REVIEW ARTICLE

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Targeting the tumor microenvironment in chronic lymphocytic leukemia

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ABSTRACT

The tumor microenvironment (TME) plays an essential role in the development, growth, and survival of the malignant B-cell clone in chronic lymphocytic leukemia (CLL). Within the proliferation niches of lymph nodes, bone marrow, and secondary lymphoid organs, a variety of phenotypically and functionally altered cell types, including T cells, natural killer cells, monocytes/macrophages, endothelial and mesenchymal stroma cells, provide crucial survival signals, along with CLL-cell-induced suppression of antitumor immune responses. The B-cell receptor pathway plays a pivotal role in mediating the interaction between CLL cells and the TME. However, an increasing number of additional components of the multifactorial TME are being discovered. Although the majority of therapeutic strategies employed in CLL hitherto have focused on targeting the leukemic cells, emerging evidence implies that modulation of microenvironmental cells and CLL-TME interactions by novel therapeutic agents significantly affect their clinical efficacy. Thus, improving our understanding of CLL-TME interactions and how they are affected by current therapeutic agents may improve and guide treatment strategies. Identification of novel TME interactions may also pave the road for the development of novel therapeutic strategies targeting the TME. In this review, we summarize current evidence on the effects of therapeutic agents on cells and interactions within the TME. With a growing demand for improved and personalized treatment options in CLL, this review aims at inspiring future exploration of smart drug combination strategies, translational studies, and novel therapeutic targets in clinical trials.

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Introduction

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy characterized by the clonal expansion of $CD5^+/CD19^+$ malignant B cells, and displays a heterogeneous pathology with chromosomal aberrations, recurrent mutations, and microenvironmental involvement.[1] Although characterized by an accumulation of malignant cells in peripheral blood, CLL develops in protective niches and proliferation centers within the bone marrow, lymph nodes, the spleen and, more rarely, the liver.[2] These tissues allow close interactions between malignant cells and various host cells constituting the tumor microenvironment (TME). The survival and growth of CLL cells is highly dependent on support from these surrounding microenvironmental cells that include T cells, monocytes/macrophages, endothelial and mesenchymal stroma cells, and natural killer (NK) cells.[2-5] The complex crosstalk between CLL cells and these essential microenvironmental components is still poorly defined but studies have revealed how these interactions support disease progression and drug resistance.[6-9] For an extensive and detailed overview of the CLL-TME constituents and interactions, we refer the reader to previously published reviews,[3-5] as a complete review of the CLL TME is beyond the scope of this review. However, key components and interactions relevant for the contents of this review are briefly highlighted here.

The T-cell compartment in CLL has a complex dual role since it can exert both pro-tumor as well as anti-CLL cytotoxic activity.[10] Recruited CD4+ T helper cells (T_h cells) within proliferation centers provide tumor support through CD40/CD40 ligand (CD40L) co-stimulation and cytokine signaling.[11,12] In the peripheral blood of patients, T-cell numbers are increased with skewing towards cytotoxic CD8+ T cells and enriched effector cell subpopulations.[13] Both CD4+ and CD8+ T-cell subpopulations exhibit functional defects including impaired immune synapse formation with antigen-presenting cells, impaired cytokine production, degranulation, and antitumor cytotoxicity.[14-16] Furthermore, T cells in CLL show increased expression of markers of chronic activation and exhaustion, such as programmed cell death protein 1 (PD-1),[13,16] contributing to inhibited effector function and impaired immunological synapse formation.[15,16] Patients with CLL also have elevated numbers of regulatory T cells (T_{reg}), a subset of immunosuppressive T cells that constitute significant suppressors of antitumor T-cell responses.[17] Thus, T_h cells play an important supportive role in CLL, whereas the accumulation of T_{reg} and exhausted cytolytic T cells prevent effective anti-CLL effector functions.

Similarly, myeloid cells in CLL play both tumor-supportive and immunosuppressive roles. These cells include nurse-like cells (NLC), which constitute an essential tumor-supporting component of the TME. NLC, generated *in vitro*, protect CLL cells from spontaneous and drug-induced apoptosis, promote migration, and aid recruitment of tumor-supportive T cells.[18-20] Importantly, NLC reveal a strong resemblance to tumor-associated macrophages infiltrating lymph node tissue in CLL.[21] In contrast, myeloid cells with immunosuppressive properties, termed myeloid-derived suppressor cells (MDSC), accumulate in the peripheral blood of CLL patients.[22] *In vitro*, CLL-induced MDSC suppress T-cell effector function and promote T_{reg} differentiation.[23] Thus, MDSC represent a significant immunosuppressive component within the CLL-TME.

Co-culturing CLL cells with bone marrow-derived stromal cells or endothelial cells abrogates the spontaneous apoptosis of CLL cells *in vitro*, highlighting the supportive role of stromal cells in the CLL-TME.[24] Stromal cells mediate lymphocyte trafficking and homing, and promote CLL survival and proliferation by inducing expression of pro-angiogenetic and anti-apoptotic proteins.[19,24] Thus, the CLL-TME constitutes a complex cellular and molecular network that contributes to tumor survival and immune suppression.

The B-cell receptor pathway is a central mechanism by which CLL cells maintain their crucial interaction with the TME. It consists of an antigen-binding transmembrane immunoglobulin connected to downstream regulators including spleen tyrosine kinase (SYK), Bruton tyrosine kinase (BTK), and phosphoinositide-3-kinase δ (PI3Kδ) (Figure 1A). B-cell receptor signaling, recently reviewed elsewhere,[25] promotes proliferation, survival, and migration of the malignant clone. Stimulation of B-cell activating factor receptor (BAFF-R) by its ligand B-cell activating factor (BAFF) provided by, for example, NLC in the TME, also promotes important pro-survival and growth signals.[26] Furthermore, through direct cell-cell contact by co-expressed adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1, and chemokine signaling via the CXC motif chemokine receptor (CXCR)4/CXC ligand (CXCL)12 axis, TME constituents, such as NLC and stromal cells, aid migration and homing of CLL cells into protective niches.[4,18,19,24]

Reciprocally, CLL cells release cytokines including interleukin (IL)-6 and IL-10,[27,28] chemokines such as CCL2,[12] and extracellular vesicles,[4,29] through which they recruit and alter microenvironmental cells, thus inducing a tumor-supportive niche. The above highlighted CLL-TME constituents and interactions are summarized in *Online Supplementary Figure S1*.

The immune-subversive milieu preventing the host immune system from eliminating CLL cells also entails a state of clinical immune dysfunction, manifested as an increased risk of infections and autoimmune conditions in patients with CLL.[30] Thus, the CLL-TME is not merely a "silent" support system for malignant cells, but contributes significantly to clinical presentation and disease aggressiveness.

The majority of therapeutic strategies employed hitherto have been designed to target the survival axes of CLL cells, as exemplified by the development of inhibitor drugs targeting the B-cell receptor pathway. However, as our knowledge on the mechanisms of action is expanding, there is emerging evidence that targeted agents modulate immune TME cells and interactions, which likely profoundly influences clinical responses. These effects occur both indirectly, through elimination of CLL cells and/or disruption of critical CLL-TME interaction pathways, and directly, through inhibition of targets within the specific TME cells (Figure 1B). Furthermore, some novel treatment modalities rely directly on the engagement and activation of microenvironmental cells for their anti-CLL activity (Figure 1B).

