Mechanisms of CLL Cell Stimulation via BcR

Subjects: Hematology

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The engagement of the B cell receptor (BcR) on the surface of leukemic cells represents a key event in chronic lymphocytic leukemia (CLL) since it can lead to the maintenance and expansion of the neoplastic clone. This notion was initially suggested by observations of the CLL BcR repertoire and of correlations existing between certain BcR features and the clinical outcomes of single patients. Based on these observations, tyrosine kinase inhibitors (TKIs), which block BcR signaling, have been introduced in therapy with the aim of inhibiting CLL cell clonal expansion and of controlling the disease. Indeed, the impressive results obtained with these compounds provided further proof of the role of BcR in CLL.

immunoglobulin chronic lymphocytic leukemia

1. Introduction

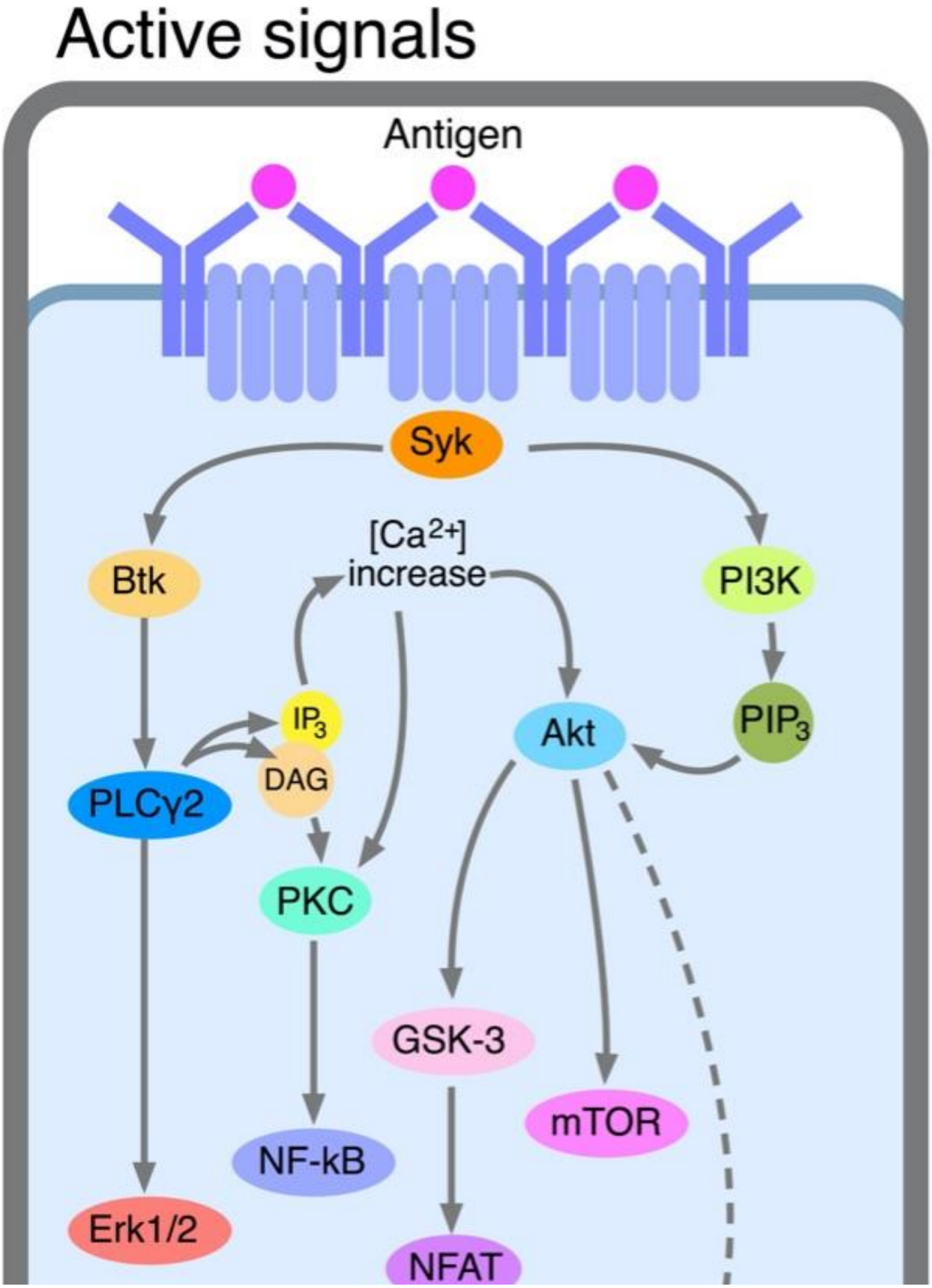
Lymphoproliferative disorders of mature T and B cells are generally believed to originate from cells that have completed their maturation process. This is unlike what has been observed for most cancers, in which the transforming events occur primarily in the stem cells, which, because of these events, become cancer stem cells, capable of proliferating and of differentiating in part into mature neoplastic cells, with low proliferative capacities [1]. This genesis is possible since mature T and B cells can be recruited into the cell cycle via external signals and propagate the transforming events to their progeny, as stem cells of other tissues do. The stimulation of mature T and B cells by antigens may represent one of the signals which facilitates the process of malignant transformation. Gastric lymphomas are accompanied by *Helicobacter pylori* (HP) infection, which promotes the formation of lymphoid tissue in the gastric mucosa and the proliferation of B cells that already carry transforming mutations or which cause the accumulation of transforming events while proliferating [2]. Antibiotic treatment results in a regression of the lymphoid gastric lesions, concomitant with the eradication of the HP infection in the early disease stages, whereas it is ineffective or partially effective at later stages, indicating the role of additional transforming events in disease progression [3]. In lymphomas arising in patients with hepatitis C virus (HCV) infection, the BcR of the malignant clone frequently has specificity for HCV epitopes and treatment of the viral infection can result in lymphoma regression [4][5]. In CLL, the analysis of the repertoire indicates the involvement of BcR in promoting clonal expansion.

2. BcR Signaling in CLL

Two types of signals can be delivered by BcRs, i.e., tonic and active signals, according to the current terminology [6], with different functions relevant to leukemogenesis.

