Int. J. Dev. Biol. 59: 379-389 (2015)
doi: 10.1387/ijdb.150204om

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Calcium signaling and cell fate: how can Ca^{2+} signals contribute to wrong decisions for Chronic Lymphocytic Leukemic B lymphocyte outcome?

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ABSTRACT **Ca^{2+} signaling is a key regulator of B lymphocyte cell fate and defects in this signaling pathway have been reported in numerous diseases such as Chronic lymphocytic leukemia (CLL). CLL is a B cell clonal disorder characterized by the accumulation of mature monoclonal $CD5^+$ B cells. Although CLL could be considered to be a proliferative disease, most circulating CLL B cells are arrested in the G0 phase of the cell cycle and present both defects in calcium (Ca^{2+}) homeostasis and signaling. The Ca^{2+} response to antigen ligation is heterogeneous and related, in part, to defects arising from the incapacity to respond to B cell receptor (BCR) engagement (anergy), to the expression of T cell kinases (e.g. Zap70), and to the presence of negative feedback regulation by phosphatases (e.g. SHP-1). Anergic $CD5^+$ CLL B cells are characterized by an elevated basal Ca^{2+} level, IgM/CD79 downregulation, a constitutive activation of BCR pathway kinases, and an activation of the nuclear factor of activated T cells (NF-AT). Based on the Ca^{2+} response, patients are classified into three groups: unresponders, responders with apoptosis, and responders with entry in the cell cycle. Moreover, internal and direct interaction between leukemic BCR-HCDR3 epitopes at the plasma membrane and interaction between Bcl-2 and the IP3-receptor at the endoplasmic reticulum are also suspected to interfere with the intracellular Ca^{2+} homeostasis in CLL-B cells. As a whole, the Ca^{2+} pathway is emerging to play a key role in malignant CLL-B survival, disease progression, and last but not least, in the therapeutic response.**

KEY WORDS: *calcium, chronic lymphocytic leukaemia, CD5, cell fate*

Introduction

In all cell types including immune cells, calcium (Ca^{2+}), is an essential and universal second messenger controlling a wide and diverse range of cellular functions such as migration, cell adhesion, apoptosis, proliferation, cell cycle, protein kinase signaling, mitochondrial and endoplasmic reticulum physiology, protein nucleocytoplasmic trafficking of transcription factors, and many others (Berridge *et al.,* 2003, Feske 2007, Berridge 2012). Deregulation of this processes associated with defects in calcium signaling have been involved in many cancers (Bergmeier *et al.,* 2013, Stewart *et al.,* 2014) and in particular in Chronic Lymphocytic Leukemia (CLL) (Stevenson *et al.,* 2011, Burger *et al.,* 2013, Chiorazzi *et al.,* 2013).

CLL is the most common adult B cell malignancy in the Western world and is characterized by the accumulation of mature monoclonal $CD5^+$ B cells characterized by defective apoptosis (Burger *et al.,* 2013, Chiorazzi *et al.,* 2013, Zhang *et al.,* 2014). CLL B cells are flowed into peripheral blood where they accumulate, and for some of them migrate to the lymphoid tissues and bone marrow (BM)

Abbreviations used in this paper: Ag, antigen; BAX, Bcl-2 associated X protein; BCR, B cell receptor; Ca^{2+}, calcium; CD, cluster of differenciation; CLL, chronic lymphocytic leukemia; CLL-B cell, chronic lymphocytic leukemic-B cell; DAG, diacyl glycerol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; HCDR3, heavy chain complementary-determining region 3; Ig, immunoglobulin; InsP3, inositol-1,4,5,-triphosphate; NFAT, nuclear factor of activated T cells; NFkB, nuclear factor kB PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC γ2, phospholipase C γ2; SIg, surface immunoglobulin.

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Accepted: 22 July 2015.

ISSN: Online 1696-3547, Print 0214-6282

Printed in Spain

to proliferate and survive. Although CLL could be considered as a proliferative disease, most of circulating CLL B cells are arrested in G0 phase the cell cycle. CLL clinical course is heterogeneous from indolent to aggressive forms with a poor prognostic outcome for some patients. In order to characterize this heterogeneity, many attempts have been made to propose biological prognostic markers such as immunoglobulin (Ig) heavy chain variable region (IgVH) mutation status, cytogenetic abnormalities, cell membrane expression of CD38 or intracellular expression of the ζ-chain-associated protein kinase 70 kDa (ZAP-70).

Transient variations in the cytosolic concentration of calcium ions ($[Ca^{2+}]i$) occurring after cell stimulation directly through the B cell receptor, chemokine receptors, or indirectly through co-stimulatory molecules, transmit information that is crucial to multiple cell-fate decisions arising during B-cell ontogeny, including B cell maturation in the bone marrow, activation of mature B cells in response to antigen presentation or selection into germinal centers (Niiro *et al.,* 2002, Hoek *et al.,* 2006, Feske 2007, Kurosaki *et al.,* 2010, Baba *et al.,* 2011, Seda *et al.,* 2014). In some cases, the B cell receptor (BCR) signal could be activated independently of antigen (Ag) ligation, a process termed tonic signaling that occurs during B cell development and selection (Duhren-von Minden *et al.,* 2012). In normal B cells, BCR responses and Ca^{2+} signals vary with signal strength and are modulated by co-receptors, with outcome ranging from a low level, Ag independent 'tonic' signal essential for survival, to strong Ag-mediated signals which drive the cell toward activation, differentiation or apoptosis (Fig. 1).

In CLL B cells, antigen stimulation seems to be able also to deliver both pro-survival and pro-apoptotic signals (Efremov *et al.,* 2007, Stevenson *et al.,* 2011, Chiorazzi *et al.,* 2013).

The balance between BCR induced 'positive' signals leading to proliferation/survival, 'negative' signaling in favor of cell death and BCR unresponsiveness such as in anergy will determine the B cell fate.

Changes in cytosolic Ca^{2+} concentration are driven by a balance of active and passive Ca^{2+} fluxes driving Ca^{2+} respectively against or in the sense of its electrochemical gradient, both of which are subject to the influence of multiple receptors and environmental sensing pathways (Engelke *et al.,* 2007).

As in many other cases, it is quite difficult to distinguish between causes and consequences for disturbances of Ca^{2+} signaling in B cell fate decision and CLL. However we will try to decipher in this review the nature of Ca^{2+} signaling deregulation and their consequences on B cell fate in CLL.

