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Old and new targets for immunotherapy of B cell acute lymphoblastic leukemia

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Abstract

B cell-specific antigens such as CD20 and CD19 are the leading examples of clinically utilized targets for cancer immunotherapy. Rituximab, the anti-CD20 monoclonal antibody (mAb) approved for the treatment of B cell lymphoma in 1997, was the earliest mAb drug ever registered in cancer immunotherapy. The clinical success of chimeric antigen receptor (CAR)-modified T cells has been demonstrated in patients with B cell acute lymphoblastic leukemia (B-ALL), and CD19-directed CAR-T cells were the first CAR therapy ever approved to treat cancer patients. While surface antigen-targeting immunotherapies play a significant role in the therapy of B-ALL, in particular in the treatment of relapsed and refractory patients, they have some limitations and face continuous challenges. Herein, I review the types of antigen-specific immunotherapies that are used in the treatment of B-ALL, including naked mAbs, antibody-drug conjugates, B cell-specific T cell engagers, and CAR-modified T cells. I discuss the requirements for good immunotherapy targets and summarize the main methods used to identify novel putative targets. I present an overview of B cell-specific and non-B cell-specific target antigens, both already used in clinics and tested in preclinical models. I also discuss limitations of current B-ALL immunotherapy, attempts to overcome these limitations, and future directions of immunotherapy research.

Key words: acute lymphoblastic leukemia, B cell, immunotherapy, antigen, target, CD19, CD22, CD20, CD72

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Introduction

Surface antigen-targeted immunotherapies were first introduced in the treatment of B cell malignancies. The availability of B cell-specific target antigens such as CD19, CD20 and CD22 that are not expressed in other tissues has greatly contributed to success. CD19 is the main target for chimeric antigen receptor (CAR) T cell immunotherapy and for blinatumomab, a bispecific T cell engager (BiTE). Both therapies are already approved for the treatment of relapsed/refractory (R/R) B cell acute lymphoblastic leukemia (B-ALL). Although CD19 CAR-T cells have shown unprecedented response rates, exceeding 80% in R/R B-ALL patients, the durability of response is limited, and many patients relapse with CD19-negative disease [1, 2]. Other B cell-specific antigens such as CD22 and CD20 are already available as second-line therapies of CD19-negative relapses and their efficacy has been tested in clinical trials [3, 4]. However, emerging results of these trials are revealing at best transient responses, hence novel target antigens need to be identified and verified.

B-ALL is a heterogeneous disease with dozens of genetic abnormalities identified to date, and it develops in both children and adults. Although the survival prognosis is good for pediatric B-ALL (~90%), the treatment outcome in adults is much worse (40–50% overall survival) [5]. Patients harboring specific genetic translocations respond poorly to conventional therapy but also to modern

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immunotherapies. The subtype with the poorest outcome is **mixed lineage leukemia**-rearranged B-ALL (MLLr B-ALL), which frequently escapes CD19-targeted immunotherapy due to lineage switch from MLLr B-ALL to MLLr acute myeloid leukemia (AML), with a loss of B cell phenotype-associated markers [6–9]. This precludes the use of B cell-specific antigens as immunotherapy targets and encourages the selection of novel candidates as alternative targets for immunotherapy.

In this review, I describe the types of antigen-specific immunotherapy currently used in the treatment of B-ALL. I define the features of appropriate immunotherapy targets, and list ways to identify novel targets for immunotherapy. In addition, I summarize the target antigens already utilized in R/R B-ALL therapy as well as novel, alternative targets tested in preclinical models. Finally, I discuss the limitations and challenges of B-ALL immunotherapy.

Types of antigen-specific immunotherapy employed in B-ALL

Conventional B-ALL therapy is composed of intensive, multiagent chemotherapy delivered in several cycles of treatment over 2–3 years, and is associated with numerous side effects and long-term consequences [5]. Naked monoclonal antibodies (mAbs), such as rituximab, can be added to the chemotherapy of a subset of adult B-ALL patients at the induction phase, due to their low price, broad availability and mild side effects. Other, more advanced, immunotherapies such as BiTe and CAR-T cells play an important role in the treatment of R/R B-ALL patients, as a bridge therapy to allogeneic hematopoietic stem cell transplantation (allo-HSCT). Some attempts are also being made to apply CAR-T cells to treat relapse after allo-HSCT [10].