2.1. Tonic Signals

These signals allow the survival of mature B cells in vivo and an active engagement of the BcR with a specific antigen epitope is not required. This conclusion stems from the observation on the conditioned inactivation of surface IgM or of CD79a, the BcR accessory chain, which is part of a disulfide-linked heterodimer (CD79a/CD79b) subunit capable of mediating BcR membrane expression, signal transduction and receptor internalization [7][8]. In mice, this inactivation causes a progressive and prolonged reduction in the total number of circulating B cells over a period of weeks. The mechanisms of the delivery of tonic signals are not fully understood, although it has been proposed that BcR is expressed at the cell surface in the form of stable auto-inhibited oligomers which re-organize into clusters in the presence of the appropriate BcR ligand, which are capable of driving cell activation [9][10]. Stable oligomers can still deliver tonic signals by activating the PI3K pathway which is one of the pathways that is dependent on the engagement of BcR (see **Figure 1**). The activation of this pathway may imply Syk activation or may be operated directly via CD79b activation [11]. The requirement of tonic signals explains why the cells from most lymphoproliferative disorders of mature B cells express fully assembled surface IgM molecules, the Ig isotype most suitable to deliver tonic signals [6]. The majority of CLL clones express surface IgM, irrespective of whether they belong to the U- or M-CLL group. This finding is somewhat unexpected since M-CLL cells are likely to have passed through germinal centers where somatic IGHV hypermutation (SHM) and isotype switching take place, although it may relate to the capacity of surface IgM to deliver more efficient tonic signals than IgG or IgA [6]. Although intra-clonal isotype switching from IgM to IgG or IgA is observed in many CLL cases, it is confined to a minor sub-clonal component, with a few exceptions, as it may not offer a special survival advantage to the leukemic cells [12][13]. Certain CLL cases express stereotypes invariably connected with an IgG isotype, such as subset #4 or #8 stereotypes, suggesting that tonic signals are substituted for by other more advantageous signals in these clones [14]. However, unlike normal resting B lymphocytes, CLL cells frequently have some IgM clusters on their surface, suggesting that tonic signals are delivered together with active signals in many circumstances, as outlined below [15].


Active signals
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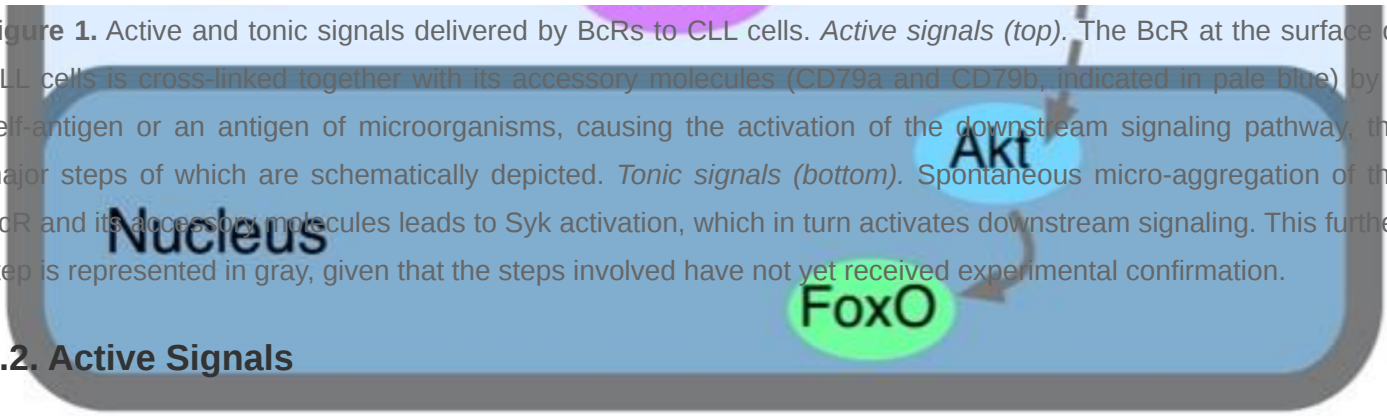

Figure 1. Active and tonic signals delivered by BcRs to CLL cells. *Active signals (top).* The BcR at the surface of CLL cells is cross-linked together with its accessory molecules (CD79a and CD79b, indicated in pale blue) by a self-antigen or an antigen of microorganisms, causing the activation of the downstream signaling pathway, the major steps of which are schematically depicted. *Tonic signals (bottom).* Spontaneous micro-aggregation of the BcR and its accessory molecules leads to Syk activation, which in turn activates downstream signaling. This further step is represented in gray, given that the steps involved have not yet received experimental confirmation.

2.2. Active Signals

These have the function of promoting cell proliferation and clonal expansion and include classic antigenic stimulation (extrinsic BcR engagement) (**Figure 1**) and BcR self-recognition (intrinsic BcR engagement) (**Figure 2**). These signals can be further subdivided as follows:

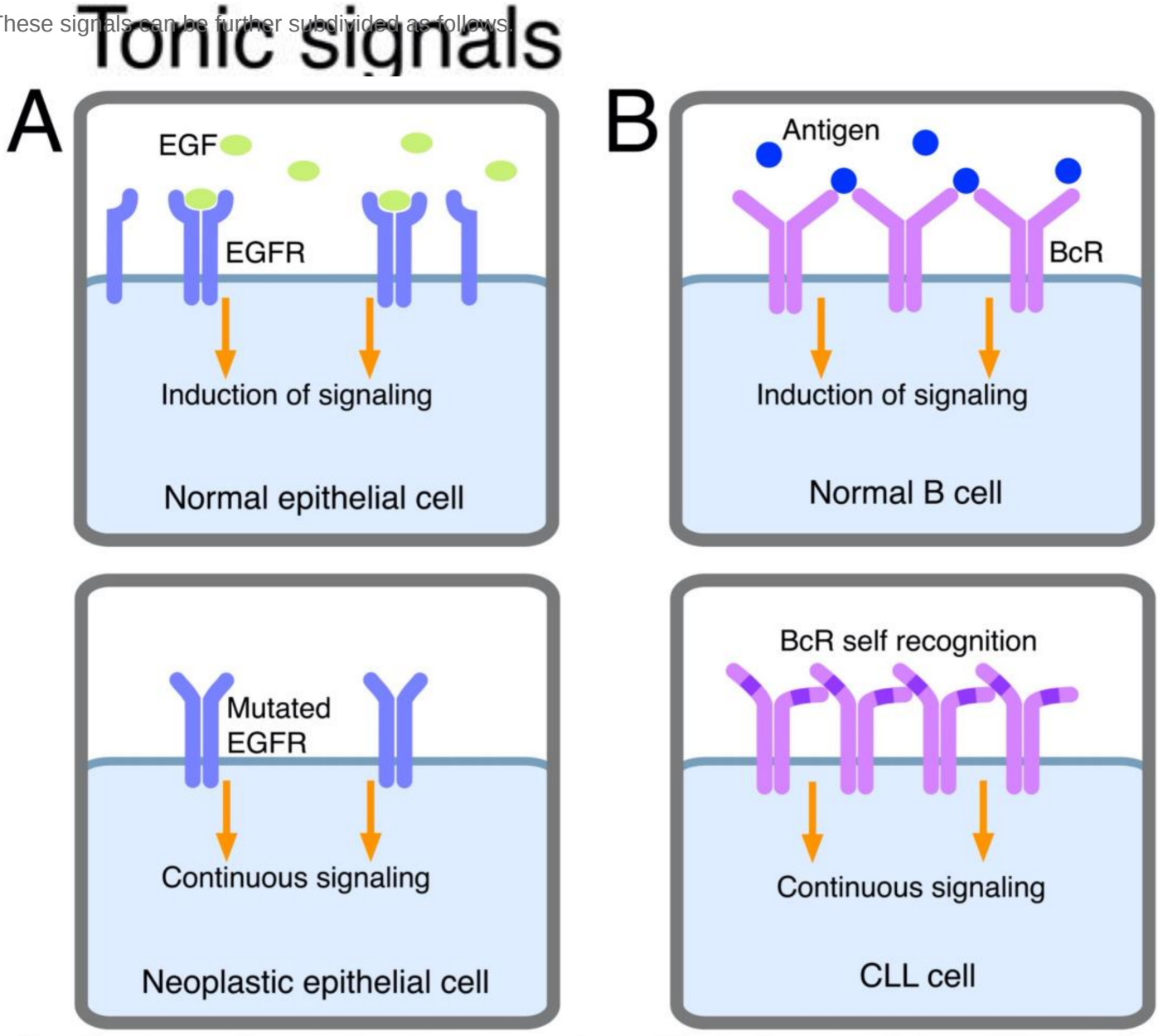

Figure 2. Autonomous signaling in CLL cells. The mechanisms leading to autonomous signaling ((**B**), right) are depicted and compared with the continuous signaling delivered by mutated EGFR in cancer epithelial cells ((**A**),

left). In normal epithelial cells, dimerization of a proportion of the membrane's EGFR molecules by EGF causes the activation of the downstream pathway, which is transient and lasts until EGF is present in the micro-environment (top). In neoplastic cells, "mutated" EGFR molecules aggregate spontaneously, providing continuous signaling to the cells which are induced to proliferate (bottom). In normal B cells, BcR stimulation is caused by the presence of a specific antigen and is transient (top). In CLL, in which autonomous signaling occurs, BcR signaling is constantly activated since the BcR binds to a specific structure of the BcR itself (marked in blue).

(i *Stimulation by self-antigens.* The presence of frequent auto-immune manifestations, such as auto-immune hemolytic anemia or thrombocytopenia, suggested a connection between CLL and auto-immunity since the early studies [16]. This notion was substantiated more recently by the observation that a considerable number of CLL clones expressed a BcR characterized by poly-reactivity, a definition indicating that each monoclonal antibody could react with low affinity with a variety of different (auto)antigens, including platelets, aggregated IgG, nuclear antigens, double-strand (ds) and single-strand (ss) DNA, insulin, etc. Antibodies with these features are found among the "natural antibodies", a family of antibodies mostly of the IgM isotype, of all mammalian species, representing one of the first lines of defense against assaulting pathogens [17][18]. These concepts were further refined by showing that U-CLL clones produce polyreactive IG very frequently, whereas this occurs rarely for M-CLL clones [19]. However, autoantibodies from patients with auto-immune manifestations, observed in both U- and M-CLL patients, are not produced by leukemic cells, since they are of the IgG isotype, they utilize both K and lambda light chain types and they are polyclonal [20]. Therefore, auto-immunity is not directly caused by the leukemic clone, although models in which leukemia and autoimmunity are part of the same pathogenetic process can be proposed, as researchers shall discuss later. Rare patients with cold agglutinin disease or cryoglobulinemia and CLL represent a notable exception. In these conditions, leukemic cells produce monoclonal low-affinity auto-antibodies to red cells or to the Fc portion of IgG [20][21][22], a feature consistent with the limited capacity of CLL cells to mature into plasma-cell-secreting antibodies [23]. Additional information came from the observation that poly-reactive antibodies often recognize antigens at the surface of apoptotic cells. Although normally located intracellularly, certain self-antigens can be expressed at the cell surface, when apoptosis is activated, and can be modified by metabolic processes, such as oxidation associated with apoptosis [24][25][26]. One physiologic function of poly-reactive antibodies is the clearance of apoptotic cells [27]. The expression of a BcR with polyreactive features, capable of recognizing apoptosis-related antigens, may become instrumental in promoting CLL clonal expansion, particularly in the presence of abundant CLL cell apoptosis. A substantial proportion of IG molecules cloned from or secreted by CLL cells react with intracellular proteins such as vimentin, tubulin and filamin B exposed at the cell surface following the induction of apoptosis [26]. Additional proteins to which these IGs have reactivity are those with which the sera of systemic lupus erythematosus (SLE) patients have reactivity, including Sm, snRNPA, Ku and other molecules which are also recognized both in the native or oxidized form by IG from the sera of SLE patients [24]. IG molecules with these reactivities are produced predominantly by U-CLL cases and many have stereotypic features, particularly those encoded for by the VH1-69 genes [24][25][26]. However, the absence of IGHV mutations and/or the utilization of a given stereotype do not classify these BcRs as specific for apoptosis-related antigens, since several of them fail

to show any reactivity and different reactivities have been detected for the different BcR or BcR families investigated.