In order to improve tailored treatment options for patients with CLL, and ultimately improve the clinical course of the disease, a better understanding of how current novel therapies affect the CLL-TME is warranted. Here we review the current knowledge on how novel targeted therapies modulate CLL-TME cells and their interactions. We discuss implications for future treatment strategies and the development of combination therapy, and highlight potential novel therapeutic targets that warrant future exploration.

BTK inhibitors

The introduction of small molecule inhibitors of BTK, a TEC family kinase that plays a crucial role downstream of B-cell receptor signaling, has shifted the paradigm for CLL treatment during the past decade. Ibrutinib (PCI-32765) was the first oral covalent BTK inhibitor to be approved for CLL by the Food and Drug Administration. Second-generation BTK inhibitors, acalabrutinib and zanubrutinib, are currently being introduced into clinical use.[31,32] BTK inhibition by ibrutinib inhibits activation-induced proliferation and induces apoptosis of CLL cells.[33] However, a growing number of studies describe effects of ibrutinib on several components of the TME.

Changes in total T-cell numbers induced by ibrutinib are controversial, as studies have documented both increased and decreased total T-cell numbers in patients treated with ibrutinib.[34-36] This discrepancy may be due to differences in treatment duration and disease status at the time of fol-

low-up, as well as differences between cohorts of patients. Increased T-cell numbers were observed during the first 6 months of treatment in one study,[35] while a decrease and normalization of T-cell numbers were found in studies with longer follow-up.[33,34,36] This may suggest a correlation between T-cell dynamics and CLL tumor burden during ibrutinib treatment. It was previously demonstrated that T-cell receptor repertoire diversity increased in patients upon ibrutinib treatment, which correlated with disease response and lower infection rates.[34] Interestingly, an increase in clonal T cells during ibrutinib treatment, which could be linked to residual CLL disease persistence and the co-occurrence of anti-CLL T-cell clones, was reported recently,[37] suggesting that residual disease may maintain certain, specific anti-CLL T-cell clones. Thus, reduced tumor burden as an indirect effect of ibrutinib likely contributes significantly to normalization of the majority of the T-cell repertoire along with T-cell numbers. Ibrutinib exhibits off-target activity against IL-2-inducible T-cell kinase (ITK), a TEC kinase signaling downstream of the T-cell receptor, which plays a role in T-cell activation, cytokine release, and proliferation.[38] The second-generation BTK inhibitors, acalabrutinib and zanubrutinib, have increased BTK selectivity but an insignificant inhibitory effect on ITK.[39,40] In contrast to ibrutinib, treatment with acalabrutinib and zanubrutinib did not alter patients' T-cell numbers; however, the follow-up time in these studies was limited to 6-7 months, when residual disease may still be present.[35,40] Thus, further studies are warranted to clarify the potential contribution of direct ITK inhibition to the changes in T-cell numbers seen with ibrutinib.

It was also demonstrated that ibrutinib restored T-cell proliferation and degranulation,[36] enhanced T-cell lytic immune synapse function,[41] and reversed the