Antigen dependent BCR signaling in normal mature B-cells

The physiology and cell fate of B cells is intimately connected with the function of their BCR. In normal B cells, binding of external Ags to the variable (V) region of the Ig present at the cell surface mediates the formation a BCR complex with the two co-activators CD79a/Igα and CD79/Ig. Next, the BCR complex translocates to the lipid raft in order to initiate the signaling cascade from the BCR to the nucleus leading to proliferation, survival, differentiation, anergy or apoptosis (Fig. 2). Within the lipid rafts, the protein tyrosine kinase (PTK) Syk and the SRC-family PTK Lyn allow the phosphorylation of tyrosines present in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD79 chains (pathway 1) and of the CD19 molecules (pathway 2).

In the first pathway, CD79 activation by Syk and Lyn leads to the rapid recruitment of the adaptor molecule B cell linker (BLNK). Phosphorylated BLNK binds then to the Bruton's tyrosine kinase (Btk) and the phospholipase Cγ2 (PLCγ2) kinase through SRC homology 2 (SH2) domains. Activated PLCγ2 hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) leading to inositol-1,4,5,-triphosphate (InsP3) and diacylglycerol (DAG) production (Niiro *et al.,* 2002, Engelke *et al.,* 2007, Kurosaki *et al.,* 2010, Baba *et al.,* 2011, Baba *et al.,* 2014).

In the second pathway and downstream BCR engagement is the phosphorylation of CD19 by Lyn and its association with the Syk-activated adaptator BCAP (B cell adaptor molecule for PI3 K) allowing the activation of the p110 isoform of PI3K. Next, P13K induces the local phosphorylation of phosphatidylinositol-4,5-bisphosphate ($PtdIns(3,4,5)P_2$; PIP_2) to produce and accumulate phosphatidylinositol-3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$; PIP_3) (Baba *et al.,* 2014). PIP_3 transmits signals downstream the BCR and in parallel the PI3K pathway positively regulates BCR-elicited Ca^{2+} flux (Okada *et al.,* 2000, Okkenhaug *et al.,* 2003). Pathways 1 and 2 are interconnected: through its interaction with Btk, promoting membrane targeting of the kinase, PIP_3 positively regulates PLCγ2 activation and BCR-mediated Ca^{2+} mobilization (Scharenberg *et al.,* 1998).

InsP3 binds therefore to its specific InsP3 receptor (InsP3R) located on the endoplasmic reticulum (ER)

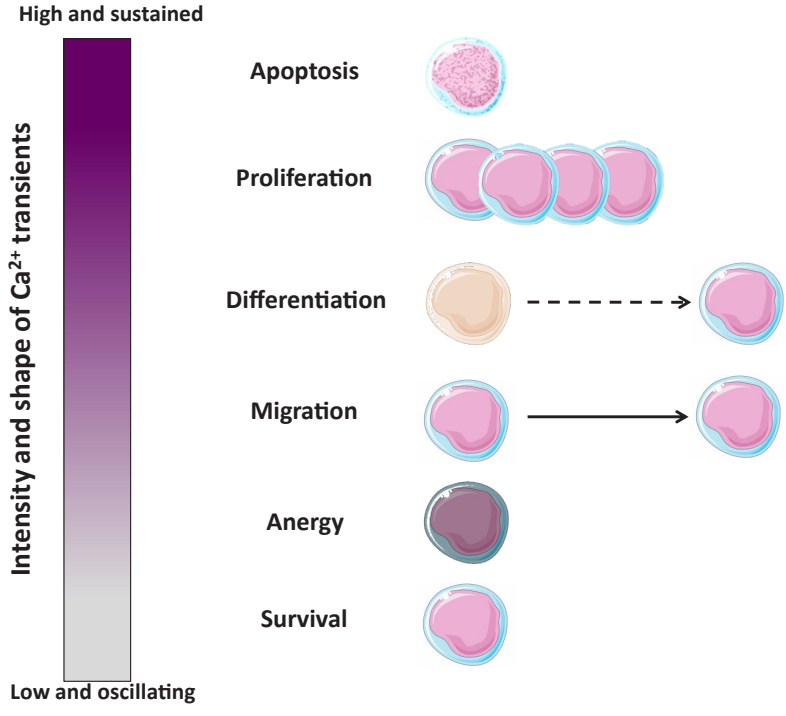

Fig. 1. Calcium intracellular concentration rules B Cell fate. *Shape and intensity of calcium transients are decisive for B-cell fate decision: Ca^{2+} signal ranges from a low amplitude-, oscillating pattern to a high amplitude-,sustained profile of transients leading to either survival, anergy, migration, differentiation, proliferation or apoptosis.*