(ii) *Stimulation by microbial antigens.* In the absence of a clinically evident infection, such as the HP infection in gastric lymphoma, it is difficult to determine whether the BcRs of CLL cells may have specificity for antigens of a given pathogen. Nevertheless, researchers were able to trace a BcR with specificity for certain microorganisms in CLL. Hoogeboon and colleagues [28] analyzed 82 CLL patients, whose cells expressed an IGHV3-7-encoded BcR. The choice of this cohort was suggested by the reported over-representation of this gene in CLL and by the observation of a frequent SHM in these CLL clones, indicating antigenic stimulation and passage through germinal centers. A further selection within the cohort led to the choice of four patients in whom the BcR was characterized by a very short HCDR3 sequence of 5–6 amino acids (aa) instead of the canonical 15 aa. These BcRs were characterized by the utilization of nearly identical IGKV2-24-encoded Ig light chains and for sharing a glutamic acid at position 106 of HCDR3, which was not detected in any of the other sequences utilizing the IGHV3-7 gene. Cloning of genes and the expression of fully assembled IgM molecules led to the observation that the antibody bound 4/33 commensal yeast species and presented a specific binding to the β-(1,6)-glucan of the yeast. The substitution of the glutamic acid at position 106 of the HCDR3 via site-directed mutagenesis caused inhibition of the high-affinity binding to glucan, as did the substitution of certain aa of the short HCDR3. Finally, in vitro exposure of CLL cells with this BcR to the β-(1,6)-glucan resulted in specific cell proliferation. Together, these observations suggest a process of antigen selection, which may be more frequent than possibly thought, particularly if the stimulating antigens are carried by commensal rather than pathogenic microorganisms. Autoreactivity, described in the preceding section, and reactivity with microbial antigens may represent two aspects of the same phenomenon. Antibodies reacting with molecules exposed on the surface of apoptotic cells and/or with molecules oxidized following apoptosis show cross-reactivity for several microbial components. Therefore, this cross-reaction, which can be demonstrated in vitro, may also operate in vivo in CLL patients and certain CLL BcRs can concomitantly recognize antigens of micro-organisms and self-antigens [26].

(iii) *Autonomous signaling.* This definition relates to the capacity of CLL cell BcRs to autonomously deliver activation signals to leukemic cells, as discovered in a very elegant system in vitro. Using retroviral gene transfer, BcRs from CLL clones were expressed in murine cells lacking endogenous BcR components and were thus unable to be signaled via their own BcR [29]. Positive signaling, revealed by Ca^{++} mobilization, was consistently noted following cell transfection with the BcRs from 17/17 CLL clones and not with the BcRs from other lymphoproliferative disorders including mantle cell, marginal zone and follicular lymphoma and myeloma (15/15 cases, collectively). This phenomenon was observed with the BcRs from U- and M-CLL cases and from cases expressing BcRs with/without poly-specific reactivity. Autonomous signaling also was observed with the BcRs from leukemic cell clones from mice that were transgenic for the *T cell Leukemia 1* or *tcl1* gene. These mice, obtained following the observation that U-CLL clones showed high TCL1 protein levels, expressed a *tcl1* transgene under the control of the IGHV promotor and the E*μ*-enhancer [30]. They developed a lymphoproliferative disorder characterized by the expression of CD5 and the utilization of unmutated IGHV genes, thus resembling human U-CLL [31]. Autonomous signaling was not observed when transfecting the BcRs from murine non-leukemic clones specific for several antigens, indicating that the phenomenon occurs

preferentially in malignant cells [29]. Autonomous signaling can be reminiscent of that observed in other cancers, such as breast, lung or gastrointestinal tract cancers, in which activating mutations of receptors for growth factors enable the receptors to deliver signals similar to those of normal receptors following interaction with specific ligands [32][33]. Because of mutations, the receptor acquires the property of self-aggregation or dimerization or undergoes conformational changes, which can activate downstream signals (see **Figure 2**). However, there are no activating mutations in the BcR transducing the downstream signaling, and the system is more complex in comparison to these models. Ca^{++} mobilization is observed when the transfected BcR displays a HCDR3 motif which enables binding to a conserved epitope in the framework region 2 (FR2) of the IGHV domain of the same BcR molecule [29]. An alternative epitope for BcR autologous binding is found in FR3 [34]. Although these mechanisms have the same effect as the activating mutations of growth factor receptors present in other cancers, the underlying molecular mechanisms are different, as the recognition of a specific epitope of the "leukemic" BcR by the antigen-combining site of the same BcR expressed by the leukemic clone is required for receptor activation. In other words, autonomous signaling is intra-clonal and implies a specific interaction between BcR molecules on the surface of the same cell (or the BcRs of different cells from the same clone), given that the BcR recognizes a self-epitope (see **Figure 2**) [29]. A good example of the relevance of autonomous signaling is provided by a subgroup of CLL of the subset #2 stereotypes. These cases carry a single-point mutation, termed R110, at the junction between the variable and the constant region of the light chain. Although subset #2 stereotype is itself a poor prognosis indicator, $R110^{+}$ cases display an even worse outcome. Light-chain mutation has been found to facilitate homotypic BcR-BcR interactions which result in a more robust activation of autonomous signaling [35].

Not all the types of BcR engagement occur concomitantly or operate in all CLL clones. Some of the signals depend on the BcR type. Poly-reactivity is frequent in U- and rare in M-CLL clones; hence, the BcR engagement is different in the two groups, explaining in part their differing clinical behavior. Some of the BcR engagements operate constantly during the disease, as is the case for self-antigens delivering signals to a polyreactive BcR or for autonomous signaling, whereas others are intermittent, as is the case for antigens of pathogens, which can deliver stimulatory signals during infections, although cross-reacting epitopes of apoptosis-related antigens can continue to stimulate the leukemic clone in the absence of pathogens. The presence and quantity of a given antigen may represent a limiting factor in BcR engagement, as is perhaps the case for CLL patients whose cells express a BcR with rheumatoid factor-like specificity, since aggregated IgG, in the form of immune complexes, may not be constantly available for stimulation [21]. A successful BcR engagement may require support that only particular anatomical sites can offer. The presentation of self-antigens to a polyreactive BcR may be facilitated by the cellular/molecular structure of the proliferating centers of lymphoid tissues and prevented in circulating CLL cells [36]. Autonomous signaling may not have these topographical limitations, although the differing availability of accessory cells in the various sites may impose different constraints in the outcome.