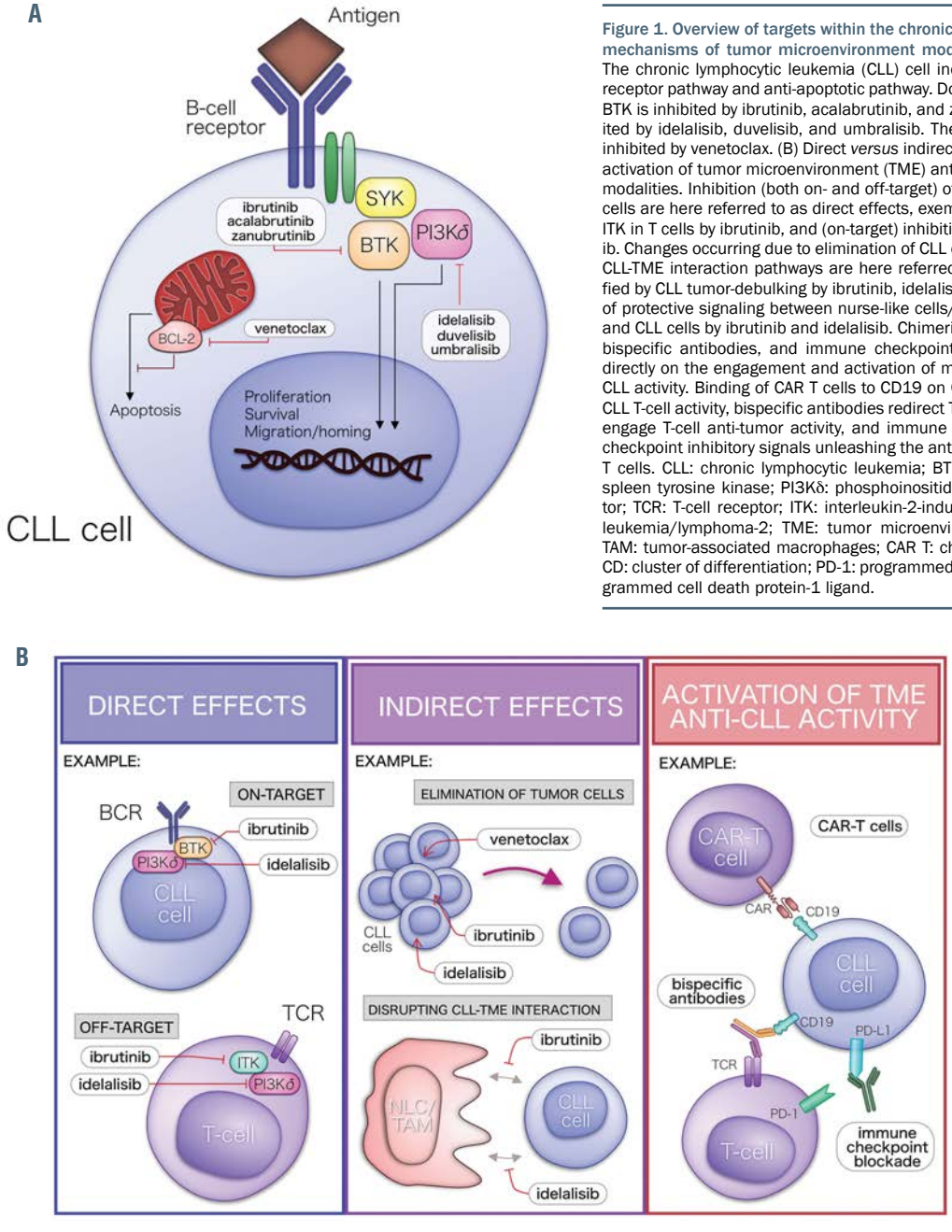

Figure 1. Overview of targets within the chronic lymphocytic leukemia cell, and mechanisms of tumor microenvironment modulation by targeted agents. (A) The chronic lymphocytic leukemia (CLL) cell including targets within the B-cell receptor pathway and anti-apoptotic pathway. Downstream of the B-cell receptor, BTK is inhibited by ibrutinib, acalabrutinib, and zanubrutinib, and PI3Kδ is inhibited by idelalisib, duvelisib, and umbralisib. The anti-apoptotic protein BCL-2 is inhibited by venetoclax. (B) Direct *versus* indirect effects of targeted agents, and activation of tumor microenvironment (TME) anti-CLL activity by novel treatment modalities. Inhibition (both on- and off-target) of targets within the specific TME cells are here referred to as direct effects, exemplified by off-target inhibition of ITK in T cells by ibrutinib, and (on-target) inhibition of PI3Kδ in T cells by idelalisib. Changes occurring due to elimination of CLL cells and/or disruption of critical CLL-TME interaction pathways are here referred to as indirect effects, exemplified by CLL tumor-debulking by ibrutinib, idelalisib, or venetoclax, and disruption of protective signaling between nurse-like cells/tumor-associated macrophages and CLL cells by ibrutinib and idelalisib. Chimeric antigen receptor (CAR) T cells, bispecific antibodies, and immune checkpoint blockade immunotherapy rely directly on the engagement and activation of microenvironmental cells for anti-CLL activity. Binding of CAR T cells to CD19 on CLL cells activates cytolytic anti-CLL T-cell activity, bispecific antibodies redirect T cells into CLL cell proximity and engage T-cell anti-tumor activity, and immune checkpoint blockade abrogates checkpoint inhibitory signals unleashing the anti-CLL activity of tumor-infiltrating T cells. CLL: chronic lymphocytic leukemia; BTK: Bruton tyrosine kinase; SYK: spleen tyrosine kinase; PI3Kδ: phosphoinositide-3-kinase δ, BCR: B-cell receptor; TCR: T-cell receptor; ITK: interleukin-2-inducible T-cell kinase BCL-2: B-cell leukemia/lymphoma-2; TME: tumor microenvironment; NLC: nurse-like cells; TAM: tumor-associated macrophages; CAR T: chimeric antigen receptor T cells; CD: cluster of differentiation; PD-1: programmed cell death protein-1; PD-1L: programmed cell death protein-1 ligand.

exhaustion/chronic activation T-cell phenotype illustrated by PD-1 downregulation,[33,35] supporting the concept that ibrutinib improves T-cell function. Similarly, treatment with acalabrutinib and zanubrutinib downregulated T-cell PD-1 expresssion.[35,40] Thus, reduced exhaustion phenotypes could be due to indirect removal of tumor burden by all three BTK inhibitors. However, improved T-cell functionality may also be due to differential effects on $CD4^+$ and $CD8^+$ subsets, which could be linked to direct off-target activity of ibrutinib.

ITK has particular importance for T_h2 T-cell polarization as well as for the development of T_{reg}. Ibrutinib promoted T_h1 polarization in a CLL mouse model,[38] but this has been more challenging to detect in patients receiving therapy.[35] Furthermore *ex vivo* ibrutinib treatment of γδ T cells from CLL patients promoted a T_h1 phenotype leading to improved antitumor effector function, indicating effects due to direct off-target ITK inhibition.[42] Ibrutinib treatment also reduced the fraction of T_{reg} in CLL patients,[43] while treatment with acalabrutinib did not affect T_{reg} numbers, further indicating direct off-target ITK inhibition by ibrutinib.[35,43] Reduced numbers of $CD4^+$ IL-17 producing T_h cells (T_h17 cells) in ibrutinib-treated patients, as well as reduced T_h17 differentiation *in vitro*, have also been demonstrated, recapitulating findings from ITK knockout mice.[33] As for T_{reg}, acalabrutinib did not affect T_h17-cell numbers.[35] However, contradictory findings, with increased T_h17 T cells in patients with CLL receiving ibrutinib, have been reported.[35] This is perhaps due to complex $CD4^+$ subset changes which are related to time on therapy and prior treatment history in study cohorts. Additionally, although current data support ibrutinib-mediated direct ITK inhibition in both T_h17 and T_{reg} subsets, given their antagonizing roles,[44] indirect effects on T_h17 T cells due to reductions of T_{reg} may "dominate" the direct effects, and contribute to this compartment expanding.

Inhibition of B-cell receptor signaling leading to redistribution of CLL cells from sanctuary niches into the peripheral blood is a hallmark of ibrutinib treatment,[45] the mechanism of which is, in part, disruption of microenvironmental interactions. Bone marrow specimens from ibrutinib-treated patients revealed disruption of (tumor-associated) macrophage-CLL cell contacts, with macrophage cellular protrusions contracting during therapy, likely reflecting a loss of NLC pro-survival signaling.[33] Ibrutinib has been shown to block BTK and downstream transcription factors within macrophages, resulting in downregulated expression of the chemokines CXCL12 and CXCL13, thus suggesting a direct effect of ibrutinib on macrophages.[46] The reduced levels of these chemokines further compromised adhesion and migration of malignant B cells *in vitro*.[46] In accordance, ibrutinib-mediated inhibition of the migratory response of CLL cells towards these chemokines was demonstrated.[33,47] Thus, direct effects of ibrutinib on macrophages seem to mediate inhibited CLL-cell chemotaxis and adhesion, thereby likely contributing significantly to the clinical reduction in lymph node and spleen size, and concomitant peripheral lymphocytosis.[45] Contrariwise, unfavorable effects of ibrutinib, including impaired phagocytosis in macrophages and neutrophils, and inhibited NK-cell activation and suppressed antibody-dependent cellular cytotoxicity by NK cells have been demonstrated, likely related to direct inhibition of BTK and ITK by ibrutinib in these cells. This may have important clinical implications for combination treatment with CD20 antibodies.[48]

Reduction of MDSC and a concomitant increase in classical monocytes were recently demonstrated in patients with CLL after 12 months of ibrutinib treatment,[36] and were likely due to both direct effects of BTK inhibition in MDSC,[49] and indirect effects induced through reduced tumor burden. Given their suppressive effect on T-cell function,[23] a reduction of MDSC may also further contribute indirectly to improved T-cell/immune functions. Moreover, ibrutinib abrogates the adherence of vascular cell adhesion molecule-1-positive CLL cells to fibronectin on stromal cells, thereby further reducing the ability of CLL cells to remain in the protective tissue niches.[47,50] Although ibrutinib produces impressive clinical results, treatment resistance is emerging,[6] and residual disease remains a challenge. *In vitro* studies have demonstrated a protective effect of NLC in the presence of ibrutinib, thereby implying a role for NLC in contributing to residual disease and the development of ibrutinib resistance.[7] Furthermore, it was demonstrated that ibrutinib-resistant subclones harboring BTK mutations promote proliferation of BTK wild-type cells during ongoing ibrutinib treatment through paracrine stimulation, further implying a role of microenvironment crosstalk in the development of resistance.[8]

A number of studies seem to point toward improved clinical immune function due to the TME modulations mediated by ibrutinib.[51,52] This issue, however, remains controversial, as there is still a lack of data demonstrating reduced risk of infections compared to prior ibrutinib treatment. However, studies do indicate that restoration of immune phenotypes and function establish after long-term treatment.[33,34,36,51] This is in line with previous real-world data demonstrating that infectious adverse events in patients with CLL treated with ibrutinib are most frequent during the first 6 months, after which infection rates decline.[53] Thus, the long-term indirect effects of ibrutinib due to reduced tumor burden and disrupted CLL-TME crosstalk may allow the various immune cell compartments to re-establish normal host immunity; however, further studies on this matter are warranted.