Currently available immunotherapy options in the treatment of B-ALL are extensively described in [11]. Briefly, the vast majority of registered immunotherapies target surface antigens specific for B cells, namely CD19, CD20, CD22, with mAbs recognizing these antigens. Apart from naked mAbs that work mainly through the induction of host's effector cell-dependent mechanisms (immunophagocytosis, antibody-dependent cytotoxicity), mAb derivatives such as antibody-drug-conjugates (ADC) or BiTE are also used in clinical practice [12]. Additionally, cellular therapy using autologous, patient-derived T cells genetically modified with chimeric antigen receptors (CARs) has been available since 2017 [13]. CARs are synthetic constructs composed of several domains: 1) an extracellular domain responsible for the recognition of tumor-specific targets, which is derived from mAb; 2) a transmembrane part; and 3) intracellular domains responsible for the transmission of activating signals and co-stimulation, CD3ζ, CD28, 4-1BB.

During CAR-T cell therapy, autologous T cells are collected from a B-ALL patient by leukapheresis, genetically modified *ex vivo* with CARs, and infused back into the patient's circulation, where they specifically recognize cells expressing target antigens, mainly leukemic cells. Importantly, the recognition of malignant cells by CAR-T cells and induction of the cytotoxic responses are major histocompatibility complex (MHC)-independent. The construction of CAR molecules, e.g. the choice of costimulatory domains as well as types of hinge and transmembrane domains, significantly affect CAR-T cell functionality [14].

Although antigen-targeted immunotherapy is more specific than conventional chemotherapy, adverse side-effects associated with CAR-T cell and BiTE therapy have been frequently reported. B cell aplasia, a direct consequence of on-target off-tumor toxicity, impairs antibody production and increases susceptibility to infection, but is manageable with immunoglobulin infusion. A treatment-induced life-threatening complication is cytokine release syndrome (CRS), which leads to multiple organ dysfunction and neurotoxicity [15]. This can be mitigated with the use of corticosteroids and tocilizumab, an antibody blocking interleukin 6 (IL-6) receptor. Further information about the efficacy, challenges and ways to address obstacles to CAR-T cell therapy can be found in [16, 17].

Immunotherapy targets

What makes a good target for cancer immunotherapy?

An ideal target for cancer immunotherapy should be expressed exclusively on malignant cells and should be essential for cancer cell proliferation and survival. However, none of the targets currently in use meets these stringent criteria. The vast majority of antigens utilized in the immunotherapy of B-ALL are B cell-specific proteins that occur both in malignant and normal B cells, but are rarely present in other tissues. This usually ensures sufficient efficacy and safety of the targeted immunotherapy. The resulting on-target off-tumor toxicity to normal B cells is an unavoidable but manageable side effect. However, B-ALL subtypes derived from early stages of B cell development, with more stem cell-like features such as MLLr- or TCF3-ZNF384 fusion-B-ALL, in response to CD19-directed immunotherapies were shown to undergo lymphoid-to-myeloid lineage switch [7, 8, 18]. This resulted in the loss of B cell phenotype and precluded further immunotherapy targeting, not only CD19 but also other B cell-specific antigens.

Stability, sustainability, and abundance of a target antigen in all leukemic clones are other important features. Indeed, the outcome of immunotherapy usually correlates with high antigen density on malignant cells [1, 19]. Another important issue is the lack of expression of the target antigen on activated T cells. This is particularly important for CAR-T cell immunotherapy targets. Fratricide

elimination of CAR-T cells expressing the corresponding antigens has been observed in the case of CD38 [20], and is a major obstacle to CAR-T cell manufacturing and their subsequent efficacy.

