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REVIEW ARTICLE

Lymphoma

New roles for B cell receptor associated kinases: when the B cell is not the target

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Abstract

Targeting of B cell receptor associated kinases (BAKs), such as Bruton's tyrosine kinase (BTK) or phosphoinositol-3-kinase (PI3K) delta, by specific inhibitors has revolutionized the therapy of B lymphoid malignancies. BAKs are critical signaling transducers of BCR signaling and seem relevant in B cell lymphoma pathogenesis. The functional relevance of BTK for lymphoid malignancies is strongly supported by the observation that resistance to therapy in CLL patients treated with BTK inhibitors such as ibrutinib is often associated with mutations in genes coding for BTK or Phospholipase-C gamma (PLCγ). In some contrast, next generation sequencing data show that BAKs are mutated at very low frequency in treatment-naïve B cell lymphomas. Therefore, it remains debatable whether BAKs are essential drivers for lymphoma development. In addition, results obtained by targeted deletion of BAKs such as Lyn and Btk in murine CLL models suggest that BAKs may be essential to shape the dialogue between malignant B cells and the tumor microenvironment (TME). Since BAKs are expressed in multiple cell types, BAK inhibitors may disrupt the lymphoma supportive microenvironment. This concept also explains the typical response to BAK inhibitor treatment, characterized by a long-lasting increase of peripheral blood lymphoid cells, due to a redistribution from the lymphoid homing compartments. In addition, BAK inhibitors have shown some efficacy in solid tumors, probably through mediator cells in the TME. This review summarizes and validates the evidence for BAK inhibitors being part of a class of agents that modulate the (hematopoietic) microenvironment of cancers.

Introduction

Non-Hodgkin lymphoma, of which around 93% derive from B cells, comprise the most common hematologic malignancy with estimated 73,000 new cases and 20,000 deaths in the US in 2016 [1]. Most common subtypes include chronic lymphocytic leukemia (CLL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL) and mantle cell lymphoma (MCL). Within B cell lymphoma, aggressive and indolent lymphoma with unique biological and clinical characteristics can be distinguished.

Indolent B cell lymphomas are usually not curable. In recent years, tremendous progress regarding the therapy of B lymphoid malignancies has been achieved by introducing small molecule kinase inhibitors, which were believed to target kinases associated with the B cell receptor (BCR) [2, 3]. The development of drugs inhibiting BCR associated kinases (BAKs) comprising LYN, SYK, BTK and PI3K was inspired by the concept that BCR signaling is a key pathway for the pathogenesis of B cell lymphoma [4]. BAKs are localized in proximity of the BCR and form a signalosome complex upon BCR activation.

More recent evidence suggests that therapeutic effects of BAK inhibitors are not only mediated by direct targeting of the malignant B cell population but also by disrupting the bi-directional dialogue of tumor cells with the microenvironment. Accordingly, inhibitors such as idelalisib or ibrutinib were shown to interfere with additional kinases in various cell types of the lymphoid microenvironment. Moreover, both inhibitors have shown

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some efficacy against solid tumors or in other diseases [5–10]. These observations illustrate that the precise mechanisms of action and the definitive cellular targets of inhibitors known to target BAKs are not fully understood. This incomplete mechanistic understanding of these highly active drugs has inspired the authors to compile the current evidence regarding the role of BAKs and their inhibitors for modulating the tumor microenvironment (TME).

Role of BAKs in lymphoma pathogenesis

BAKs are critical components of the BCR signaling pathway

BAKs are crucial signaling transducers that play a key role for B cell maturation, as well as for the initiation and progression of B cell lymphoma. BAKs are activated by binding of antigen to the BCR. Upon antigen ligation, the BCR becomes rearranged and translocated into lipid rafts, initiating the binding of the BCR to LYN that is densely located within these rafts [11, 12]. LYN phosphorylates the Ig heterodimers of the BCR, leading to the recruitment and phosphorylation of SYK [13]. Thereafter, activated LYN and SYK induce the formation of the BCR signalosome leading to the phosphorylation and activation of SYK, BLNK, BTK, and PLCγ. The BCR signaling complex is anchored to the raft by LYN and additional adaptors such as CBP/PAG [14, 15]. Activation of LYN is also one of the earliest events that facilitate the phosphorylation and recruitment of PI3K to the BCR complex at the plasma membrane [16]. Together, these early events trigger a signaling cascade, which in turn activates a series of kinases and pathways including MAPK/ERK, AKT/mTOR, PKCβ, BCL10/CARD11/MALT1 and the mobilization of calcium ions. As a result, BCR signaling may initiate transcription processes via transcriptional factors such as NF-κB, MYC, and NFAT. Depending on the nature of the BCR stimuli (tonic or antigen-depending), on the duration and strength of BCR activation and subsequence calcium release, signaling through the BCR may result in the specific activation of one of these transcriptional factors, thus leading to distinct outcomes such as B cell proliferation, differentiation or apoptosis [3, 4, 17, 18]. Among these, LYN modulates the signal strength and responsiveness of BCR signaling pathway via activation of phosphatases and inhibitory signaling pathways [19]. This function distinguishes LYN from other SRC kinases or from SYK. LYN activates phosphatases such as SHIP1 or SHP1/2, which subsequently inactivate substrate proteins such as BTK, thereby maintaining BCR signaling homeostasis [20].