Some signals may be operative only in certain periods of the history of a leukemic clone. The BcR of M-CLL can be made to revert to the U status using site-directed mutagenesis and can regain poly-reactivity, suggesting that M clones are derived from a U progenitor B cell [19]. Although not universally accepted, this hypothesis opens the possibility that the change from U to M features during leukemogenesis also requires a change in the type of BcR

stimulation, possibly with a switch from a poly-reactivity-type to an autonomous-signaling-type BcR engagement. The transforming CLL clones could adapt the type of BcR engagement appropriate to their needs during leukemogenesis. For example, leukemogenesis could be promoted initially by cell proliferation induced by a pathogen that also induces somatic hyper-mutation (SHM). When infection ends, SHM could be selected based on autonomous signaling rather than the affinity maturation requirements. In M-CLL, the quality/quantity of IGHV mutations does not affect the clinical course, possibly suggesting that SHM is driven more by autonomous signaling than by affinity maturation needs [37][38]. The differences in the strength of autonomous signaling reported in clones expressing different stereotyped BcRs may reflect a similar selection process [39].

The stimuli illustrated above provide a sufficient array of mechanisms whereby the BcR can keep CLL cells in an activated status in vivo. This condition is documented by the expression of activation markers detected at different extents depending upon the subgroup of origin (e.g., U- or M-CLL) and by the presence in unmanipulated CLL cells of a number of phosphorylated molecules belonging to the BcR-dependent signal transduction pathway [40][41].

A final note of caution concerns the fact that most BcR engagements proposed for the survival/expansion of CLL cells have been deduced from in vitro observations and the results may not reflect the real situation in vivo, although some of the mechanisms have found a further demonstration in vivo in murine models of lymphoproliferative diseases.

3. Outcome of BcR Engagement

The outcome of BcR engagement is heterogeneous, ranging from activation to anergy, and from proliferation to apoptosis, and can be modulated by the microenvironment. Several aspects of these regulatory mechanisms are analyzed below.

3.1. Identification of the Proliferating Cell Fraction of the CLL Clone

Since the early studies on CLL cell surface markers, it was clear that CLL clones with high numbers of $CD38^+$ cells responded to surface BcR stimulation, whereas those with low $CD38^+$ cell numbers could be classified as low responders or anergic [42][43]. The same was observed with CLL clones classified as ZAP-70^{high} and ZAP-70^{low} or U and M, respectively [44][45]. BcR cross-linking was achieved by exposing the cells to a divalent anti-IgM antibody and cell stimulation was measured via Ca^{++} mobilization and/or protein tyrosine phosphorylation. These data could suggest that some CLL clones only responded to stimulation via BcR, whereas the expansion of the others followed different pathways. Intra-clonal fractionation of cells according to CD38 expression indicated a different interpretation, since the $CD38^+$ cells only proved capable of a response via BcR stimulation, whereas the $CD38^-$ cell fractions were invariably anergic and this was observed irrespectively of whether the two fractions were obtained from clones classified as $CD38^{high}$ or $CD38^{low}$ [46]. In line with this result was the observation that $CD38^+$ cells were comprised of more Ki67- and ZAP-70-expressing cells and had higher telomerase activity than $CD38^-$ cells from the same clone, suggesting that $CD38^+$ cells were those with the highest proliferating capacity [47]. This

finding was confirmed by observations with deuterated water labeling in vivo, demonstrating that the circulating $CD38^+$ cells were enriched for recently divided cells [48][49].

Tests with deuterium labeling in vivo and gene expression profile (GEP) methodologies on lymphocytes from different sites showed that the proliferation of CLL cells takes place predominantly in the peripheral lymphoid tissues [50][51]. Moreover, immunohistochemical observations indicated that the proliferating centers in peripheral lymphoid tissues were the sites of the highest leukemic cell proliferation, given the enrichment in the expression of $Ki67^+$ leukemic cells co-expressing activation markers [36]. Here, the CLL cells have the opportunity of being in close contact with accessory cells such as macrophages, nurse-like cells (NLC) and T cells and with the cytokines that they release [52][53][54][55]. Thus, the combination of effective BcR engagement, through one or more of the mechanisms described above, facilitated by the environment of proliferating centers, together with the assistance of a variety of accessory cells, appears to promote the process of clonal expansion. Leukemic cells in lymphoid tissues are characterized by high CXCR4 expression, which facilitates their homing by interacting with its ligand, CXCL12, on NLC [53][55]. In addition, chemokines such as CCL3 and CCL4 released by the leukemic cells exert a chemo-attractant function for lymphocytes and monocytes with specific receptors (CCR1 and CCR5) [54]. The activation of CLL cells in proliferating centers can result in the lowering of the CXCR4 levels, which facilitates the cells' exit from proliferating centers into the circulation, where they continue to express activation markers at an extent proportional to the activation in the proliferating centers [56]. CD5, an activation marker capable of regulating BcR activity, is also upregulated [57]. However, the diminished chances of stimulation in the circulation contribute to a decrease in the activation of leukemic cells and to lower levels of CD5, while increasing CXCR4 levels. This makes the cells ready to re-enter the peripheral lymphoid organs and to initiate a new cycle of proliferation [58]. The levels of CD5 and CXCR4 expression have been used to define three groups of CLL cells: the $CD5^{high}$ and $CXCR4^{low}$ cells, which are recently divided cells that express high levels of activation markers including CD38; the $CD5^{low}$ and $CXCR4^{high}$ cells, resting CLL cells which are prone to re-enter lymphoid tissues; and the $CD5^{intermediate}$ $CXCR4^{intermediate}$ cells, which are in between the two other categories and are likely cells at various activation states and/or on their way to becoming quiescent [59][60]. These distinct groups can be identified in both M- and U-CLL clones, confirming that both subsets have proliferating compartments, as anticipated by the initial fractionation studies conducted using surface CD38. Observations on circulating CLL cells, fractionated according to the density of sIgM, provided another means of cell subdivision. Four subgroups were identified and named SG1–4, with increasing sIgM density from SG1 to SG4. Cells from SG1 had the highest levels of activation markers (CD25 and CD38) and the lowest capacity to be stimulated by sIgM cross-linking, determined based on protein tyrosine phosphorylation [61]. The other SGs progressively lost their activation status and regained the capacity of expressing sIgM, together with a more efficient response to sIgM cross-linking. The cells from SG4 also had the highest CXCR4 levels and were classified as "dangerous cells" based on their presumed capacity to re-enter lymphoid tissues and to re-initiate a proliferative cycle, feeding the proliferating compartment of CLL cells. Again, these distinct cell fractions were detected in both U- and M-CLL clones, albeit in different proportions. The two models present differences (not unexpected in view of the complexity of the fractionation procedures) related to the definition of the cell subsets that were responsive to BcR engagement. Nevertheless, these studies have the merit of disclosing heterogeneity within CLL clones, with cells that represent the proliferating compartment and with

others that form a sort of reservoir of “stem cells” capable of re-entering the cycle to make up for the continuous cell loss. **Figure 3** presents a hypothetical model of CLL cell turn-over based on the data described.