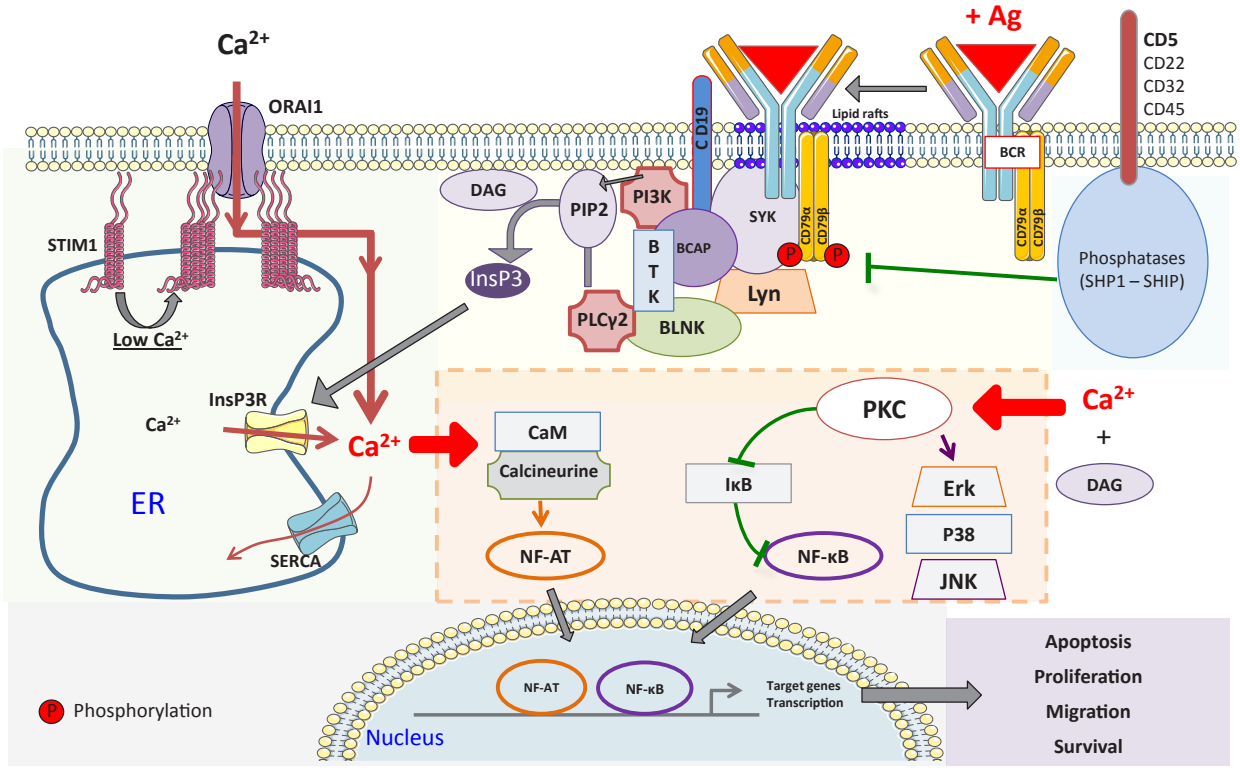

Fig. 2. The B-cell receptor (BCR) signaling pathway in normal B-cells induces apotosis, proliferation, migration or survival. *Antigen (Ag) binding to surface immunoglobulins (sIg) triggers BCR (B-Cell Receptor) activation by inducing its translocation to lipid rafts, where the co-stimulating immunoglobulins heterodimere (CD79α/β) is phosphorylated by Syk and Lyn kinases. Next, the recruitment of the B-Cell Linker (BLNK) with Phospholipase Cγ2 (PLCγ2) and its co-activator Bruton's tyrosin kinase (Btk) induces Phosphatidylinositol-4,5-diphosphate (PIP2) cleavage into diacyglycerol (DAG) and inositol-1,4,5,-triphosphate (InsP3). In parallele, phosphorylation of CD19 by Lyn and B Cell Adaptator molecule for phosphoinositide 3-Kinase (PI3K) (BCAP) by Syk allows the activation of PI3K that positively regulates PIP2. InsP3-induced activation of InsP3 Receptors (Insp3R) allows the release of calcium (Ca^{2+}) from the endoplasmic reticulum (ER) and triggers Stromal Interaction Molecule 1 (STIM1) oligomerization. This leads to the activation of Ca^{2+} Release-activated channels (CRAC) encoded by ORAI1 multimers, responsible for Store Operated Ca^{2+} Entry (SOCE). The increase in the cytoplasmic Ca^{2+} concentration activates Calmoduline (CaM) and Calcineurine, which triggers the nuclear translocation of Nuclear Factor of Activated T-cells (NFAT). Ca^{2+} along with DAG production activates Protein Kinase C (PKC). Downstream signaling of PKC initiates Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38 activation, and the nuclear translocation of the transcription factor Nuclear Factor-κB (NF-κB) by inhibiting its inhibitor, IKK. CD5, CD22, CD32 or CD45 coreceptors participate to a negative control of BCR signaling by inducing SHP-1 and SHIP phosphatases. SERCA: Sarco/endoplasmic reticulum Ca^{2+} ATPase.*

membrane. Opening of this InsP3-gated Ca^{2+} channel leads to the release of ER Ca^{2+} stores supporting a transient increase in cytosolic Ca^{2+}. Sufficient decrease in ER Ca^{2+} concentration triggers the activation of an influx of Ca^{2+} from the extracellular space. As this calcium entry is subsequent to the release of Ca^{2+} from ER stores, this influx was named Store Operated Calcium Entry (SOCE) (Putney 2005, Putney 2009). In lymphocytes as in other cell types, once InsP3R opening induces sufficient ER Ca^{2+} pool depletion, the reduced ER Ca^{2+} concentration is sensed by the pool of stromal interaction molecule (STIM1) located in the ER membrane via their EF-Hand domains (Putney 2009, Soboloff *et al.,* 2012, Prakriya 2013). STIM1 molecules undergo Ca^{2+}-dependent conformational changes and oligomerization to form punctae close to the plasma membrane. This allows direct interaction of STIM1 with plasma membrane Ca^{2+}-release activated Ca^{2+} (CRAC) channels, encoded by multimers of ORAI1 (Engelke *et al.,* 2007, Feske 2007, Feske 2010, Feske 2011). Genetic deletion of the Orai1 or Stim1 genes results in almost completely abolished SOCE signal, defective, proliferative response and reduced IL-10 production in B cells (Baba *et al.,* 2008, Oh-Hora *et al.,* 2008, Picard *et al.,* 2009, Matsumoto *et al.,* 2011, Fuchs *et al.,* 2012, Shaw *et al.,* 2012, Baba *et al.,* 2014) (Baba *et al.,* 2009, Matsumoto *et al.,* 2011, Feske *et al.,* 2012, Baba *et al.,* 2014). However, given the great importance of Ca^{2+} dependent signaling cascades and transcription factors activation in B cells, it is quite surprising that no significant defects in B cell development are observed in SOCE deficient B cells in mice and human patients lacking ORAI1 or STIM1 (Feske 2007, Feske 2011, Shaw *et al.,* 2012, Baba *et al.,* 2014). We can hypothesize that only small increases in $[Ca^{2+}]_i$ are sufficient for efficiency of signal transduction or that other Ca^{2+} influx pathways mediate Ca^{2+} signals of first importance for B cells cell fate and development.

Finally, increase in intracellular Ca^{2+} and DAG accumulation lead to the activation of protein kinase C (PKC) that are involved in the activation of mitogen-activated protein kinases (MAPKs), such as the extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38 MAPK. Elevation of [Ca^{2+}]i

also promotes nuclear translocation of two important transcription factors for B cell fate decisions and development: NF-AT (nuclear factor of activated T cells) and NF-KB (nuclear factor κB) (Healy *et al.,* 1997, Gallo *et al.,* 2006).