BAK expression and activation is relevant in B cell lymphoma

Alterations of the BCR cascade, particularly regarding the expression and activation status of BAKs are common events in B-lymphoid malignancies as highlighted by studies using gene expression profiling (GEP) and high-throughput proteomics: In MCL, several kinases including SYK, PI3K, LYN, and BTK are highly active in primary tissues and cell lines [21–26]. Activation of these BAKs correlates with NF-κB activity and is linked to the proliferation rate of MCL cells [27]. Similarly, the NF-κB pathway is constitutively active in activated B cell type of DLBCL (ABC-DLBCL) [28]. Knockdown experiments in cell lines showed that BTK is an important pro-survival factor in ABC-DLBCL [29]. Recently, studies using phospho-proteomics identified a crucial role of BCR signaling for the survival of Burkitt lymphoma (BL) cells, with highly phosphorylated levels of LYN, SYK, PI3K, and PLCγ [30]. Constitutive phosphorylation of LYN, SYK, PKCβ, BTK, and PI3K has also been described in CLL, followed by enhanced expression and activation of NF-κB upon interaction with microenvironmental stimuli [31–35]. Furthermore, comparative GEP data revealed BCR signaling as the most activated pathway in proliferation centers of CLL lymph nodes [36, 37].

In addition to this experimental evidence, there is also *clinical evidence* to support the notion that BCR signaling is essential for lymphoid malignancies. The mutational status of *IGVH* gene shows prognostic relevance in CLL [38, 39]. Moreover, the existence of a restricted repertoire of BCRs in certain lymphoma subtypes suggests a role of specific antigens during lymphomagenesis. Stereotyped groups of BCR have been demonstrated in several types of lymphoma including CLL, marginal zone lymphoma (MZL), and DLBCL with indications of clinical correlation [40–42]. Cellular expression of ZAP70, a receptor associated tyrosine kinase that enhances SYK activation, also correlates with the signaling capacity of the BCR to promote survival and migration of CLL cells [43, 44]. Collectively, the activation of BAKs and NF-κB seems to play a key role for B cell lymphoma pathogenesis.

Inhibition of BAKs is efficient in B cell lymphoma

In light of the above-described role of BAKs for B cell lymphoma pathogenesis, BAK inhibitors were developed and tested in clinical trials. These inhibitors showed impressive clinical efficacy leading to the approval of the first in-class drug ibrutinib, an inhibitor of BTK [45]. Additional inhibitors are now being developed. A detailed review of these drugs is beyond the scope of this paper. Instead, we briefly summarize the clinical activity and

effects on malignant B cells of some clinically relevant BAK inhibitors.

Ibrutinib inactivates BTK and is currently the most broadly used inhibitor in B cell lymphoma. It is licensed for frontline and second line treatment of CLL, treatment of relapsed or refractory (R/R) MCL, pretreated, rituximab-refractory Waldenström's macroglobulinemia (WM) and MZL [2]. In addition, ibrutinib also showed some efficacy in ABC-DLBCL [46]. In FL, single agent ibrutinib showed only moderate activity [47]. Finally, ibrutinib showed some efficacy in primary CNS lymphoma [48]. As ibrutinib inhibits multiple alternative targets such as EGFR that potentially compromises its therapeutic index, second-generation BTK inhibitors such as acalabrutinib have been developed that are currently compared to ibrutinib in a randomized protocol in previously treated CLL patients (NCT02477696) [49, 50].