Continued investigation of the impact of BTK inhibitors on the TME compartments is warranted in order to provide tailored treatment strategies to improve clinical outcome (residual and progressive disease) and immune function in patients with CLL, while evading emergence of drug resistance. The most important effects of BTK inhibitors on the TME are summarized in Table 1 and illustrated in Figure 2.

PI3K inhibitors

In addition to BTK, PI3K constitutes another critical component of the B-cell receptor signaling pathway (Figure 2). Idelalisib is a selective inhibitor of PI3Kδ, the PI3K isoform generally restricted to hematopoietic cell types,[54] and was the first PI3K inhibitor approved for CLL treatment. In preclinical studies, idelalisib induced caspase-dependent apoptosis of primary CLL cells and also reduced their chemokine secretion, independently of cytogenetics or IgHV mutational status.[55,56] Although treatment of autologous T cells and NK cells with idelalisib does not induce apoptosis in these cells, it does decrease their production of inflammatory cytokines (IL-6, IL-10, tumor necrosis factor-

α [TNF-α], interferon [IFN]-γ) and activation-induced molecules (CD40L).[55] These changes could potentially have effects on both pro-tumor and antitumor immune functions. In addition, idelalisib antagonizes the CLL pro-survival functions of TNF-α and CD40L.[55] The effect of idelalisib on the T_{reg} subset has been a focus of previous studies, as inactivation of PI3Kδ in mice impaired T_{reg}-mediated immune tolerance, enhancing $CD8^+$ T-cell mediated cytotoxic responses towards tumor cells.[57] Interestingly, PI3Kδ inhibition in a CLL mouse model resulted in reduced numbers and maturation of T_{reg}; however, this did not result in enhanced antitumor $CD8^+$ T-cell function, likely due to concomitant direct inhibition of PI3Kδ downstream of T-cell receptor signaling.[58]

Similar to the effects of ibrutinib, idelalisib seems to interfere with CXCL12-mediated chemotaxis, and abrogates adhesion of CLL cells to stromal cells, suggesting an indirect mechanism through disrupting the protection of CLL cells provided by the TME.[9,56] This correlates with clinical findings of reduced lymphadenopathy and splenomegaly concomitant with lymphocytosis and significantly reduced levels of CLL-related chemokines.[59] It has been demonstrated that idelalisib impairs neutrophil function *ex vivo*,[60] which together with changes in cytotoxic T-cell subsets and strong suppression of T_{reg}, likely contribute to the increased immune-related adverse events and increased risk of infections observed upon idelalisib treatment in clinical trials.[61] The next-generation PI3K inhibitor, duvelisib, a dual inhibitor of PI3K isoforms δ and γ, was recently approved for the treatment of

Table 1. Effect of novel therapeutic agents on the microenvironment in chronic lymphocytic leukemia.

Population	Agent	Functional changes
T cells		
	BTK inhibitors -ibrutinib (ibr) -acalabrutinib (aca) -zanubrutinib (zan)	Increased T-cell receptor diversity[34,37] (ibr) Enhanced T-cell lytic immune synapse function[52] (ibr) Skewing towards T_h1 polarization[23,53,54] (ibr) Reduced T-cell PD-1 expression/exhaustion phenotype[49,51,67] (ibr, aca, zan) Reduced number of T_{reg}[51,55] (ibr)
	PI3K inhibitors -idelalisib (id) -duvelisib (duv)	Reduced secretion of inflammatory cytokines[70] (id) Inhibition of T_{reg} functions[73,79] (id, duv)
	BCL-2 inhibitors -venetoclax (ven)	Reduced number of T cells[70] (ven) Reduced number of T_{reg}[70] (ven) Decreased T-cell PD-1 expression[70] (ven)
	IMiD/CELMoD -lenalidomide (len) -avadomide (ava)	Immune activation, repaired T-cell dysfunction[20,24,90,91] (len) Suppressed T-cell proliferation[93] (len) Promotion of T.1 polarization[92] (len) Induction of inflammatory IFN type I and II signaling in previously exhausted T-cells[27] (ava)
Myeloid cells		
	BTK inhibitors -ibrutinib (ibr)	Abrogation of the protective contact[49] (ibr) Inhibited chemokine signaling and mediation of CLL cell homing[58,59] (ibr) Impaired phagocytosis by macrophages and neutrophils[48] (ibr) Reduced number of MDSC and increased classical monocytes[36] (ibr)
	PI3K inhibitors -idelalisib (id)	Impaired neutrophil inflammatory responses[76] (id)
	IMiD -lenalidomide (len)	Impaired migration/chemotaxis, abrogated CLL cell protective capability, increased phagocytosis[93] (len)
Stromal cells		
	BTK inhibitors -ibrutinib (ibr)	Revoked adherence to stromal cells in protective niches[59,60] (ibr)
	PI3K-inhibitors -idelalisib (id)	Reduced chemotaxis and impaired adhesion[71,74] (id)
NK cells		
	BTK inhibitors -ibrutinib (ibr)	Inhibited NK-cell activation[48] (ibr) Suppressed ADCC[48] (ibr)
	PI3K inhibitors -idelalisib (id)	Reduced secretion of inflammatory cytokines[70] (id)
	BCL-2 inhibitors -venetoclax (ven)	Decreased number of NK cells[70] Improved NK-cell function[70]

CLL: chronic lymphocytic leukemia; BTK: Bruton tyrosine kinase; ITK; interleukin-2-inducible T-cell kinase; BCL-2: B-cell lymphoma 2; T_h: T helper; PD-1: programmed cell death protein 1; T_{reg}: regulatory T-cell; PI3K: phosphoinositide 3'-kinase; IMiD: immunomodulatory drugs; CELMoD: cereblon E3 ligase modulators; IFN: interferon; MDSC: myeloid derived suppressor cells; ADCC: antibody-dependent cellular cytotoxicity.

relapsed/refractory CLL. Similar to idelalisib, treatment with duvelisib entails increased risk of immune-related toxicities and infections in patients with CLL,[62] likely also due to strong direct inhibitory effects on T_{reg} and cytotoxic T-cell effector function as demonstrated in a CLL mouse model.[63] In contrast, another next-generation PI3K inhibitor, umbralisib, with dual PI3Kδ/casein kinase-1-ε (CK1ε) inhibitory activity, did not modulate T_{reg} function, which was associated with lower toxicity in a murine model.[63] Thus, the disadvantageous direct effects of idelalisib and duvelisib on T-cell subsets contributing to a risk of infections and toxicity, which have hampered their clinical usage, could potentially be mitigated with the use of umbralisib due to altered PI3K specificity. The most important effects of PI3K inhibition on the TME are summarized in Table 1 and illustrated in Figure 2.