CLL B cells defects in Ca^{2+} signalling (activated by anti-IgM)

Since the years 90, it is clearly established that a prominent feature of CLL B cells is the heterogeneity of proximal BCR signals induced by surface IgM crosslinking such as phosphorylation of Syk, PLCγ2 activation and Ca^{2+} response (Hivroz *et al.,* 1988, Hivroz *et al.,* 1990, Michel *et al.,* 1993, Lankester *et al.,* 1995, Semichon *et al.,* 1997, Potter *et al.,* 2006, Song *et al.,* 2010, Le Roy *et al.,* 2012). As a consequence of this heterogeneity, BCR engagement has been reported to induce prolonged survival (Hivroz *et al.,* 1988, Bernal *et al.,* 2001) proliferation or either accelerated apoptosis of CLL B cells (Zupo *et al.,* 1996, Zupo *et al.,* 2000). Since these mechanisms are clearly linked to Ca^{2+} signaling, variability in Ca^{2+} responses may conduct to heterogeneity in B cell fate and subsequently to diversity in CLL ontogenesis and progression.

Numerous people working on CLL put tremendous efforts to decipher this heterogeneity and to link the different observed patterns in BCR induced Ca^{2+} signals with B CLL cell fate. In a study from Hivroz *et al.,* three patterns of response to BCR crosslinking are distinguished: One third of the CLL cells proliferate and mobilize Ca^{2+} upon BCR activation behaving like resting B cells; in another one third of the cases, antigen stimulation leads to Ca^{2+} mobilization without cell proliferation and in the last third neither Ca^{2+} mobilization and $Insp_3$ production, nor cell proliferation were observed (Hivroz *et al.,* 1988, Hivroz *et al.,* 1990).

Non responding (anergy) versus responding CLL B cells

Even if CLL B cells are characterized by a low level of surface immunoglobulin (sIg) expression, B cells from at least 70 % of CLL patients respond to *in- vitro* cross-linking of sIg with effective activation of signaling pathways. In these cells, BCR stimulation leads to a robust $[Ca^{2+}]_i$ increase, whereas B cells are unresponsive to such BCR cross-linking stimulation in the remaining cases. B cells from BCR non-responding CLL patients display constitutively phosphorylated extracellular signal-regulated kinase (ERK)1/2, constitutive phosphorylation of MEK1/2 (Mitogen activated Kinase Kinase 1/2) and an increase in nuclear factor of activated T cells (NF-AT) NF-AT2/NF-ATc1 translocation to the nucleus associated to a greater resistance to spontaneous apoptosis similar to anergic B cells (Petlickovski *et al.,* 2005, Kurosaki *et al.,* 2010, Packham *et al.,* 2014).

Anergy is one of the mechanisms adopted by the immune system to silence auto-reactive B cells upon low-affinity recognition of self Ag subsequent to BCR desensitization induced by chronic Ag binding (Gauld *et al.,* 2006, Cambier *et al.,* 2007, Muzio *et al.,* 2008, Kurosaki *et al.,* 2010, Packham *et al.,* 2014). BCR-mediated signaling is deregulated in anergic B cells and these cells also exhibit an elevated basal $[Ca^{2+}]_i$ by approximately 50-120 nM, but no further increase in $[Ca^{2+}]_i$ upon BCR stimulation (Cooke *et al.,* 1994, Healy *et al.,* 1997, Benschop *et al.,* 2001, Merrell *et al.,* 2006, Yarkoni *et al.,* 2010, Packham *et al.,* 2014). As an example, BCR aggregation failed to evoke a large $[Ca^{2+}]_i$ transient in HEL (Hen Egg Lysozyme)-specific B cells from MD4 ML5 mice, although these cells display a constitutive increase in basal intracellular Ca^{2+} (Healy *et al.,* 1997). In these HEL-anergic B-cells, BCR engagement induces low Ca^{2+} oscillations and activates NF-AT and ERK, but does not activate NF-KB or JNK. Chronic signaling by occupied BCR receptors may explain the fact that anergic B cells exhibit elevated intracellular free Ca^{2+} and constitutive activation of the Erk kinase pathway. Return to normal basal Ca^{2+} concentration, reduction of Erk phosphorylation, extension of lifespan, and restoration of the ability of BCR cross-linking to induce Ca^{2+} mobilization can be obtained indeed by disengagement of the anergic BCR (Gauld *et al.,* 2005, Gauld *et al.,* 2006).

Comparing human CLL unresponsive B cells' phenotypes with anergic B cells (blunted BCR-mediated protein-tyrosine-phosphorylation, basal phosphorylation of Erk, basal NFAT nuclear translocation), human CLL unresponsive B cells may be considered as anergic B cells aberrantly expanded and protected from death. Their phenotype recapitulates the signaling pattern of anergised murine B cells after chronic stimulation by exposure to mono- or oligovalent soluble antigens and of naturally occurring anergic B cells (Healy *et al.,* 1997, Macian *et al.,* 2002, Niiro *et al.,* 2002, Jun *et al.,* 2003, Merrell *et al.,* 2006, Quach *et al.,* 2011).

Mechanisms involved in the basal increase in Ca^{2+} concentration have still to be clarified and deciphering the links between constitutive activation of some signaling pathways such as ERK and NFAT and the defects in Ca^{2+} signals may conduct to a better understanding of the defects in B CLL cell fate.

CD5 and SHP-1 as a link between anergy and CLL?

Part of the anergic effect is related to the expression of the cell surface molecule CD5 that defines the B1 cell phenotype (Hippen *et al.,* 2000). It has been demonstrated that CD5 antagonizes early signaling events mediated by the BCR both in murine and human B cells (Bikah *et al.,* 1996, Gary-Gouy *et al.,* 2000, Gary-Gouy *et al.,* 2002). Ca^{2+} signals activated by IgM cross-linking are significantly decreased in $CD5^+$ B cells along with an inhibition of ERK phosphorylation. Upon IgM stimulation, splenic B2 lymphocytes proliferate and display normal Ca^{2+} responses, whereas peritoneal B1 cells undergo apoptosis and do not develop Ca^{2+} transients following BCR engagement (Bikah *et al.,* 1996). Moreover, anergic B1 cells from transgenic HEL mice lacking CD5 also display enhanced proliferative responses *in vitro* and elevated intracellular Ca^{2+} levels at rest and after IgM cross-linking (Hippen *et al.,* 2000). CD5 expression seems to result from chronic BCR stimulation of normal B2 cells and accumulation of $CD5^+$ B cells is the main CLL characteristic (Berland *et al.,* 2002). Moreover, it is suggested that constitutive activation of NF-AT could be responsible for the high expression of CD5 in human $CD5^+$ B-CLL cells and that Ca^{2+} signaling deregulation may be responsible for this. However, even if NFAT nuclear translocation is clearly Ca^{2+} dependent, any data have been provided yet to clearly link the constitutive activation of this transcription factor, CD5 expression and expansion of $CD5^+$ cells with Ca^{2+} deregulations. Altogether and along with the unresponsiveness of a portion of CLL B cells displaying an anergy-like phenotype, these observations lead people to spend attention on the role of CD5 in resting and BCR stimulated anergic B cells (Hippen *et al.,* 2000, Gary-Gouy *et al.,* 2007).