With regard to the direct effects of ibrutinib on B cell lymphoma cells, peripheral blood CLL and MCL cells showed decreased ex vivo proliferation when analyzed longitudinally during treatment [35, 51]. This is in line with in vitro data obtained with some CLL and MCL cell lines cultured in the presence of clinically relevant ibrutinib concentrations [25, 52]. However, the apoptotic rate of peripheral blood CLL cells ex vivo or in vitro was not significantly increased under ibrutinib treatment [35, 52]. Similarly, MCL cell lines underwent apoptosis only when treated with very high concentrations of ibrutinib [25]. Direct in vivo measurements with isotopic labeling revealed that ibrutinib has immediate effect on CLL cell proliferation and death rate, with cell death rates being higher in tissues than in the blood. Ibrutinib was also shown to inhibit BCR and NF-κB signaling in lymph node and bone marrow resident CLL cells, suggesting that the effects of ibrutinib may depend on the analyzed compartment [53, 54]. Taken together, the short-term clinical effects of ibrutinib in CLL patients are characterized by a rapidly reduced lymphadenopathy accompanied by long-lasting lymphocytosis [45].

A particularly relevant observation that supports the importance of BTK signaling in neoplastic B cells relates to the acquisition of mutations conferring resistance, such as BTK-C481S, under prolonged therapeutic ibrutinib use in CLL patients with high-risk genetic features [55]. Together with PLCγ mutations these BTK mutations represent the most frequent mechanism of ibrutinib resistance and underscore the relevance of the effects of ibrutinib on BAKs in B-CLL cells [56].

The selective PI3Kδ inhibitor *idelalisib* is approved for the treatment of relapsed or high-risk CLL (only in Europe), relapsed FL and small lymphocytic lymphoma (SLL) [2, 57]. Idelalisib showed modest activity in heavily pretreated MCL, but seems to have significant activity in R/R WM and MZL, although there is only limited experience [2, 57]. Idelalisib promotes apoptosis in primary CLL and multiple myeloma (MM) cells, and inhibits DLBCL, FL and MM cell lines at clinically relevant concentrations in vitro, and human MM cell growth in vivo (murine xenograft model) [58–60]. In primary CLL cells, the activation of the PI3K pathway through CD40L, TNFα, fibronectin and BCR activation can be inhibited by idelalisib [58, 59, 61].

Dasatinib is a second generation SRC/ABL inhibitor that inhibits LYN and other SRC family kinases. Dasatinib inhibits the survival and proliferation of CLL, DLBCL and MCL primary cells and cell lines in vitro [62–65]. CXCR4 signaling and CXCL12 migration is reduced by dasatinib in primary CLL cells ex vivo [66]. ERK activation and calcium flux of CLL cells from ibrutinib resistant patients harboring PLCγ mutations can be blocked by dasatinib in vitro [67]. However, dasatinib showed only modest efficacy in R/R or fludarabine-resistant CLL [68, 69]. Due to its relevant adverse effects and the advent of more efficient kinase inhibitors, dasatinib does not play a substantial role in B lymphoma therapy.

The competitive, second generation SYK-inhibitor *entospletinib* showed efficacy in R/R FL, lymphoplasmacytoid lymphoma, MZL, MCL,and CLL [2, 70, 71]. The combination of entospletinib with idelalisib led to severe pneumonitis in a phase II study in B cell lymphoma [72]. Entospletinib treatment inhibits proliferation of BCR-dependent DLBCL cell lines [73]. It increases apoptosis and reduces chemokine secretion in primary CLL cells in vitro [71, 74, 75]. Entospletinib also down-regulates MCL1 inducing CLL cell death in vitro [76].

How relevant are BAKs for B cell lymphoma initiation?

BAK mutations are rare events in de novo B cell lymphoma

Data obtained through extended next generation sequencing allowed to create a genomic landscape of most B cell lymphoma. Remarkably, the results shed some doubt regarding a dominant functional role of BAKs in lymphoma pathogenesis. In CLL, whole-exome sequencing (WES) of leukemia cells in the largest cohort of patients investigated so far has revealed an array of diverse, recurrent driver mutations [77]. Interestingly, this comprehensive analysis of the mutational landscape did not detect mutations of genes coding for BAKs, suggesting that BAK mutations do not function as dominant drivers of CLL initiation [77, 78]. Similarly, a comprehensive WES of MCL did not allow to detect any BAK mutation [79, 80]. In ABC-DLBCL, although mutations of genes coding for proteins acting

downstream of the BCR were identified as putative driver mutations, such as *CARD11* or *CD79a/b*, leading to chronic BCR signaling and constitutive NF-κB activation [81]. But again, no mutations of BAK genes were found in ABC-DLBCL [82]. It is reasonable to assume that activation of BAKs in *CARD11*-mutated DLBCL becomes irrelevant due to a constitutive downstream-activation of NF-κB [29]. In summary, BAK mutations are not found in the majority of B cell lymphoma cases. Instead, it can be speculated that tonic or chronic activation, or autonomous pathway of the BCR functions as an oncogenic driver at the protein level in some lymphoma, for example in BL and CLL, thereby reducing the need to create BAK mutations [83–85]. Alternatively, it cannot be excluded that due to the redundant influx of various signals into B cells during the initiation of lymphomagenesis, signaling of specific BAKs may be readily bypassed and not be essential for this process.