BCL-2 inhibitors

The anti-apoptotic regulatory protein B-cell lymphoma 2 (BCL-2) is constitutively upregulated in several lymphomas including CLL, hence playing a dominant role in blocking apoptotic signaling and promoting survival in these malignancies.[64] Venetoclax (ABT-199), a selective BCL-2 inhibitor, demonstrated the ability to induce rapid apoptosis in primary CLL cells *in vitro* and in xenograft models[65] (Figure 3). In clinical trials, venetoclax alone or combined with an anti-CD20 antibody has achieved deep and durable undetectable minimal residual disease in patients with CLL.[64,66] However, while leukemic cells are highly dependent on BCL-2, the dependence of non-leukemic cells on this protein seems to vary substantially. The high prevalence of grade 3/4 neutropenia among patients treated with venetoclax likely reflects the relatively marked dependency of granulopoiesis on BCL-2.[67] T-cell homeostasis also depends on BCL-2, however with variable impact on different T-cell subsets. While murine naïve T cells were found to be highly dependent on BCL-2, the protein was dispensable for memory T cells.[68] Coherently, a decrease in naïve T-cell subsets and increased memory T cells have also been reported in both mice and healthy human subjects receiving venetoclax[69] (Figure 3). A study on CLL patients treated with venetoclax and the CD20 antibody obinutuzumab documented decreased numbers of normal B cells, NK cells, and T cells, including T_{reg}, in the peripheral blood. In addition, a decrease in the exhausted/chronically activated $PD1^+$ T-cell phenotype was observed, along with improved NK-cell function, and reductions of the levels of elevated inflammatory cytokines[70] (Figure 3). The authors interpreted these changes as being indirect effects due to eradica-

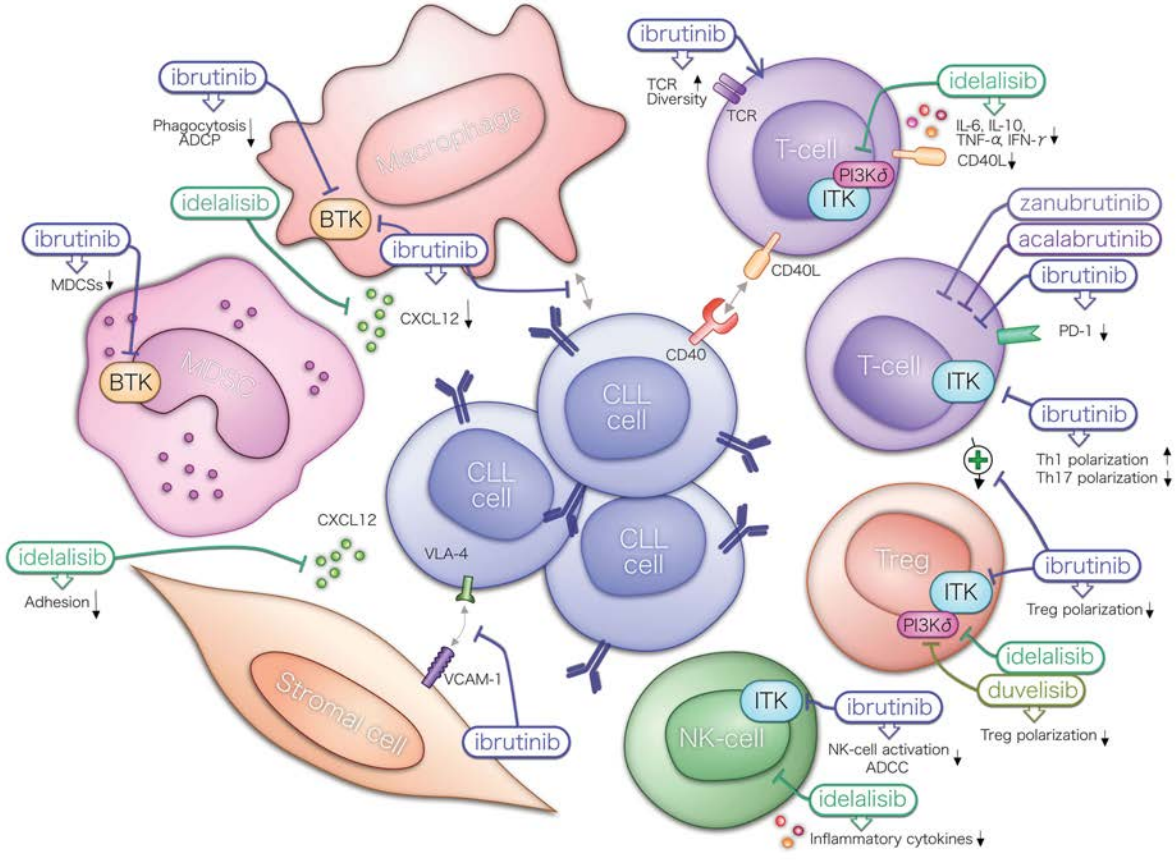

Figure 2. Effects of BTK inhibitors on the chronic lymphocytic leukemia tumor microenvironment. Inhibitory effects are represented by bars, stimulatory effects are represented by arrows. Upward arrows indicate increases, downward arrows indicate decreases. CLL: chronic lymphocytic leukemia; TME: tumor microenvironment; BTK: Bruton tyrosine kinase; BTKi: BTK inhibitor; ITK:interleukin-2-inducible T-cell kinase; PI3Kδ: phosphoinositide-3-kinase δ; TCR: T-cell receptor; CD: cluster of differentiation; CD40L: CD40 ligand; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; PD-1: programmed cell death protein 1; T_h: T helper; T_{reg}: regulatory T cell; ADCP: antibody-dependent cellular phagocytosis; VCAM: vascular cell adhesion molecule; VLA, very late antigen; CXCL: CXC motif chemokine; MDSC: myeloid-derived suppressor cells.

tion of the leukemic cells, and any direct effects on these cells by venetoclax were not investigated. Critically, the TME also appears to play a role in venetoclax resistance. In a previous study, *in vitro* CD40/CD40L co-stimulation strongly reduced sensitivity to venetoclax through upregulation of other anti-apoptotic proteins, such as myeloid cell leukemia 1 (MCL-1) and B-cell lymphoma extra large (BCL-XL), in CLL cells.[71] The varying dependency on BCL-2 among different microenvironmental cell types, as well as between patients, warrants further investigation, in order to optimize the advantages of targeting the apoptotic pathway in malignant cells, and utilize potential immunomodulatory effects in the immune TME while minimizing disadvantageous on-target-but-off-leukemic effects leading to adverse events.