B-1 cells express a high level of the membrane glycoprotein CD5

that is only expressed in a subset of B cells and in T cells. These $CD5^+$ B cells (or B1) are a unique subset of B cells that are distinguishable from the conventional B or B2 cells in terms of functional responses to external stimuli, their phenotype and self-renewal properties (Hayakawa *et al.,* 2000, Su *et al.,* 2000). In B1 cells, the CD5 molecule is constitutively phosphorylated on its tyrosine (Y) 429 enhancing the interaction with the phosphatase SHP-1 which is responsible of the inhibitory effect on BCR signaling (Sindhava *et al.,* 2012). Cytosolic tyrosine phosphatase SHP1 acts a negative regulator of BCR signaling through its inactivation of Syk and BLNK by dephosphorylation (Yarkoni *et al.,* 2010, Packham *et al.,* 2014). In SHP1 knockout mice an elevated number of B1 cell displaying impaired BCR-induced Ca^{2+} mobilization was observed (Pao *et al.,* 2007). BCR stimulation of B2 cells ($CD5^-$ B cells) induces normal Ca^{2+} signaling and the activation of the different kinases pathways (MAPKs, JNK, and p38, MAPK) and transcription factors (NF-AT, NF-kappa B) (Berland *et al.,* 2002). In contrast, B1 cells display constitutive activation of ERK and NF-AT and BCR cross-linking fails to activate p38 MAPK and NF-kB. PLCγ2 activation is also significantly reduced in B1 cells resulting in a decreased of Ca^{2+} signaling (Wong *et al.,* 2002). Moreover, BCR engagement leads to proliferation of B2 cells, whereas B-1 cells are blocked in The G0 phase of the cell cycle. Considering B1 cells phenotype and their functional characteristics, it is largely proposed that B1 cells may be a special class of anergic or tolerant B cells and $CD5^+$ B (or B1) cells may be considered as the normal precursors of B CLL.

In a portion of CLL B cells, CD5 crosslinking result in apoptosis of a portion of B CLL cells and as for other markers, patients can be classified in two groups based on their sensitivy to CD5-triggered apoptosis (Pers *et al.,* 1998, Pers *et al.,* 2002, Renaudineau *et al.,* 2005). A higher expression level of CD38 is observed in CD5 crosslinking responsive cells associated also with elevated Zap70 expression. Resistant B cells to CD5 induced apoptosis are also unable to modulate spontaneous *in vitro* apoptosis induced by IgM stimulation (Nedellec *et al.,* 2005). In CLL B cells sensitive to CD5 (and IgM) induced apoptosis, CD5 loss its association with SHP-1 and is recruited in lipids rafts, along with CD79, sIgM and Syk. CD5 and the BCR colocalization in lipid rafts may be necessary for the early signaling events leading to apoptosis (Renaudineau *et al.,* 2005). Similar pattern of survival and apoptosis responses following anti-CD5 stimulation were obtained in the study of Perez-Chacon and collaborators (Perez-Chacon *et al.,* 2007). Moreover, authors showed that in unstimulated and resting CD5 transfected B cells, it was observed that $CD5^+$ B cells produce more IL-10 than $CD5^-$ B cells enhancing survival of B cells (Gary-Gouy *et al.,* 2002, Garaud *et al.,* 2011).

CD5 may also protect CLL and anergic B cells from apoptosis after BCR stimulation by concurrently exerting negative feedback on cell death promoting signals such as BCR-induced Ca^{2+} transients. CD5 expression level may set the threshold level for activation of survival or pro-apoptotic signals through its action on BCR induced signals such as intracellular Ca^{2+} transient increases.

Non responding (anergic) versus responding CLL B cells

In responding CLL B cells, BCR stimulation leads to the entry into the cell cycle (Lankester *et al.,* 1994, Lankester *et al.,* 1995, Lankester *et al.,* 1996). Recently, Le Roy *et al.,* confirmed the existence of BCR responding and unresponsive CCL-B cells. As for previous reports, a higher rate of phospho-Syk and PLCγ2 activation is observed in responding cells associated with Ca^{2+} release from the ER compared with non-responding cells (Le Roy *et al.,* 2012). IgM stimulation systematically increases phospho-Syk in responding cells. Authors pointed out that NF-AT2 is over-expressed and constitutively activated in both responsive and unresponsive CLL B cells. Constitutive activation of NF-AT2 seem to be a hallmark of unstimulated CLL B cells and may contribute to B CLL cell survival (Schuh *et al.,* 1996). However, NFAT2 is preferentially activated after BCR stimulation in responsive cells favoring cell survival after BCR crosslinking.

Non-responsiveness is also linked to decreased levels of BCR-associated PTK activity associated with a decreased expression of the PTK Syk. Semichon and collaborators also pointed out in their study this defect in SYK tyrosine phosphorylation triggered by BCR ligation in B-CLL cells with low Ca^{2+} responses induced by Ag stimulation (Semichon *et al.,* 1997). However similar expression of the kinase was observed regardless of CLL B cell responsiveness. In early studies from Bismuth group, CLL B cells from patients with a defective Ca^{2+} response also present an altered pattern of protein tyrosine phosphorylation after Ag stimulation in comparison with normal human B cells and CLL responding B cells (Michel *et al.,* 1993, Semichon *et al.,* 1997). The defect in PLCγ2 phosphorylation suggests that the interruption of the phosphoinositide pathway in CLL nonresponsive B cells is proximal to the BCR receptor, at a level in the signaling cascade between activated surface Ig receptors and protein tyrosine kinases activation.

As CLL B cells from both non responsive and responsive groups showed similar Ca^{2+} influx and NF-AT2 activation in response to the ionophore ionomycin, Le Roy and collaborators suggest that Ca^{2+} influx pathways are not altered in non-responding CLL B cells. However, in Semichon's study, some low responding patients exhibit a decreased in the Ca^{2+} response to thapsigargin, a SERCA pump inhibitor known to release intracellular Ca^{2+} without inositol 1,4,5-trisphosphate production and to induce SOCE activation. This latter result suggests the existence of a BCR independent alteration of SOCE activation pathway in CLL B cells that could also be involved in the decreased Ca^{2+} response observed in B-CLL cells (Semichon *et al.,* 1997). Unfortunately, this interesting hypothesis has not been explored further.