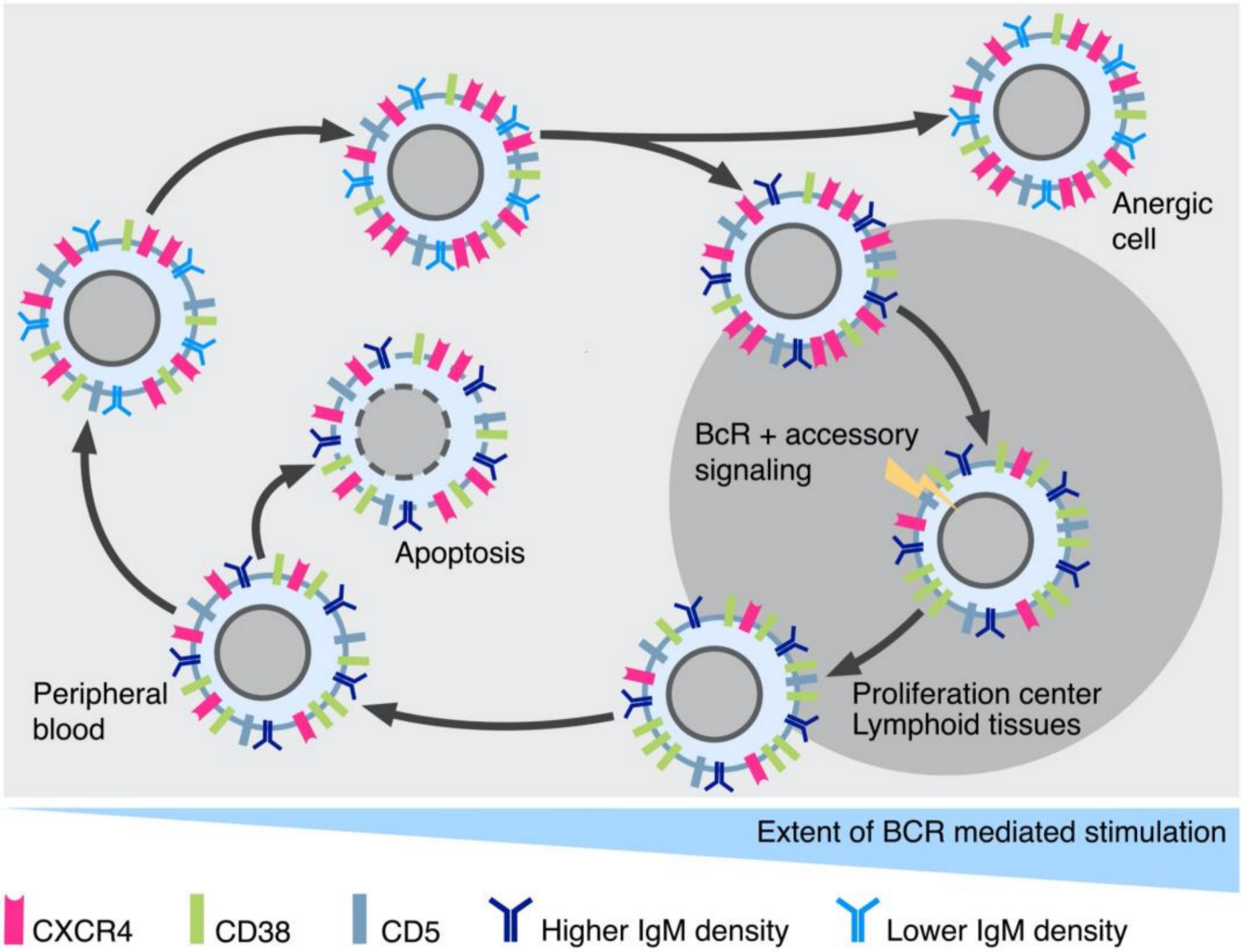

Figure 3. Hypothetical model of CLL cell turn-over. CLL cells are stimulated via BcR through the mechanisms depicted in **Figure 1** and **Figure 2**. The cells proliferate with the assistance of accessory cells and cytokines that are abundant in proliferating centers. Cell activation consequent to stimulation causes the downregulation of adhesion molecules, particularly of CXCR4, so that the cells can leave the proliferating centers and reach the circulation. Here, there is a progressive downregulation of adhesion molecules and upregulation of CD5. Concomitantly, activation molecules are downregulated, confirming the progressive tendency of CLL cells to reach a more resting state (here only CD38 is depicted among the activation molecules). A number of CLL cells undergo apoptosis outside the proliferating centers in the absence of accessory signals (this is particularly true for U-CLL cells). The CLL cells, progressively more quiescent because of the absence of stimulation, may become tolerant (particularly M-CLL cells) and/or upregulate a new set of adhesion molecules which allow re-entry into lymphoid tissues and initiate a new proliferation cycle in proliferating centers. The sIgM density varies across the different stages.