Lymphoma initiation occurs in the absence of BAKs in vivo

The use of genetically engineered murine model systems (GEMMS) represents a powerful tool to determine the functional roles of oncoproteins. Consequently, numerous GEMMS have been generated recently to gain insights regarding the role of BAKs in B cell lymphoma.

The *Eμ-TCL1* mouse is one of the most frequently used models for CLL. *Eμ-TCL1* transgenic mice develop a CLL-like disease with 100% penetrance after a few months [86]. We and others have crossed *Eμ-TCL1* mice with different mouse strains that are deficient for BAKs such as *Lyn, Btk, Pkc-β*, or Nf-κb signaling such as *p50-NF*. In all these experiments, a significantly delayed onset and reduced burden of leukemia was observed [87–90]. Interestingly, despite the clear delay in disease progression, some BAK-knockout *Eμ-TCL1* mice still developed CLL in the complete absence of these kinases, suggesting that CLL cells may grow in vivo without a fully functional BCR signaling or even without Nf-κb. Recently, we could demonstrate that in the absence of Lyn and despite a strongly reduced activity of Syk and Btk activity in B cells, CLL cells could still transform and proliferate efficiently [87]. Even in a complete knockout of Btk, some *Eμ-TCL1* mice still develop fatal CLL (own unpublished data). Apparently, B cells can exploit BCR-independent signaling pathways to induce the full picture of overt CLL in this mouse model. Together, the results from these animal models and WES data from big patient cohorts challenge the current concept that the activation of BAKs in B cells is of essential importance for the initiation and survival of all lymphoma, and in particular for indolent lymphoma such as CLL.

A new role for BAKs in the lymphoma microenvironment

The absence of BAKs leads to a dysfunctional tumor microenvironment

Until recently, studies on the roles of BAKs in promoting B cell lymphoma progression were strongly focusing on the intrinsic signaling within malignant B cells. As a consequence, these studies partially neglected that most BAKs are also widely expressed in a broad variety of other cell types, both in the hematopoietic and in the non-hematopoietic compartment. For example, LYN is expressed almost ubiquitously in all hematopoietic cell lineages except T cells [20]. The important function of LYN has been extensively studied in cells of the immune system. Moreover, the role of LYN in non-immune cells has also been recently recognized in several diseases, rendering LYN kinase a relevant player in the tumor microenvironment [20]. Similarly, SYK is highly expressed in all hematopoietic cells, and also in additional cell types such as fibroblasts, endothelial cells, hepatocytes and neuronal cells [91, 92]. All isoforms of PI3K are also ubiquitously expressed, with the δ and ɣ isoforms being highly enriched in the hematopoietic compartment [93, 94]. BTK is broadly expressed in hematopoietic cells, with the exception of T cells and plasma cells [95, 96].

Given the broad expression and function of BAKs in many tissues, studies using BAK inhibitors need to consider their effects in a rather wide spectrum of tissues, instead of focusing on B cells alone. This view has been supported by a recent series of experiments, where CLL cells from *Eμ-TCL1* mice were adoptively transplanted into BAK-knockout mice. These experiments revealed that BAKs may be crucial for the formation of the leukemic niche that fosters CLL development. In experiments where leukemic cells were transplanted into wild type versus *Lyn-* or *Btk*-deficient mice, the absence of Lyn or Btk in recipient mice significantly delayed leukemic progression (Fig. 1), leading to prolonged survival of the knockout recipient mice. This effect was explained in part by the reduced capacity of *Lyn*-knockout macrophages and fibroblasts, and of Btk-knockout macrophages to support the survival of primary CLL cells (own unpublished data and [87]). Lyn-mediated support from macrophages to leukemic cells was dependent on direct cell-cell contact but not on humoral factors [87].

In a similar way, the Pkcβ/Nf-κb axis in bone marrow stromal cells (BMSC) represents an important component of a supportive TME for CLL cells. Again, a *Pkc-β* knockout mice failed to support the growth of TCL1-induced leukemic cells [97]. Moreover, the overexpression of PKCβII in stromal cells was induced upon interaction with various malignant B cells such as CLL, ALL and MCL, illustrating