The usefulness of a surface protein as an immunotherapy target also depends on the type of immunotherapy in which it is employed. Comparisons of the intracellular transport and efficacy of CD22- and CD19-targeted immunotoxins revealed that CD22 is much more efficiently internalized and hence may serve as a better target for ADC [21, 22]. In contrast, antigens that internalize slowly, such as CD20, are better targets for naked antibodies due to prolonged exposure of the Ab crystallizable fragment (Fc) and therefore efficient activation of Fc-dependent effector mechanisms [23].

Methods of target identification

To date, combined transcriptomic and proteomic approaches have been successfully used to select candidates for novel immunotherapy targets in various cancer models including B-ALL [24–26]. Identifying protein-coding mRNAs that are specifically expressed in cancer cells is feasible by comparing malignant primary cells and cancer cell lines to normal counterparts. However, as the correlation between mRNA and protein expression on the cell surface is moderate, transcriptomic data must be integrated with cell surface proteomics. Quantitative mass spectrometry has been successfully employed to generate human cell surface proteome [24, 26]. The integrated proteomic and transcriptomic approach has been recently used to identify CD72 as an optimal target in MLLr B-ALL by comparing cell surface proteins in cell lines representing MLLr to other subtypes of B-ALL [25].

B cell-specific targets used in B-ALL immunotherapy

B-ALL arises from B cell lineage-committed progenitors at various stages of their differentiation, such as pro-B or pre-B cells. The use of B cell-specific antigens including CD19, CD22, and CD20 as targets in B-ALL therapy has already proved very successful in clinical studies. On the other hand, CD72 was recently proposed as an alternative B cell-specific target, and proved its efficacy in preclinical models. The main features of these antigens and the corresponding immunotherapies are summarized in Table I and briefly described below.

CD19

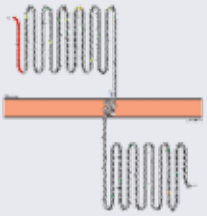
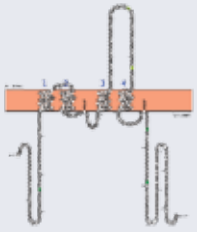
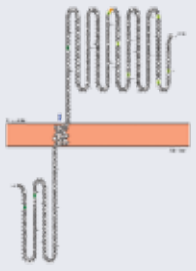
CD19 is a B cell-specific molecule considered as a marker of B cells. Its expression starts during the B lineage commitment from hematopoietic progenitors, and continues throughout all stages of B cell development up to plasma cells. CD19 is a type-I transmembrane protein, with a single transmembrane domain belonging to the immunoglobulin superfamily. It is a co-receptor of the B-cell receptor (BCR) and is involved in modulating BCR signaling [28]. Although its role in the promotion of the proliferation and survival of mature B cells is well-documented, its role in immature B cells is unclear. Importantly, CD19 expression is neither crucial for B-ALL cells viability and proliferation rate *in vitro* nor for B-ALL lymphoblasts' engraftment and progression *in vivo* [29].

Nevertheless, as CD19 is ubiquitously expressed in B-ALL cells independent of the genetic subtype, it is utilized as the main target for B-ALL immunotherapy. Upon binding to an antibody, CD19 internalizes, which makes it a suitable target for immune-conjugate therapy rather than for naked mAb [12]. Denintuzumab mafodotin and coltuximab ravtansine are ADC that have been already tested in clinical trials, but initial results have revealed low clinical responses in patients with R/R B-ALL [30]. Much better clinical responses have been achieved with the use of blinatumomab, a BiTE. Blinatumomab is a single-chain, dual-specificity construct with the ability to recognize CD3 molecules on T cells and CD19 molecules on B cells, thus activating T cells to kill proximal B cells. The efficient renal clearance of blinatumomab results in its short half-life and enforces continuous infusions over several days [31]. Blinatumomab provides clear benefits over conventional consolidation chemotherapy [32] and is effective even in patients with therapy-related and congenital T cell impairments [33].