Immunomodulatory drugs

Lenalidomide is an immunomodulatory drug (IMiD) widely used to treat multiple myeloma. Despite having no direct cytotoxicity against CLL cells *in vitro*,[72] clinical activity in patients with CLL has been demonstrated,[73,74] supporting anti-CLL immunomodulatory effects in the TME as a principle mode of action. *In vitro*, lenalidomide induces downregulation of CLL immune checkpoint receptors on T cells, suggesting treatment-induced immune activation or reversal of exhaustion.[15] Moreover, lenalidomide treatment of autologous T cells and CLL cells triggers repair of T-cell dysfunction. This results in improved synapse formation, granzyme B- and IL-21-mediated cytotoxicity, enhanced $CD8^+$ T-cell effector killing, and restored LFA-1-mediated T-cell motility.[14,75–77] Supporting this, *in vivo* samples from treated patients revealed changes in the composition of the T-cell subpopulations and their cytokine production.[78] Lenalidomide also affects CLL monocytes/NLC. The presence of lenalidomide impaired migration of CLL-supportive monocytes towards CCL2, CCL3, and CXCL12 in *in vitro* chemotaxis assays.[79] The same study demonstrated downregulation of genes associated with pro-survival signals for CLL cells and impaired protective ability of NLC.[79] Moreover, CLL-induced immunosuppression was reversed by lenalidomide, with improved phagocytotic activity, cytokine production, T-cell stimulatory and proliferative activity.[79] Lenalidomide has produced clinical responses as monotherapy,[74] in combination with rituximab or with chemotherapy,[80] and as maintenance following chemotherapy.[73] However, increased risk of toxicities and infections with treatment remains a concern,[73] potentially reflecting potent activation of the immune TME with this class of drug. Thus, the place and dosing regimen for lenalidomide in clinical practice remain unclear. A novel option emerging for CLL therapy are next-generation cereblon E3 ligase modulators (CELMoD), with avadomide recently investigated in a preclinical study. Avadomide stimulated T-cell activation, the expression of immunostimulatory chemokines, and the formation of lytic synapses with CLL cells by triggering inflammatory IFN type I and II signaling in previously exhausted T cells from patients.[81] The potential and optimal roles of IMiD and CELMoD in the context of the CLL-TME remain to be determined; however, the favorable immunomodulatory effects on the T-cell/NK-cell compartments imply a role for IMiD and CELMoD in developing novel combination treatment strategies. The most important effects of IMiD/CELMoD on the TME are summarized in Table 1 and illustrated in Figure 3.

Immune checkpoint blockade

The PD-1:PD-L1 is an immune checkpoint pathway used by tumor cells to inhibit T cells and escape immune surveillance. Thus, this pathway constitutes an attractive therapeutic target (Figure 4).[82] Blocking PD-L1 in CLL-transplanted mice resulted in repressed disease development and restored T-cell immune effector functions including improved cytotoxicity, cytokine production, and immune synapse formation.[83] Despite this, the sparse clinical data on immune checkpoint blockade (ICB) in CLL are disappointing. In a phase II study of the PD-1 blocking antibody drug, pembrolizumab, four out of nine patients with Richter transformation showed clinical response to treatment, whereas none of the 16 CLL patients responded.[84] The clinical efficacy of ICB-based therapy correlates with upregulated levels of tumor PD-L1 expression that is associated with an "inflamed" microenvironment with the presence of activated cytotoxic tumor-infiltrating T cells attempting to engage tumor cells, which can be unleashed as checkpoint inhibitory signals are abrogated.[85] PD-L1 expression on CLL cells is relatively low, likely reflecting low activity of cytolytic T cells.[81,82] Furthermore, the immunosuppressive state of the TME in CLL, with profoundly exhausted effector T cells that exhibit multiple functional defects, likely contributes significantly to the lack of clinical response to checkpoint inhibitor monotherapy in CLL patients. Consistent with this, a recent study of patients' lymph node biopsies has provided evidence for a non-inflamed microenvironment in CLL, incorporating low numbers of $CD8^+$ T cells, low PD-L1 expression and profound T-cell exhaustion.[81] Thus, strategies that can subvert the strong immunosuppressive pressure of the CLL-TME and overcome T-cell dysfunction may be necessary to sensitize CLL to ICB immunotherapy and develop therapeutic options for CLL patients. Further research to unravel the complex immunosuppression in the CLL-TME is warranted in order to develop and optimize immuno-oncology treatments.

T-cell-based therapy

Chimeric antigen receptor (CAR) T cells have emerged as a powerful therapeutic option designed to transfer high numbers of tumor-targeted effector T cells into the TME to overcome a paucity of endogenous cytolytic T cells. Briefly, autologous T cells are genetically modified to express CAR with specificity for specific tumor antigens, such as CD19 in B-cell malignancies, thus creating an adoptive T-cell-mediated cytotoxic response (Figure 4).[86] CAR T cells combine the effects of T-cell and antibody-mediated immune responses by triggering T-cell activation with granule exocytosis upon antigen binding.[87] Despite the first successful CAR T-cell trial being reported in CLL, few clinical trials have subsequently reported efficacy of CAR T cells in CLL.[88] CLL-induced T-cell dysfunction, as well as understudied lymphoid TME barriers, likely reduce the efficacy of this approach in CLL. A

recent study revealed that CAR T cells from CLL patients responding well to CAR T-cell therapy expressed upregulated genes associated with T-cell memory. Furthermore, enriched T-memory subsets prior to CAR T-cell generation correlated with sustained remissions. Meanwhile, CAR T cells from non-responders had upregulated genes associated with effector T-cell differentiation, apoptosis and exhaustion, thus emphasizing that T-cell fitness is crucial for the efficacy of CAR T cells.[89] Due to the multitude of (successful) treatment options for CLL currently, CAR T-cell therapy may first become a relevant option in treating multi-relapsed disease, and preliminary reports from current clinical trials of CD19-targeted CAR T-cell therapy in patients with multi-relapsed CLL show somewhat encouraging results.[90] However, paradoxically, T-cell exhaustion in CLL is demonstrated to worsen with progressive disease,[13] thus pointing towards a need for options that improve T-cell function prior to the manufacture of CAR T cells or during treatment. Furthermore, it was recently elucidated that CLL cells can directly impair CAR T-cell function and induce an exhausted phenotype through the release of plasma extracellular vesicles.[91] Thus, a meaningful role for CAR T-cell therapy in CLL may rely on the ability of current and/or future therapies to successfully target the TME and improve T-cell fitness in patients with CLL, prior to the CAR T-cell treatment, during preparation of the product, and after its administration.

A novel therapeutic approach that could constitute an alternative to CAR T-cell therapy is off-the-shelf bispecific CD19/CD3 or CD20/CD3 antibody treatment. Bispecific antibodies simultaneously engage CD3 on T cells and CD19 or CD20 on target B cells, and thereby redirect T cells to recognize CLL cells, facilitating synapse formation and, thus, T-cell-mediated antitumor responses (Figure 4). Preclinical studies using bispecific antibodies have demonstrated antileukemic activity against CLL cells *in vitro* and in xenograft models.[92] Thus, bispecific antibodies may constitute a promising T-cell-based immunotherapeutic approach for CLL, alone, or in combination with TME-modulating agents that help improve T-cell function.