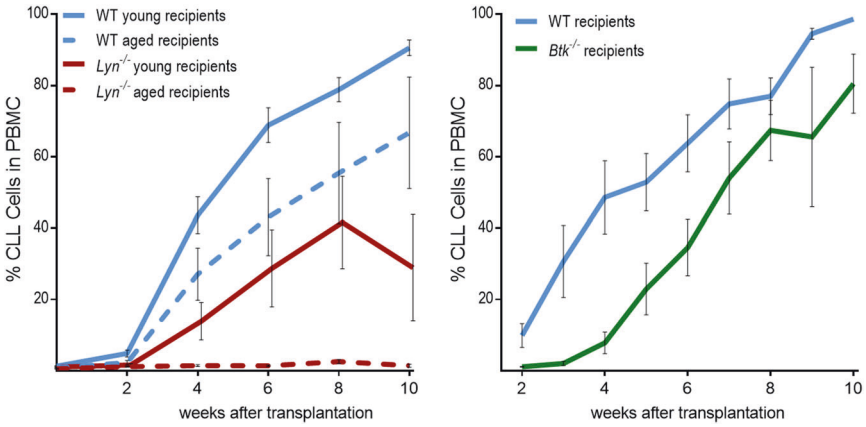

Fig. 1 Lyn and Btk deletion in the microenvironmental cells hindered CLL progression in vivo. Flow cytometric analysis of CLL ($CD5^+$ $CD19^+$) development in the peripheral blood of wildtype (WT) versus *Lyn*-knockout (left) and *Btk*-knockout (right) mice after transplantation of CLL cells. CLL progression was significantly delayed in *Lyn*-knockout and *Btk*-knockout recipients compared to WT counterparts. Eμ-TCL1 donors spleens containing 80–90% CLL cells were injected intraperitoneally into at least two recipients of each genotype. Blood samples were taken from recipient mice every two weeks from the tail vene and subjected to flow cytometry analysis to determine CLL burden. Data represent Mean ± SEM. (Modified from ref. [87])

the bi-directional interplay between tumor and microenvironmental cells.

These findings have so far not been broadly reproduced in other B cell lymphoma. However, our own work using the *Eμ-Myc* lymphoma model suggests that *Lyn*-deficient mice also fail to fully support the growth of aggressive lymphoma [87]. Since B cell lymphoma may depend on interactions with the TME at different levels, future studies of BAK-deficient hosts should use additional B cell lymphoma models to further validate these findings [98–100].

BAK inhibitors exert substantial effects on the lymphoma microenvironment

As described above, the class effect of BAK inhibitors in B cell lymphoma treatment is not caused by a rapid, efficient tumor killing by disrupting essential proliferative pathways, but rather by a slow redistribution of malignant cells from lymph nodes to the blood or marrow [101, 102]. From all B cell lymphoma, the most thorough experimental and clinical observations have been obtained in CLL. In fact, ibrutinib and idelalisib show very modest cell killing activity when treating malignant B cells with these reagents in vitro [35, 52, 58, 103–105]. In comparison, dasatinib that also potently inhibits BTK, induced significant cell killing and apoptosis in vitro, potentially by targeting a wide spectrum of proteins [62, 106]. Interestingly, we recently demonstrated that dasatinib-induced apoptosis in B cells was independent of LYN or BTK, as shown by a chemico-genetic strategy in B cell lines engineered to harbor *LYN*-gate-keeper (GK) and *BTK*-GK mutations with a greatly reduced binding to dasatinib. These *LYN-GK or BTK-GK* carrying B cells did not show relevant differences in survival and metabolic activity when exposed to dasatinib treatment [107]. Similarly, in B cell lines transfected with the ibrutinib-resistant *BTK-C481S* mutation or wild type *BTK*, incubation with ibrutinib did not change the cellular rate of apoptosis or cell growth, but altered the capacity to secrete cytokines such as CCL3, CCL4, and TNFα [107]. Therefore, a major function of BTK seems to establish the dialogue of B cells with the microenvironment rather than the activation of cell proliferation or the direct inhibition of apoptotic pathways. In agreement with these in vitro experiments, serum levels of CCL3, CCL4, and TNFα are decreased in CLL patients treated with ibrutinib [108].

Moreover, ibrutinib inhibits migration of CLL and MCL cells to chemoattractants such as CXCL12 and CXCL13 in vitro [4, 17, 51]. Similarly, idelalisib inhibits CLL cell chemotaxis towards CXCL12 and CXCL13 and decreases migration beneath mesenchymal stroma cells (MSC), a process called pseudoemperipolesis [103].

Some of the effects of BAK inhibitors are caused by effects on cells of the TME, which express the specific target kinases. For example, BAKs are highly expressed in *macrophages*. Macrophages seem to function as nurse like cells (NLC) for CLL cell survival and proliferation. In CLL patients, macrophages exhibit a M2-like phenotype and are found in lymphoid organs [105]. A study with bone marrow-resident macrophages from CLL patients showed a decrease of direct macrophage-CLL cell interaction during ibrutinib treatment [108]. In some contrast, survival of freshly isolated CLL cells could be rescued by NLCs when co-cultured under addition of ibrutinib in vitro [109, 110].