CAR-T cells recognizing CD19 antigen are pioneering, breakthrough therapy [2] and since 2017, CD19-CAR-T cells (tisagenlecleucel, kymirah) have been approved for the treatment of R/R B-ALL. However, despite remissions reaching up to 90% in some studies, the durability of this treatment is limited, with overall survival reaching only 12.9 months [2]. There are several reasons behind CAR-T cell therapy failure and both CD19-positive and CD19-negative relapses have been detected. CD19-positive relapses occur due to insufficient CAR T-cell expansion, lack of memory T cell formation resulting in poor CAR-T cell persistence, and immunosuppressive microenvironment. Extensive studies are underway aimed at optimizing CAR construction and their *ex vivo* manufacturing in order to increase their persistence and overcome the inhibitory environment [17].

CD19-negative relapses are the most frequent causes of CD19 CAR-T cell treatment failure in B-ALL. As mentioned, CD19 is dispensable for B-ALL cell survival, therefore various processes leading to CD19 loss, such as selection of CD19-negative clones, downregulation of CD19 mRNA, antigen masking, and trogocytosis have been detected in patients undergoing CD19-targeted therapy [34]. The challenges surrounding CAR-T cell therapy have been summarized in a recent review [35].

Table I. B cell-specific surface antigens used in clinics and tested in preclinical models as targets in B cell acute lymphoblastic leukemia (B-ALL) immunotherapy. Visualization of transmembrane topology performed with Protter [27]

Target antigen	Membrane topology	Function	Available immunotherapy	Stage of development/ /references
B cell-specific targets				
CD19	Single-pass type I membrane protein 	Co-receptor for the BCR B cell differentiation and proliferation	BiTE — blinatumomab ADC Denintuzumab mafodotin Coltuximab ravtansine CAR-T cells — tisagenlecleucel	Registered drug Clinical trials Registered cellular therapy
CD22	Single-pass type I membrane protein 	Involved in regulation of BCR signaling, both positive and negative	ADC Inotuzumab ozogamycin CAR-T cells mAbs Epratuzumab	Registered drug Clinical trials Cinical trials
CD20	Tetraspanin 	Development, differentiation, activation of B cells Ca^{2+} signaling	mAbs Rituximab Ofatumumab CAR-T cells	Registered drug Registered drug Clinical trials
CD72		Negative regulation of BCR signaling Interaction with T cells	CAR-T cells	Preclinical studies *in vitro* and *in vivo*

ADC — antibody-drug-conjugates; BiTE — bispecific T cell engagers; mAbs — monoclonal antibodies

CD22

CD22, like CD19, is a B cell-restricted protein. CD22 expression starts in the early stages of B cell development. In pro-B cells, it occurs as a cytoplasmic protein. At the late pre-B cell stage, CD22 appears on the cell surface, where it persists during all subsequent stages of B cell differentiation. CD22 is a type-I, single-pass membrane protein with the ability to bind sialic acid, and therefore it is also known as sialic acid-binding immunoglobulin-like lectin (SIGLEC-2). Interactions with sialylated ligands regulate the ability of CD22 to modulate B cell function. CD22 contains intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) and plays a role in the negative regulation of BCR signaling, by recruiting a cytoplasmic SRC homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) [36]. Upon mAb binding, CD22 is promptly internalized [37], which makes it an ideal target for ADC. Indeed, it has been demonstrated that CD22 may shuttle between endosomal compartment and a cell surface, enabling continuous transportation and intracellular accumulation of pH-sensitive cargo, which contributes to the efficacy of CD22-targeting immunotoxins [38–40].

CD22 is expressed in the majority of B-ALL subtypes, hence it is employed as a target in B-ALL immunotherapy. Inotuzumab ozogamycine, a conjugate of cytotoxic drug ozogamycin with anti-CD22 mAb, is approved for the treatment of adult and pediatric B-ALL patients who do not respond to conventional chemotherapy [41]. Although it has a role in the treatment of R/R B-ALL, it can cause severe, life-threatening hepatotoxicity, which limits its use [42]. CARs targeting CD22 have also been developed [43–46]. Importantly, CD22-specific CAR-T cells exert cytotoxicity also in CD19-negative R/R B-ALL. The results of clinical trials testing the efficacy of CD22-CAR-T cells conducted mainly on B-ALL patients who relapsed from previous CD19 CAR--T cell therapy revealed more than70% complete remission, but only 13.4 months overall survival [47]. In contrast, the very recent results of another two clinical trials (NCT02588456, NCT02650414) have reported very low response rates [48]. Both the previous and the more recent clinical studies employed CARs with single chain variable fragment derived from the same antibody, and the length of the linker between the heavy- and the light-chain variable domains was the only difference. Detailed preclinical investigations have confirmed the impact of the linker length on tonic CAR-T cell signaling and consequent clinical efficacy, indicating that only fine differences in CAR construction can significantly affect the clinical outcome [48].