Developing combination strategies targeting the chronic lymphocytic leukemia – tumor microenvironment

It is becoming evident that improving clinical responses (residual and progressive disease), overcoming toxicity, infection risk, as well as drug resistance, likely require strategies aimed at reshaping the immunosubversive, protumor TME state. Our improved understanding of the direct and indirect CLL-TME modulations by novel therapeutic agents in recent years provides a unique opportunity to optimize CLL treatment with strategic drug combinations that target multiple CLL-TME interactions to achieve therapeutic synergy while controlling toxicity.

Monoclonal antibodies targeting the B-cell surface protein CD20 have been the backbone of standard chemoimmunotherapy regimes used to treat CLL for decades, although they are rarely used as a monotherapy in CLL.[1]

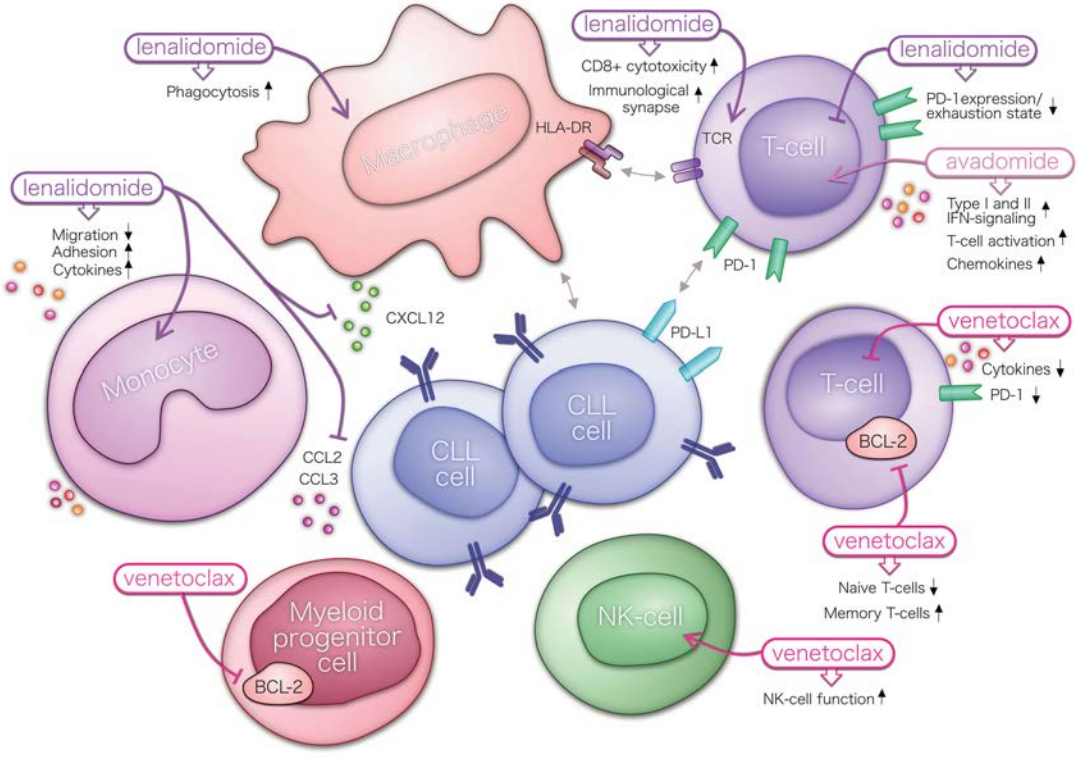

Figure 3. Effects of BCL-2 inhibitors, immunomodulatory drugs, and cereblon E3 ligase modulation on the tumor microenvironment. Inhibitory effects are represented by bars, stimulatory effects are represented by arrows. Upward arrows indicate increases, downward arrows indicate decreases. CLL: chronic lymphocytic leukemia; TME: tumor microenvironment; BCL-2: B-cell lymphoma 2; BCL-2i: BCL-2 inhibitor; IMiD: immunomodulatory drug; CELMoD: cereblon E3 ligase modulator; TCR: T-cell receptor; HLA-DR: human leukocyte antigen DR-isotype; IFN: interferon; PD-1: programmed cell death protein 1; PD-1L: programmed cell death protein-1 ligand; CCL: chemokine ligand.

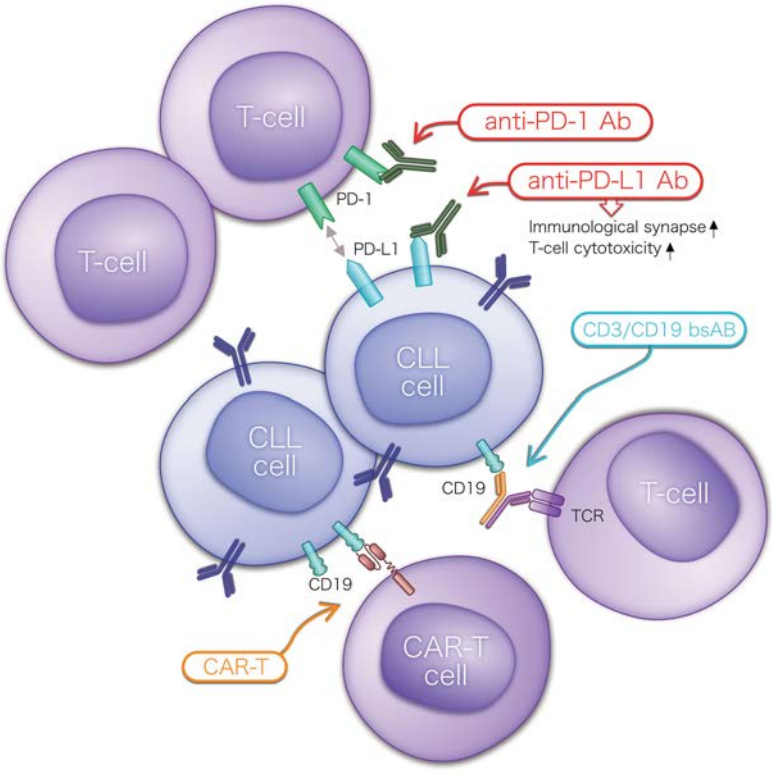

Figure 4. Effects of immune checkpoint blockade, chimeric antigen receptor T cells, and bispecific antibodies. Arrows point out the actions of immune checkpoint blockade, chimeric antigen receptor T cells and bispecific antibodies, leading to engagement and activation of the tumor microenvironment for anti-chronic lymphocytic leukemia activity. Upward arrows indicate increases. CLL: chronic lymphocytic leukemia; PD-1: programmed cell death protein 1; PD-1L: programmed cell death protein-1 ligand; Ab: antibody; CAR-T: chimeric antigen receptor T cells; bsAB: bispecific antibodies; TCR: T-cell receptor; CD: cluster of differentiation.