Macrophages also express PI3Kδ, and idelalisib impaired the phagocytosis of rituximab-coated CLL cells by human macrophages [52, 111]. A similar phenomenon was observed for entospletinib [112].

Analysis of ibrutinib-treated patients revealed that the inhibition of BTK and VLA4-dependent adhesion of CLL cells to *stroma cells* might serve as another mechanistic explanation for the detachment of leukemic cells from lymphoid homing organs [113]. Idelalisib also inhibits VLA4 and VCAM1 mediated interaction of CLL cells with different cell lines of endothelial and BMSC origin. Idelalisib decreases the pro-survival signals provided by these cells in vitro [114].

The *T cell* compartment is highly dysfunctional in CLL patients [105]. The dialogue CLL cells with T cells creates a state of immune suppression [115]. T cells express ITK, which is inhibited by ibrutinib [116]. In vitro, ibrutinib provides a selective advantage for Th1 and $CD8^+$ T cells compared to Th2 cell depletion, which represent potential anti-tumor responses [116]. During ibrutinib treatment, the expression of PD-1 is decreased on peripheral blood $CD4^+$ and $CD8^+$ T cells, while the skewed Th17 cell fraction becomes normalized [108, 117, 118]. In CLL patients, the diversity of the T cell repertoire increases significantly after one year of ibrutinib therapy, resulting in a lower rate of infections [119]. Long et al. reported changes of T cell subsets in CLL patients receiving ibrutinib: $CD4^+$ and $CD8^+$ T cell numbers increased, more differentiated subsets such as T effector memory cells were expanded, the ratio of CLL-promoting T regulatory cells was decreased and there was no reduction of the absolute number of naive T and T central memory cells [118]. Idelalisib also decreases the production of inflammatory cytokines in T cells that may enhance survival of CLL cells [58].

The very interesting interactions of BTK inhibition with T cell functions are starting to enter clinical trials. For example, as synergistic effects of ibrutinib with PD-1/PD-L1 inhibition were observed in preclinical studies, ibrutinib is currently tested in combination with the PD-1 antibody nivolumab in CLL (NCT02420912) [10]. Moreover, ibrutinib is also explored in combination with chimeric antigen receptor- (CAR-) T cell therapy, one of the most promising new strategies for lymphoma therapy. In a phase I/II study with 24 high-risk CLL patients of whom most were ibrutinib-resistant, CAR-T cell therapy was highly effective [120]. Interestingly, prolonged ibrutinib treatment enhanced the generation of effective CAR-T cells from CLL patients ex vivo [121]. Preliminary data on a pilot trial of anti-CD19 CAR-T cells in combination with ibrutinib in R/R CLL (NCT02640209) showed a complete bone marrow remission in all patients [122]. These results demonstrate the potential of using ibrutinib as a modifier of T cell function in the clinical setting.

NK cells show reduced effector functions in CLL [105]. BTK and ITK play important roles in NK cell maturation and activation [123]. Likewise, PI3Kδ-specific signaling plays a key role in NK cell maturation and cytokine production [123]. Ibrutinib and idelalisib do not seem to affect the viability of NK cells [58, 124]. However, ibrutinib is counteracting the anti-CD20 antibody induced Fc receptor (FcR) stimulation of NK cells, as well as the antibody-dependent cell-mediated cytotoxicity (ADCC) of NK cells directed against CLL cells [52, 125]. Similarly, in whole blood assays in CLL patients, ibrutinib strongly inhibited obinutuzumab-induced NK cell degranulation [52]. In contrast, idelalisib did not decrease rituximab-induced NK cell degranulation and cytotoxicity [124]. Idelalisib also decreased NK cell production of IFNγ when co-incubated with alemtuzumab but not rituximab [58, 124].

Taken together, treatment with drugs such as ibrutinib, idelalisib, or entospletinib induces substantial changes in various hematopoietic cell types that have functional relevance for the creation of the neoplastic niche for lymphoid tumors (Table 1).

BAKs in the tumor microenvironment are relevant targets in solid cancers

One of the interesting aspects regarding the modulation of non-malignant cells by BAK inhibitors is to provide potential therapeutic options in malignancies other than B cell lymphoma. Several recent, clinical and non-clinical observations support this idea.

Dasatinib was shown to exert a bone-protective effect in preclinical studies, which may be therapeutically useful in osteolytic bone metastasis or MM bone disease [126, 127].