Other factors involved in Ca^{2+} regulation

Along the past 20 years, researchers tried to correlate this heterogeneity in BCR response with different prognostic indicators of disease progression, including CD38, ZAP-70 and V_H gene mutation status (Zupo *et al.,* 1996, Chen *et al.,* 2002, Lanham *et al.,* 2003, Mockridge *et al.,* 2007). Patients with an aggressive form of CLL seem to exhibit an intact sIgM transduction pathway (Zupo *et al.,* 1996, Zupo *et al.,* 2000). CLL B cell responsiveness to Ag stimulation by pro-survival signals is associated with a decreased sensitivity to apoptosis and contributes to poor prognosis whereas BCR unresponsiveness is clearly restricted to stable CLL cases.

The clinical course of CLL differs significantly between patients with mutated (M-CLL) and unmutated (U-CLL) Ig variable heavy-chain (V_H) genes. Patients with unmutated genes have a worse prognosis and their CLL B cells show stronger activation of proximal BCR signaling pathways, such as Ca^{2+} signaling, whereas activation of this pathways is usually weaker or absent

in the mutated cases (Mockridge *et al.,* 2007). The cell surface expression of CD38 is also considered to be a prognostic marker in CLL. A relatively high level of CD38 surface expression by CLL cells has been shown to be a marker of poor prognosis. CLL B cell sensitivity to apoptosis induction in response to surface IgM (sIgM) cross-linking is usually associated with the expression of CD38 (Zupo *et al.,* 1996, Zupo *et al.,* 2000, Ghia *et al.,* 2003) and the lack of mutations in surface *IgVH* genes (Damle *et al.,* 1999, Lanham *et al.,* 2003). Cross linking of BCR in B cells from CLL patients with unmutated V_H genes, with a high percentage of ZAP-70-positive cells and/or a high percentage of CD38-positive cells, better induces intracellular Ca^{2+} mobilization and is able to trigger apoptosis. In these CLL responding cells, anti-IgM stimulation does not induce cell proliferation whereas normal peripheral blood B cells, which expressed low to absent CD38, are able to produce Ca^{2+} signals and to proliferate after IgM stimulation.

It has been demonstrated that CLL B cells with unmutated V_H genes generally express ZAP-70, in contrast to normal B cells or most patients with CLL that use mutated *IGHV (Chen et al., 2002).* Recruitment of ZAP-70 to BCR phosphorylated ITAMs can induce the activation of PLCγ2 and subsequently Ca^{2+} transients along with the Ras/MAPK pathway (Kane *et al.,* 2000). B cells generally lack ZAP-70, but a related PTK, called $p72^{Syk}$, play similar roles in BCR signaling pathways. Chen and collaborators nicely demonstrated that ZAP-70 acts as an adapter protein that enhances BCR signaling and notably Ca^{2+} signaling independently of its tyrosine kinase activity or its ability to interact with c-Cbl (Chen *et al.,* 2008)

In a study from our group, heterogeneity of Ca^{2+} responses were also linked to modulation of spontaneous apoptosis in CLL B cells stimulated by sIgM cross-linking (Nedellec *et al.,* 2005). Absence of Ca^{2+} response was observed in a subgroup of CLL patient's cells (named I) that do not present modulation of *in vitro* spontaneous apoptosis after BCR stimulation. In contrast, a more sustained increase in intracellular Ca^{2+} was seen in cells from another subgroups (referred as IIa) that display a decrease in apoptosis and an increase in cell proliferation associated with higher activity of ERK, relative to p38, in the presence of antigen stimulation. Patients classified in this subgroup IIa express more CD38 and present unmutated BCR. This subpopulation may correspond to CLL B cells where an increase in cell survival has been observed after anti-IgM stimulation. In the remaining cells (subgroup IIb), more transient Ca^{2+} signals were observed and associated with an increase in apoptosis and a greater activity of p38, relative to ERK. Activation of PI3K was constitutive in subgroup IIa, but not in subgroup IIb. Expression of Zap70 was restricted to B cells from subgroup IIb patients. CLL agressivity seems also to be higher in group IIb compare to group IIa. These results reflect once more the potential link between Ca^{2+} signals heterogeneity and B cell fate even if we would predict in this latter study that sustained Ca^{2+} signals may be associated with induction or modulation of apoptosis.

Although CLL B cells accumulate and are resistant to cell death *in vivo*, they undergo apoptosis during *in vitro* culture. However some CLL B cells evade from apoptosis *in vitro* through an enhanced survival response after BCR stimulation (Deglesne *et al.,* 2006, Efremov *et al.,* 2007, Stevenson *et al.,* 2011). Induction of anti-apoptotic and survival signals after BCR triggering are usually associated with prolonged activation of the MEK-ERK and PI3K-AKT pathways and activation of NF-KB (Ringshausen *et al.,* 2002, Petlickovski *et al.,* 2005, Longo *et al.,* 2008). All these studies point to the fact that sustained BCR signaling including enhanced PLC activity lead to a greater apoptosis resistance and that BCR responsiveness and consequently Ca^{2+} signals may set the threshold of B CLL cell fate.