Major mechanisms of action of anti-CD20 antibodies are activation of antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis, which rely on engaging the antitumor activity of NK cells and monocytes/macrophages within the immune TME.[1,48,93] The direct inhibitory effects of ibrutinib on macrophage phagocytosis and NK-cell activation may therefore interfere with the therapeutic efficacy of anti-CD20 treatment.[48] Compared to ibrutinib as a single agent, adding an anti-CD20 antibody to ibrutinib was associated with faster remissions and lower levels of residual disease in a clinical trial, although it was not demonstrated that the combination improved progression-free survival.[94] Thus, whether this combination is beneficial remains debatable. In contrast, combining anti-CD20 antibodies with venetoclax seems to improve the phagocytosis of CLL cells by macrophages *in vitro*,[93] and reverse venetoclax resistance induced by TME signaling.[71] Interestingly, although venetoclax plus anti-CD20 treatment produces impressive clinical responses in clinical trials, a recent retrospective study including real-world data demonstrated comparable efficacy between venetoclax as a single agent and venetoclax plus anti-CD20 combination treatment in high risk relapsed/refractory CLL patients.[95] Thus, further validating prospective studies are warranted to determine whether the addition of an anti-CD20 antibody to venetoclax is truly necessary.

The addition of venetoclax to ibrutinib constitutes another approach aiming to provide improved duration and depth of remissions as well as to enable fixed-duration treatment, which has already, in part, demonstrated success in clinical trials.[96] Similarly, the PI3K inhibitor, duvelisib, increases sensitivity of CLL cells to venetoclax, providing the rationale for duvelisib-venetoclax combination treatment, currently being investigated in clinical trials.[97]

However, the biggest challenges ahead involve finding strategic combinations that overcome T-cell dysfunction, improve the efficacy of T-cell-based therapies and ICB in CLL, and work towards curative therapy. Data from a human xenograft model support the ability of ibrutinib to enhance CAR T-cell function when administered concurrently.[98] Similarly, another murine study indicated that PI3Kδ inhibition during CAR T-cell production may have a positive effect on engraftment and antitumor activity.[99] Consistently with this, a clinical pilot study recently demonstrated high response rates in relapsed/refractory CLL patients receiving ibrutinib concomitant with CD19-targeted CAR T-cell therapy, and lower toxicities compared to those in patients treated without concomitant ibrutinib.[100] Furthermore, T cells from ibrutinib-treated CLL patients seem to exhibit improved *in vitro* anti-CLL activity combined with bispecific antibodies.[101]

The lack of clinical activity of anti-PD-1 monotherapy in CLL,[84] has highlighted the need to incorporate ICB therapies into more powerful combinations to unleash the power of antitumor immune cells. Studies of PD-1:PD-L1 blockade combined with ibrutinib have demonstrated enhanced $CD8^+$ T-cell function along with improved disease control in a CLL mouse model.[102] However, preliminary clinical results have indicated that coupling anti-PD-1 with ibrutinib may not increase response rates in patients.[103] PI3K inhibition improved the anticancer effect of ICB through modulatory effects on MDSC in a solid cancer *in vitro* model,[104] thus highlighting additional roles of PI3K inhibition in modulation of the TME which could be exploited. The relative expansion of memory T-cell subsets due to direct effects of venetoclax on other, more BCL-2-dependent T-cell subsets, support a role for venetoclax in combination with ICB. In a recent sold cancer murine study, venetoclax augmented the antitumor effect of anti-PD-1 and anti-PD-L1 inhibitors *in vivo*.[69]

Through their potent activation of T cells, CELMoD and IMiD could represent strong complementary treatment partners for combination (immune)therapy.[14,15,73,75,76,80,81] It has been demonstrated preclinically that the CELMoD avadomide could sensitize CLL to anti-PD-1 or anti-PD-L1 immunotherapy.[81] By inducing inflammatory interferon type I and II signaling in previously exhausted T cells from patients, avadomide stimulated the proliferation and release of chemokines by T cells which recruited additional CD8+ T cells, upregulated PD-L1 in the immune TME, and enhanced lytic synapse formation.[81]

Even more powerful combinations could include pairing ICB with CAR T cells or bispecific antibodies to increase tumor infiltrating T cells, or dual ICB combinations to overcome additional inhibitory barriers. T-cell bispecific antibodies combined with an anti-PD-L1 antibody showed enhanced antitumor efficacy compared to either given alone in a solid cancer mouse model.[105] Furthermore, a recent CLL murine study demonstrated that anti-PD1 ICB combined with inhibition of the immune checkpoint receptor lymphocyte-antigen gene 3 (LAG3) was able to decrease tumor load significantly, while either as monotherapy had little effect.[106]

Thus, developing combination immunotherapy could represent a powerful strategy for deepening targeted drug (e.g., BTK inhibitor- and/or venetoclax)-induced responses and working towards curative therapy in CLL.

Future perspectives: novel targetable tumor microenvironment interactions

The CLL-TME constitutes a landscape of potential targetable pathways. Antibodies interrupting the CXCL12/CXCR4 interaction have demonstrated anti-CLL activity *in vitro* and in mouse models, and have been tested in phase I clinical trials for multiple myeloma, but have not yet been further explored in CLL.[107] The BAFF/BAFF-R axis constitutes another attractive CLL-TME interaction to target. An anti-BAFF-R antibody blocked protective survival signaling in CLL cells and enhanced antibody-dependent cellular cytotoxicity *in vitro*, and also enhanced efficacy of ibrutinib in a CLL mouse model.[108] Targeting of an IL-10-producing CD38^{hi} regulatory B cell-like CLL subset is also currently under investigation.[27] Although aimed at CLL cells, the anti-leukemic potential here would be mediated *indirectly* by abrogating IL-10-mediated immunosuppression. The "don't eat me" signal regulatory protein (SIRP)1α/CD47 axis, co-expressed by macrophages and malignant cells, respectively, in various lymphoid malignancies including CLL, constitutes a mechanism of myeloid immune tolerance. It is currently being explored as a potential target in lymphoma, and may also constitute an important macrophage-CLL interaction with targetable potential.[109] Furthermore, MDSC-derived indoleamine 2,3-dioxygenase (IDO) has been explored as a target in other cancers, as an antitumor vaccination antigen.[110] Mediating strong immunosuppressive effects in the CLL-TME, IDO may constitute another potentially attractive interaction to target in CLL.

In conclusion, the heterogeneous course of CLL is driven by (i) the genetic complexity and (ii) diverse and complex tissue TME interactions, including (iii) antigenic drive. The functionally and phenotypically skewed cell types within the TME not only promote CLL itself, but also compromise the induction of adequate immune responses towards developing and progressing CLL clones, as well as infectious agents. Thus, further exploration of the impact of different therapies on critical microenvironmental interactions is warranted. This review was intended to outline our current understanding of how CLL-TME interactions are influenced by current CLL therapies, with the view to encourage continued mapping of the CLL-TME for more specific future targeting of CLL-TME crosstalk. The inclusion of translational correlative studies assessing immunological and TME changes within clinical trials should inform development of novel combination therapies beyond BTK inhibitors and BCL-2 inhibitors. These include, but are not limited to, checkpoint inhibitors, T-cell based therapies, and TME modulation overcoming the inherent immune exhaustion in CLL. Such strategies together with further understanding of the TME should, eventually, lead to improved and more personalized treatment options aiming for a clinical cure with reduced burden of adverse events for patients with CLL.

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Contributions

RS and SJ wrote the first draft of the review under supervision of CN; the final version of the review was written by all five authors. All authors approved the final version.

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