Mast cell infiltration and degranulation in the TME correlates with tumor progression in murine models of pancreatic tumors such as insulinoma or pancreatic ductal adenocarcinoma (PDAC) [6, 128]. Interestingly, BTK inhibition with *ibrutinib* led to vasculature collapse and tumor regression in insulinoma and to an anti-fibrotic effect in PDAC, where it synergized with conventional chemotherapy to prolong survival [6, 7, 128]. These observations may be mechanistically explained by ibrutinib-mediated BTK-inhibition in B cells and macrophages, and by the restoration of T cell dependent anti-tumor responses against PDAC [8].

Myeloid-derived suppressor cells (MDSCs) are critical contributors to tumor evasion of immune responses in a variety of solid tumors [9]. MDSCs from murine carcinoma cell lines and primary metastatic melanoma patients express BTK and are inhibited by ibrutinib [9]. Inhibition resulted in reduced MDSC frequency in melanoma-bearing mice in vivo, reduced MDSC function in metastatic melanoma patient cells in vitro, improved $CD8^+$ T

Table 1 BAK inhibitors target TME cell types

BAK inhibitor	a) Possible targets b) Cell type	Disease
Ibrutinib	a) BTK b) NLCs [108–110], bone marrow macrophages [108]	CLL in vitro [109, 110], ex vivo [108]
Ibrutinib	a) ITK b) T cells [108, 116–119]	CLL in vitro [116], ex vivo [108, 116–119]
Ibrutinib	a) HCK, FGR, LYN, BTK b) monocyte-derived macrophages [52, 111]	CLL in vitro [52, 111]
Ibrutinib	a) Btk b) mast cells [128]	Mouse insulinoma in vivo [128]
Ibrutinib	a) Btk b) macrophages, B cells [8]	Mouse PDAC in vivo and in vitro [8]
Ibrutinib	a) not yet defined b) mast cells [6]	mouse xenograft PDAC in vivo [6]
Ibrutinib	a) BTK/Btk b) MDSC [9]	Mouse melanoma in vivo, human melanoma *ex vivo*, mouse mammary carcinoma ex vivo [9]
Ibrutinib (in combination with PD-L1 antibody)	a) Itk b) T cells [10]	Mouse lymphoma in vivo, mouse mammary and colon cancer *in vivo* [10]
Idelalisib	a) PI3Kδ b) monocyte-derived macrophages [52]	CLL in vitro [52]
IC87114	a) PI3Kδ b) T cells [131]	Mouse thymoma in vitro [131]
Entospletinib (+Fostamatinib)	a) LCK b) T cells [112]	CLL in vitro [112]
Dasatinib	a) PDGFRβ, c-SRC and c-KIT b) osteoblasts generated from mesenchymal stem cells [127]	Human multiple myeloma ex vivo [127]

cell proliferation in mammary carcinoma-bearing mice in vitro and reduced tumor growth when combining with PD-L1 checkpoint blockade in these mice in vivo [9]. In this context, synergy of ibrutinib with PD-L1 blocking antibodies was reported in mouse models of lymphoma, breast and colon cancer, most probably due to inhibition of Itk on T cells and in a Btk-independent manner [10]. As a consequence, clinical trials using ibrutinib in different solid tumors such as refractory melanoma, advanced pancreatic or mammary cancer are underway (summarized in ref. [7]).

Activation of PI3K signaling cascades is one of the most common events in human cancers [129]. Importantly, the PI3K family comprises several regulatory and catalytic subunits, of which only the p85α-p110δ heterodimer is part of the BCR signalosome [104, 129]. PI3K are essential components of various signaling pathways in malignant and non-malignant tissues, and effects of PI3K inhibition on non-malignant compartments as well as on diverse cancers have been described [5, 130]. Accordingly, targeting of the α-subunits, β-subunits, or ɣ-subunits of PI3K in TME cells has shown antitumor effects in various solid tumors [5, 129]. Interestingly, it was also shown that a *Pi3k-δ* knockout or inhibition modulates regulatory T cells and MDSC in murine breast cancer and thymoma models [131]. However, the use of idelalisib as a TME modifier has not been explored.