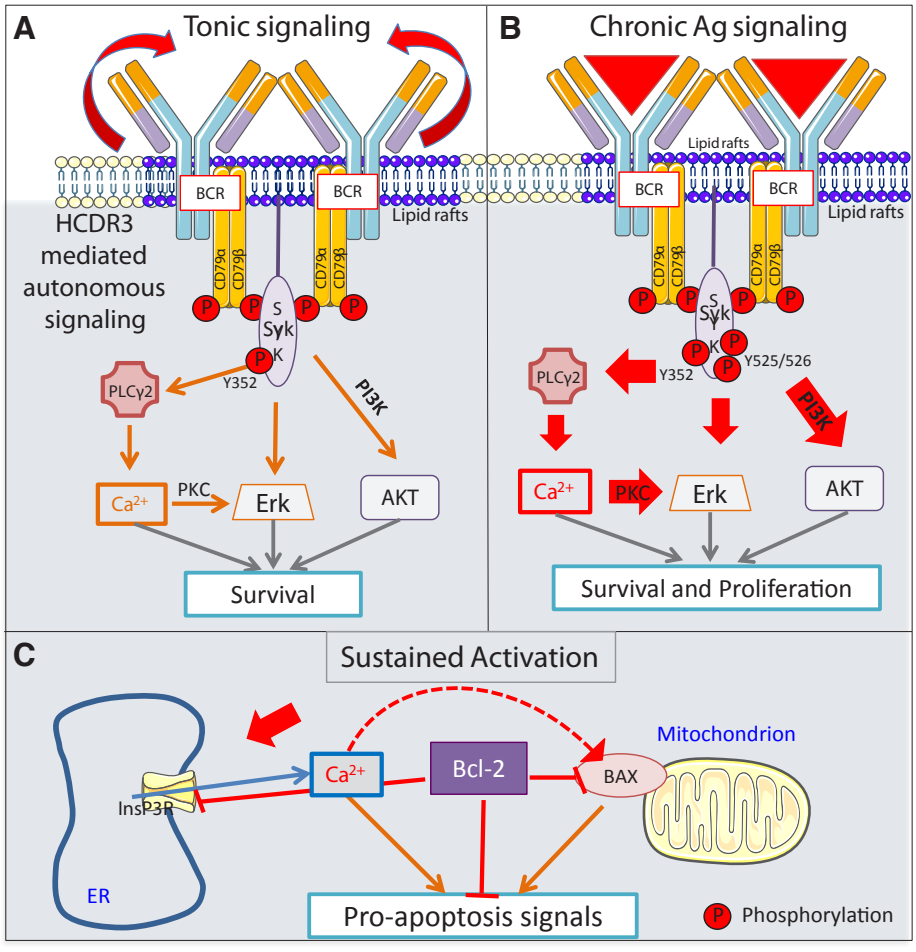

Fig. 3. B-cell receptor (BCR) activation by the phosphorylation of CD79α/β leads to enhanced Ca^{2+} influx and activation of Erk and Akt. (A) *Tonic signal is essential for survival and is promoted by intermolecular interaction between the HCDR3 of the sIg to the CDR2 of another sIg (Dühren von Minden* et al., *2012).* **(B)** *Chronic antigen-dependent BCR activation triggers a strong intracellular signal leading to proliferation.* **(C)** *BCl_2 interacts with the InsP3R to repress endoplasmic reticulum (ER) Ca^{2+} release. HCDR3, heavy chain complementary-determining region 3; Ag, antigen; Y, tyrosine; PLC γ2, phospholipase Cγ2; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; ERK, extracellular-signal related kinase; BAX, Bcl-2 associated X protein; ER, endoplasmic reticulum; InsP3R, inositol-1,4,5,-triphosphate receptor.*

The tonic BCR dependent Ca^{2+} signal

As we previously mentioned, BCR signaling is clearly involved in B CLL cell fate. Ag stimulation is of prime importance for disease progression, based on the strong association between the course of the disease and deregulations of BCR signaling. However, a number of studies brought evidences for an ongoing stimulation of CLL cells *in vivo*, indicated by constitutive activation of signaling kinases such as Syk or ERK and up regulation of associated transcription factors in unmanipulated CLL cells (Muzio *et al.,* 2008, Gobessi *et al.,* 2009). Evidences seem to support that constitutive signals could be induced by Ag binding or be considered as « tonic » and Ag independent (Fig.3 A-B).

This autonomous or tonic signaling has been recently confirmed by forcing the expression of BCRs derived from CLL B cells in a murine pre-B cell line (lacking the expression of pre-BCR and BCR). As a result, cells with CLL-BCR but not those with control-BCR display intracellular Ca^{2+} mobilization without additional BCR crosslinking (Duhren-von Minden *et al.,* 2012). According to Duhren-von Minden *et al.,* CLL-derived BCRs induce Ag-independent cell-autonomous signaling through the presence of an internal epitope present on the heavy-chain complementarity-determining region 3 (HCDR3) of the BCR. As a consequence, insertion of CLL HCDR3s converted a normal human BCR that did not signal into an autonomously active receptor. "Strength" of this BCR autonomous signal may be relatively weak as suggested by the partial activation of SYK in unmanipulated CLL B cells (Carsetti *et al.,* 2009). In this study and as also previously observed in other works on CLL and anergic B cells, basal $[Ca^{2+}]_i$ is elevated in unmanipulated CLL B cells. In murine pre-B-cell line, expressing BCRs derived from CLL cells, autonomous Ca^{2+} signaling could only occur after induction of SLP65/BLNK expression. In these conditions, enhancement of basal $[Ca^{2+}]_i$ could not be detected and return to higher levels of $[Ca^{2+}]_i$ after induction of autonomous Ca^{2+} transients induced by SLP65/BLNK expression can't obtained from the data provided in this study. Responsiveness to BCR crosslinking and the characteristics of subsequent Ca^{2+} transients after induction of autonomous signals was unfortunately not explored in this study. That could be worthwhile to determine if the decrease in BCR-induced Ca^{2+} signaling observed in CLL or anergic B cells could be at least partially explained by HCDR3-internal epitope autonomous signaling.

One of the important questions that still have to be answered is the specificity of signaling pathways activated by antigen-dependent or independent BCR signaling. Study from Carsetti and collaborators brought some answering elements. Syk phosphorylation is observed on tyrosine 352 in the case of antigen-independent activation whereas residues 525/526 and 352 are phosphorylated after BCR stimulation (Carsetti *et al.,* 2009). Syk phosphorylation on residue Y352 only is sufficient to transiently activate key regulatory proteins, such as PLCγ2, Akt and ERK but cannot sustain B-cell proliferation although prolonging survival. In contrast, Syk phosphorylated 525/526 and 352 sites induces sustained PLCγ2, Akt and ERK activation and allows growth-factor independent B-cell proliferation.

It is tempting to propose that these BCR dependent signals initiated without fixation of an external ligand on this receptor ("tonic" BCR signal) may play a role in the survival of B-cells. CLL survival would be partially explained by tonic BCR survival signals delivers continuously.

BCL2, Ca^{2+} and CLL

It has been now firmly established that the Bcl2 protein contributes through its ability to inhibit apoptosis to cancer progression mechanisms and modulation of response to cancer therapeutic agents (Akl *et al.,* 2014). Deregulation of Bcl2 expression has been link to disease occurrence in Bcl-2–positive lymphoid malignancies such as follicular lymphoma and CLL (Sanchez-Beato *et al.,* 2003, Buggins *et al.,* 2010, Scarfo *et al.,* 2013). The up-regulation of anti-apoptotic Bcl2 family members gives cancer cells a survival advantage and is a frequent event in leukemia such as CLL. One possibility is that Bcl2 contributes to the development of CLL by suppressing proapoptotic Ca^{2+} signals downstream of constitutively active BCR signals (Fig. 3C).

