermediate between a naïve B cell and antibody-secreting cells.

Analyzing in detail memory B-cell subsets we observed...
that IκBζ was preferentially expressed in conventional memory B-cell subsets, both switched and not switched (CD27+), but was absent in double-negative memory B cells (IgD CD27-) which are a special subpopulation particularly increased in systemic lupus erythematosus autoimmune disease.45,46

Interestingly, the CLL cases positive for CD38 were those that responded to stimulation with CpG with IκBζ induction; in a similar way, a high percentage of CD38- normal B cells expressed IκBζ following TLR9 stimulation.

Looking for the mechanisms regulating IκBζ protein expression, we demonstrated that while NFKBIZ mRNA was induced by CpG in all the samples analyzed, a specific post-transcriptional mechanism was operating in a selected group of CLL cases only. Of note, both transcriptional and post-transcriptional mechanisms of regulation of IκBζ have been documented.20-23 Nevertheless, this is the first example of selective regulation of IκBζ protein in CLL cases expressing CD38 and unmutated IGHV genes; this may underscore a novel biochemical framework that is specifically activated in a group of patients only, and that is highlighted by higher IκBζ induction.

Overall, our results support a non-redundant role for TLR-induced IκBζ in regulating leukemic metabolic cell activation and suggest that a novel biochemical pathway regulating its protein expression levels may be implicated in immunoglobulin secretion toward the first step of dif-

![Figure 7. IκBζ expression in normal B-cell subpopulations.](image-url)

(A, B) Flow cytometry analysis of IκBζ expression in normal B cells enriched from buffy coat (peripheral blood; panel A) and in normal tonsillar B cells (panel C), after 4 h of culture in the presence of CpG. The box plots represent the mean values and the whiskers indicate minimum and maximum values of five samples analyzed. The subpopulations analyzed in the peripheral blood were: naïve, memory (MEM), plasmablasts and plasma cells (PBs+PC, CD38bright), CD5-positive B cells (CD5+) and CD38-positive B cells (CD38+). The B-cell subpopulations investigated in the tonsil were: naïve, memory (MEM), germinal center (GC), plasmablasts and plasma cells (PBs+PC), transitional (TRANS) and activated (ACT). (B, D) Flow cytometry analysis of IκBζ expression in memory B cell subsets in the peripheral blood (panel B) or tonsil (panel D): IgM memory (M-MEM), switched memory (S-MEM) and double negative memory (DN-MEM) cells. Phenotypic features of the B-cell subsets and the gating strategy applied for the analysis are shown in Online Supplementary Figures S1 and S2.
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