3.2. Choice between Proliferation and Apoptosis following BcR Stimulation

The stimulation of unmanipulated cells from U-CLL cases with divalent anti IgM antibodies results in survival/proliferation or apoptosis in vitro, depending on the stimulus and the presence/absence of accessory cells and/or cytokines [42][44][45][62][63]. Exposure to anti-IgM-coated beads facilitates proliferation, whereas soluble antibodies favor apoptosis. In researchers' own experience, testing highly purified CLL cells favors apoptosis, whereas the presence of minor contaminants of T cells and/or monocytes favors proliferation, indicating a pro-survival role for these accessory cells, a finding in line with the observation that purified CLL cells, particularly from U-cases, do not survive in culture for long, unless accessory cells, such as NLCs, stromal cells, fibroblasts, activated T cells and macrophages, are added [64][65][66][67][68]. Collectively, CLL cells appear to be in a precarious balance between survival and death in vitro and this condition is exacerbated by BcR stimulation alone. This situation is likely to occur in vivo as well, given the abundant apoptosis observed in patients' circulation. How the balance between survival and apoptosis is regulated is not entirely clear. *Bcl-2,* which is not mutated or translocated in CLL cells [69], is generally upregulated. This finding is in part related to del13q-, which characterizes many leukemic clones and causes the deletion of the locus encoding miR-15 and miR-16, with the function of negatively regulating *bcl-2* expression, and in part due to the activation status of CLL cells, leading to the upregulation of anti-apoptotic mechanisms [70][71][72][73]. However, many pro-apoptotic molecules, particularly those of the BH3 family alone, are also upregulated, counterbalancing the anti-apoptotic effect of the molecules of the BCL-2 family [74]. This hypothesis also is supported by the therapeutic effects observed in CLL patients with BH3 mimics, which, by inhibiting the anti-apoptotic effects of the *bcl-2* family molecules, swing the balance toward the induction of apoptosis [75]. *c-myc,* which is not mutated or translocated in CLL [69][76][77], may also contribute to the apoptosis of CLL cells since it is frequently upregulated in CLL cells, possibly because of stimulation via BcR and/or other accessory cells in vivo [78]. In addition, *c-myc* has been found to be upregulated in different murine models of CLL [79][80][81]. It has been known for a long time that *c-myc* upregulation renders cells prone to apoptosis unless anti-apoptotic mechanisms are activated [82]. For example, lymphoblastoid cell lines transfected with an upregulated *c-myc* undergo apoptosis unless signals from CD40/CD40L interactions at the cell surface upregulate the cells' anti-apoptotic mechanisms [83]. Likewise, a similar, *c-myc*-controlled imbalance is created in the centro-blasts in the germinal centers (GC), where *c-myc* upregulation following antigen stimulation renders the cell prone to apoptosis. Apoptosis is prevented by interactions with T cells facilitated by antigens, favoring high-affinity maturation [84][85][86]. Therefore, *c-myc* upregulation in CLL cells may favor apoptosis but may be counterbalanced by microenvironmental signals. Finally, CLL cells have higher-than-normal levels of radical oxygen species (ROS), possibly related to BcR engagement and/or other stimuli. These ROS act as secondary messengers, driving the leukemic cells towards proliferation or apoptosis depending upon the balance between the ROS concentration and the cell's ability to prevent ROS damage [87][88][89].

The above mechanisms investigated in vitro reveal that the outcome of cell stimulation via BcR is largely dependent on the additional help that leukemic cells receive from accessory cells and cytokines and reinforce the view that CLL clonal expansion depends on close stromal interactions. BcR engagement may possibly serve to focus this help on CLL cells.

3.3. Tolerance of CLL Cells

A considerable proportion of CLL clones show a low response to sIgM cross-linking in vitro, measured based on increased protein tyrosine phosphorylation compared to the background level, or by determining Ca^{++} mobilization, or the biological effect of stimulation. The cells with these features, defined as anergic cells, represent most circulating cells from the $CD38^{-}$, $ZAP\text{-}70^{-}$ and M IGHV CLL clones. For the sake of clarity, researchers speak of anergic cells within clones, rather than anergic clones, since apparently anergic clones also comprise a minority of CLL cells that are potentially capable of proliferating following BcR engagement, as already discussed. Anergic cells exhibit low sIgM, almost normal sIgD, elevated levels of spontaneously phosphorylated Syk and Lyn in addition to ERK1/2 and MEK1/2, and increased NF-AT phosphorylation in the absence of AKT activation [90][91][92]. Excessive Lyn phosphorylation causes the phosphorylation of the immunoreceptor tyrosine inhibitory motif of surface CD5 with subsequent docking and activation of SHP-1, with an inhibitory effect on apoptosis and on any further cell stimulation [93]. Inhibition of the phosphorylation of the molecules described above results in a restoration of BcR responsiveness, generally followed by apoptosis [90][91][92][93]. The features of the tolerant CLL are reminiscent of the B cells of mice that were transgenic for a given BcR and engineered to produce the antigen to which the BcR had specificity [94][95]. These B cells failed to respond to the “transgenic” antigen when challenged with it in vitro. The same mechanism is likely to operate in regard to tolerance to self-antigens in humans. $IgM^{-/}IgD^{+}$-tolerant cells that are potentially capable of producing self-reactive antibodies have been described in normal human circulating B cells [96]. It is not clear why tolerance is present in part of a CLL clone, although it could be ascribed to the continuous stimulation of the cells via their BcR in vivo, perhaps in the absence of the appropriate accessory cells. This tolerance can be overcome in vitro, for example, via stimulation with CpG (which engages TLR-9) or by exposing the leukemic cells to CD40L-bearing cells or via cross-ligation of surface IgD [91][95][97]. The breaking of tolerance is documented by the new capacity of the cells to respond to stimuli and subsequently proliferate. Although comparisons between in vitro and in vivo testing are always difficult, the data suggest that the tolerance of leukemic cells is more transient than that observed in normal human B cells or in the B cells from experimental animal models to self-antigens. It is possible that smaller groups of anergic cells also exist in the circulation in U-CLL cases, which may be masked by the overwhelming presence of cells responding to BcR stimulation in vitro. If this is the case, the induction of transient tolerance may operate in both U- and M-CLL cases, albeit in different proportions, and tolerant CLL B cells may constitute a reservoir of cells that are inducible to cycling when required for clonal expansion.

3.4. The Function of BcR Other Than IgM

The large majority of CLL clones express both sIgM and sIgD, which share the same L chain type and the same antibody combining site. The two isotypes are assumed to have overlapping functions, although sIgD, because of its more extended hinge region (i.e., the amino-acid sequence located between the antigen binding, or Fab portion, and the tail, or Fc portion, of the molecule), is believed to have a superior flexibility and capacity for binding certain antigens with multiple epitopes compared to the more rigid sIgM, which binds antigens with fewer repeated epitopes [98][99]. These differences present a wide range of antigen binding options for B cells, offering superior defense from pathogens.