Future prospects

An increased understanding of the different targets of agents that were originally introduced as inhibitors of BCR signaling may allow to redesign their mode of action, as well as their clinical use. It is certain that these inhibitors do not only act upon B cells but also on many other cell types in hematopoietic and non-hematopoietic compartments. Their potent effects on the TME allows to explore their use in different diseases, even in solid cancers. Their mechanism of action also calls for designing more *efficient combination* therapies that use the synergy between effects on tumor cell adhesion or migration and on direct cell killing. Beautiful examples for this cooperation are the recent use of ibrutinib with obinutuzumab

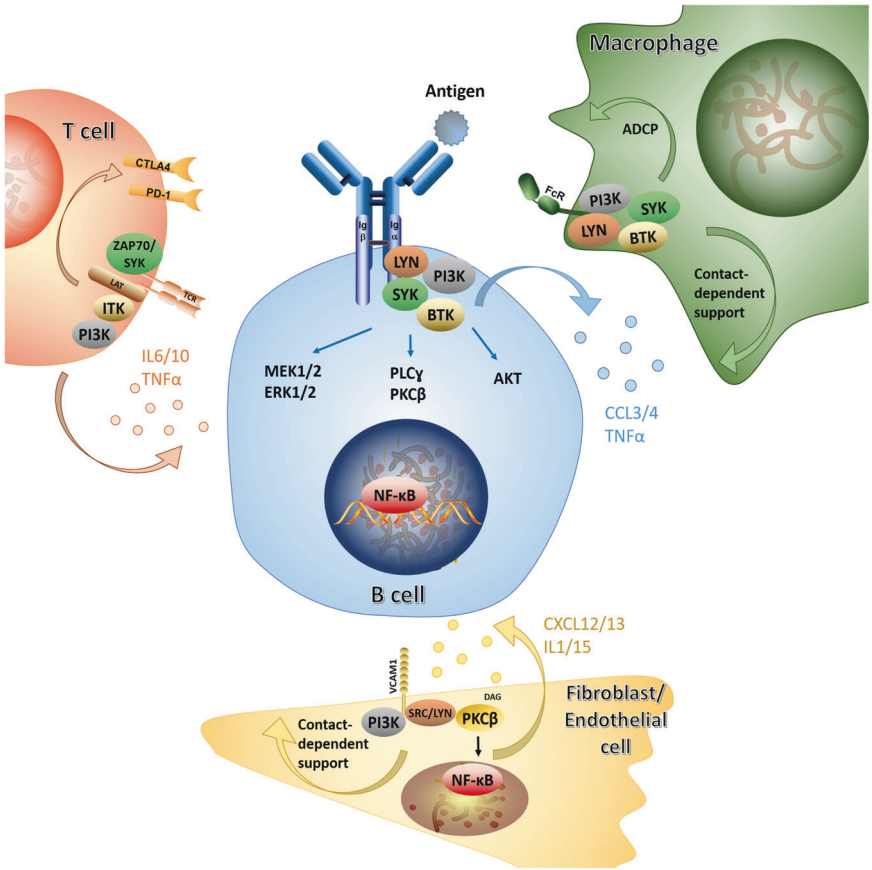

Fig. 2 Roles of BAKs (LYN, SYK, BTK, PI3K) in different cell types in B cell lymphoma and effects of their inhibition. In *B cells*, BAKs are rapidly phosphorylated and activated upon antigen binding to the BCR, leading to the formation of a signalosome in proximity of the cell membrane. Signal transduction by BAKs activates several downstream pathways, ultimately leading to the enhanced activity of transcriptional factors such as NF-κB that are critical for malignant transformation [2, 3, 17]. Activation of BAKs is also crucial for the secretion of chemokines such as CCL3, CCL4 and TNFα, which play substantial roles in the bi-directional dialogue between lymphoma B cells and the TME [107]. In the *myeloid compartment, particularly in macrophages*, BAKs are highly expressed and transduce signals from different receptors (FcR is depicted as an example). Pharmacological inhibition of BAKs either results in the loss of direct contact between macrophages and lymphoma cells or alters the antibody-dependent cell-mediated phagocytosis (ADCP) capacity [52, 108, 111, 112]. Specific knockout of Lyn and Btk in macrophages reduces the nursing capacity of this cell type in supporting malignant cell survival [87]. BAK inhibition decreases CXCL12/13-induced migration of malignant cells towards *stroma fibroblasts*, as well as affects adhesion of lymphoma cells to *endothelial cells* [4, 17, 51, 103]. Moreover, PKCβII accumulation in stroma cells leading to activation of NF-κB is important for providing support to malignant B cells, partially due to the production of IL1 and IL15 [97]. BAKs also transduce signals in *T cells* (TCR and LAT are depicted as examples). BAK inhibition in *T cells* can normalize the usually skewed T cell subsets and reduce the production of inflamatory cytokines and can synergistically enhance the efficacy of immune therapy [58, 108, 118, 121, 122]

or venetoclax, yielding very encouraging therapeutic results [132]. Taken together, we anticipate a broader application of BAK inhibitors to exploit their potential to shape the TME in a beneficial, therapeutic way both in lymphoid (Fig. 2) and non-lymphoid malignancies.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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