Bcl2 acts at two different intracellular compartments, the mitochondria and the ER. The most described mechanism for Bcl2 apoptosis block resides in part by the binding to its proapoptotic relatives such as Bax (Bcl-2-associated X protein) located in the mitochondria inducing a reduction of mitochondrial cytochrome c release (Buggins *et al.,* 2010, Akl *et al.,* 2014). Many small molecules such as BH3 mimetics that bind to the BH3 binding pocket have been developed to disrupt this interaction of BCl2 with anti-apoptotic mitochondrial BCL2 family members in order to selectively favor apoptotic cell death in cancer cells (Akl *et al.,* 2014).

Inhibition of apoptosis by Bcl2 is also mediated at the ER level through a down regulation of the $InsP_3R$ activity, leading to a decrease in ER Ca^{2+} release and subsequently to reduced elevations of cytoplasmic Ca^{2+} promoting proliferation while insufficient to trigger apoptosis (Lam *et al.,* 1994, Pinton *et al.,* 2000, Akl *et al.,* 2014). Bcl2 is supposed to inhibit high-amplitude, pro-apoptotic Ca^{2+} elevation while it promotes cell survival by enhancing Ca^{2+} oscillations in favor of cell survival (Distelhorst *et al.,* 2011). It has been established that inhibition of $InsP_3R$ activity involves a direct physical interaction with Bcl2 via its N-terminal BH4 domain, and that disruption of this Bcl2–$InsP_3R$ interaction is sufficient to induce $InsP_3R$-mediated proapoptotic Ca^{2+} elevations. These observations lead to the potential therapeutic use of targeting the Bcl2–$InsP_3R$ interaction. TAT-IDPS peptides targeting the BH4 domain of Bcl-2 reverse Bcl-2's inhibitory action on $InsP_3Rs$ and trigger spontaneous pro-apoptotic Ca^{2+} spikes and mitochondrial Ca^{2+} overload in cancer B cells, including CLL B cells and diffuse large B-cell lymphoma (DLBCL) cells (Akl *et al.,* 2014, Greenberg *et al.,* 2014).

Conclusions and therapeutic perspectives

Numerous evidences argue for a major role of BCR signaling in CLL B cell fate and chronic stimulation by antigens of CLL cells through the appropriate BCR, lead to signaling cascades play an important role in CLL pathogenesis and progression. Activation of BCR dependent pathways such as Ca^{2+} signaling, MEK-ERK and PI3K-AKT pathways has been associated with the induction of antiapoptotic and prosurvival signals. It is also clearly established for years that there is a marked case-to-case heterogeneity in BCR responsiveness in CLL, with the malignant cells being markedly less responsive. Crosslinking of the surface IgM results in a heterogeneous response, at least in terms of proximal BCR signaling events, such as PLCγ2 activation and Ca^{2+} response. Responsiveness has been correlated with progressive disease and linked to markers such as high CD38 and zap70 expression

TABLE 1

CALCIUM AND SIGNALING FEATURES OF MATURE B-CELLS VERSUS CHRONIC LYMPHOCYTIC LEUKEMIC AND ANERGIZED-B CELLS

		CLL				
	Mature B Cell	**Responders group IIb**	**Responders group IIa**	**Non responders Group I**	**B1/ CD5+ B cells**	**Anergic B Cells**
Anti-IgM BCR activation	Apoptosis	Apoptosis	Proliferation	No response	No response	No response
B Cell Receptor (IGVH)		Unmutated ?	Unmutated ?	Mutated ?		
Signalosome Syk PLCγ2 PI3K Zap70		 High P-Syk High P-PLCγ2 Constitutive High	 High P-Syk High P-PLCγ2 Low	 Low P-Syk Low		 Low P-Syk Low
Ca^{2+} transients	Yes	Yes	Yes	Absent	Absent	Absent
Kinases and Transcription Factors ERK NF-AT NF-κB		 Induced Induced	 Induced Induced	 Constitutive Constitutive	 Constitutive Constitutive No activation	 Constitutive Constitutive No activation
Co-receptors CD5 CD38	 None Low	 Medium High	 Medium High	 High Low	High	High

Table 1. The following are summerized for each different group: Main anti-IgM-induced BCR response consequences on cell fate, IgVh mutation status, activation state of several components of the proximal and distal B Cell Receptor-induced signalosome, presence of calcium transients and expression-rate of key coreceptors. Based on Nedellec *et al.,* 2005; Renaudineau *et al.,* 2005. P-Syk: Phosphorylated and activated Syk; P-PLC γ2: Phosphorylated and activated Phospholipase Cγ2; PI3K phosphoinositide 3-Kinase (PI3K); Zap 70: Zeta-chain-associated protein kinase 70; ERK: Extracellular-signal related kinase; NF-AT: Nuclear Factor of Activated T-cells; NF-κB: Nuclear Factor-κB.

and lack of extensive VH mutation. It has also been suggested that unresponsiveness may reflect an anergic state of B CLL cells induced by chronic antigen exposure (Table 1).

The dependence of CLL survival and proliferation on BCR signaling and more specifically on both PI3K and BTK signaling, has raised a new area of basic and therapeutic research interest and has conducted to preclinical studies and trials demonstrating dramatic efficacy of different SYK, BTK or PI3K kinases inhibitors (Hill *et al.,* 2013, Jones *et al.,* 2014). Modulation of BCR dependent Ca^{2+} signaling in B cell has not been proposed yet as a therapeutic target in CLL but regarding the number of deregulations observed, propositions in that sense will be certainly done in a close future.

Moreover, a number of studies report an increase in basal activity of BCR signaling independently of any external Ag in unstimulated CLL cells (Table 1). It is recognized now that some, or perhaps all, immunoreceptors can signal independently of ligand resulting in a tonic BCR signaling in the cell. Constitutive activation of signaling pathways and up-regulation of associated transcription factors in CLL cells, may contributes to their increased resistance for apoptosis and for maintaining B cell survival and proliferation. Tonic signals may not drive CLL cells fate but rather allow them to survive longer to eventually receive other expansion signals. Expansion and survival of CLL cells also depends on interactions with non-leukemic cells in lymphoid tissues. Regarding Ca^{2+} signaling, the main consequence of this tonic signaling seems to be an increase in basal $[Ca^{2+}]_i$ that might lead to enhanced survival without an accompanying proliferation. However mechanisms involved in this Ca^{2+} defect are still unknown and need to be explored. Targeting these mechanisms may represent an interesting therapeutic opportunity.

Acknowledgements

This work was supported by the "Ligue contre le cancer" and the "Région Bretagne".

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