The sIgM and sIgD density is lower in CLL than in normal B cells, a phenomenon related to in vivo endocytosis caused by chronic BcR stimulation, suggesting the participation of both isotypes to cell stimulation, although with noticeable differences. For example, tests with deuterated water in vivo demonstrated that the CLL cell birth rate (BR) correlates with the sIgM and not the sIgD density of circulating CLL cells [100][101]. This is in line with the observation that patients with the highest sIgM levels in their circulating cells have a more aggressive disease, a correlation not found for sIgD [61][100]. In normal B cells, sIgM is organized in more numerous high-density regions than sIgD [102]. Furthermore, sIgM and sIgD have different topographic relationships with membrane CD19 and CD20 co-receptor molecules during the activation of normal B cells [103]. If demonstrated for CLL cells as well, this would further stress the complementary role of the two isotypes [101][104][105]. Finally, while sIgM is efficient in eliciting autonomous signaling, sIgD is not, as shown by in vitro tests and observations in *tcl1* transgenic mice that were unable to express sIgM [101]. This possibly might occur because the recognition of internal BcR epitopes by the BcR takes place only when the epitopes of interest are on IgM and not on IgD. Alternatively, the flexibility of IgD may not allow the transduction of an effective signal when the BcR is engaged by itself [99][101][105].

The above data could indicate a predominant role of sIgM in facilitating clonal expansion, with a note of caution since most findings relate to circulating CLL cells and the correlations observed are not of univocal interpretation. Nevertheless, it has been possible to observe stimulation of CLL cells via sIgD cross-linking in vitro, although the downstream signals are more transient and less effective than those of sIgM [104][106]. These signals may be able to rescue CLL cells from anergy [91] and can drive a fraction of CLL cells into plasma cell differentiation, rather than proliferation [106], thus explaining the presence of monoclonal IgM-secreting cells in many CLL clones [23][107]. The secreted monoclonal protein may interfere with autonomous CLL signaling by preventing the interaction between the BcR and the corresponding sIgM target. An alternative and not mutually exclusive explanation may be that the secreted IgM forms aggregates with the help of the BcR itself and engages the Fcμ receptor of the CLL cells, providing additional regulatory signals [108][109]. However, the molecular mechanisms underlying this regulation must be clarified.

The stimulatory function of sIgG or sIgA, expressed by a minority of CLL cases, does not seem to differ from that of sIgM. The reason for which sIgM expression may be predominant in CLL and other lymphoproliferative disorders has already been discussed above, although it remains to be clarified why clones expressing sIgG or sIgA have a selective advantage in certain circumstances. Clones expressing certain stereotypes may provide some clues. For example, subset #4 and subset #8 stereotypes are typically associated with an IgG isotype [14]. A selection process favoring the intra-clonal emergence of sIgG-bearing cells with these stereotypes over those with sIgM molecules and the same stereotype has been reported [110]. A combination of a given stereotype with a particular isotype would be advantageous for stimulation involving certain antigens or for autonomous signaling (as apparently occurs for subset #4 stereotypes) [39], although more observations are needed.

4. Inhibition of BcR-Dependent Tyrosine Kinases (TK)

The evidence that stimulation via BcR is one of the driving forces for CLL cell expansion promoted the screening of a variety of TKIs capable of blocking BcR-dependent signaling, with potential therapeutic value. The analysis of this

research effort goes beyond the scope of this article, and we refer to the excellent ad hoc reviews presented in [111][112][113][114]. However, it is perhaps useful to add a few notes on the results obtained with widely used TKIs, such as ibrutinib and idelalisib, which covalently target the Bruton TK or the PI3K-delta isoform, respectively. These TKs are involved in the early phases of BcR signaling (see **Figure 1**) and participate in the signaling of other receptors essential for cell trafficking and homing. As is apparent from **Figure 1**, several TKs are involved in BcR signaling, some of which are located in different arms of the pathway, as is the case for Bruton TK and PI3K, raising the possibility that, in the future, different TKs can be simultaneously targeted with different specific drugs, although this strategy will have to be confronted with the issue of the amplification of adverse events [115].

Treatment with TKIs is followed by lymphoid tumor shrinking and progressive disease remission. This effect is more rapid in U-CLL cases, although clinical remission is also observed in most M-CLL cases [116][117][118][119], which is consistent with the higher hierarchical role of the BcR in promoting U-CLL cell expansion, but which also confirms a role for BcR in M-CLL. TKIs induce peripheral lymphocytosis at the start of therapy because of the redistribution of leukemic cells from lymphoid compartments to the circulation, due to many effects, including the inhibition of BcR signaling induced by exogenous or self-antigens, of autonomous signaling and of signaling from other surface structures, such as CXCR4, that contribute to the retaining of CLL cells in tissues. Notably, lymphocytosis observed following TKI administration is established more rapidly in U-CLL than in M-CLL, although it persists for a longer time in M-CLL [117]. This is consistent with the more effective role of BcR in U-CLL expansion but is also possibly related to a recently described effect of myristoylated alanine-rich C-kinase substrate or MARCKS, which is more expressed in M-CLL cells than in U-CLL cells [120]. Since the role of MARCKS is that of partially inhibiting BcR signaling by reducing BcR clustering, this observation contributes to explaining why M-CLL, receiving less effective stimulation via BcR, may be less susceptible to TKI.

When moving to the circulation under the effect of TKIs, the leukemic cells meet with conditions which are not optimal for a survival/proliferation and progressively die. Circulating CLL cells from patients treated with ibrutinib present the upregulation of surface IgM (compared to pre-treatment levels), which is not followed by an equal up-regulation of surface IgD levels [120][121][122]. Disengagement of the surface BcR caused by treatment is likely to reinstate the pre-stimulation conditions of CLL cells and is particularly evident at the level of surface IgM, the most frequently engaged isotype [121]. CD20, another molecule participating in BcR stimulation, is downmodulated following treatment with TKIs, which also induce a downregulation of CXCR4, that is responsible for leukemic/stromal cell interactions in tissues. Downregulation of CXCR4 facilitate the cells' exit from the circulation and affects the production of CXCL12 by stromal cells. Reduced signaling by CXCL12 is responsible for the lowering of CD20 [123].

The effectiveness of TKIs is reinforced by the observation of disease re-expansion in patients who have developed BTK or PLCγ2 gene mutations and acquired TKI resistance. The former mutations prevent the binding of TKIs, such as ibrutinib, to the Bruton TK, whereas the latter confer independence from Bruton TK to the BcR's signaling [117][124][125].

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