CD20

CD20 is also a B lineage-restricted antigen, but it is ubiquitously expressed only in mature B cells, hence it is extensively used as an immunotherapy target in malignancies derived from mature B cells. The expression of CD20 starts already during B cell development, at the pre-B cell stage, and persists until B cells terminally differentiate into plasma cells. CD20 also occurs in B-ALL cells, but its expression is heterogeneous, often present only in a small proportion of the leukemic population, therefore only 25% of patients qualify for treatment with anti-CD20 immunotherapy [12]. CD20 is a type II transmembrane protein with four transmembrane helices. It is localized in lipid rafts, in close proximity to BCR, CD40, MHC-II, CD53, CD81, and other receptors. Although the precise physiological role of CD20 remains unclear, both human and animal studies suggest its involvement in B cell activation, Ca^{2+} signaling, and interaction of B cells with T cells and other cells of the microenvironment. A summary of CD20 structure, function, and gene regulation has been recently published [49].

In adult, but not in pediatric, B-ALL, the expression of CD20 is associated with a poor prognosis [50, 51]. A phase III trial conducted in Philadelphia (Ph)-negative B-ALL patients with CD20 expression revealed that the outcome of young adults can be improved by a combination of chemotherapy with rituximab, the anti-CD20 mAb approved for medical use in 1997 [52]. Rituximab is one of the best-studied immunotherapy drugs with low toxicity and manageable side effects, but it is not effective in monotherapy. Hence, rituximab is being added to chemotherapy of adult B-ALL patients when at least 20% of leukemic cells are CD20-positive. In pediatric B-ALL, the benefit of the addition of anti-CD20 mAbs to chemotherapy has not been evaluated in a comprehensive way in clinics. Anti-CD20 mAbs other than rituximab have not been extensively investigated in B-ALL patients. Some preclinical studies suggest that obinutuzumab, a class II anti--CD20 mAb, is superior to rituximab *in vitro* and *in vivo* [53]. CD20 may also be targeted by CARs. CAR-T cells simultaneously targeting CD19 and CD20 antigens were designed to overcome CD19-negative relapses. Indeed, in preclinical models, CD19-CD20-bispecific CAR-T cells were more effective than any single antigen-specific CAR-T cells [54]. These dual CD19/CD20 CARs are already tested in clinical trials in patients with advanced R/R B cell malignancies (NCT04700319, NCT04007029).

CD72

Recently, Nix et al. [25] identified unique surfaceome of MLLr B-ALL subtype, with significant upregulation of adhesion-related proteins and downregulation of MHC-I and MHC-II molecules. This study also revealed significant overexpression and cell surface upregulation of CD72 in MLLr B-ALL.

CD72 is a B cell-specific protein which contains intracellular ITIM domains and is involved in negative regulation of BCR signaling. It binds CD5 molecule on T cells, suggesting its role in the crosstalk between B and T cells. CD72 is abundantly expressed in normal B cells and B cell-derived neoplasms, including B-ALL and B cell lymphomas [25]. Using an *in vitro* yeast display library, the authors developed CD72-binding nanobodies and inserted the sequences recognizing CD72 to lentiviral backbone derived from tisagenlecleucel to generate CAR-T cells targeting CD72. The CD72-directed CAR-T cells effectively killed CD72-expressing B-ALL and B cell lymphoma cell lines including CD19-negative cells, and were not toxic against normal cells including PBMC, IVECs, MSC, iPSC. Importantly, the CD72 CAR-T cells were also effective *in vivo* against MLLr B-ALL cell lines and patient-derived xenografts, without toxicity against normal tissue other that B cell ablation [25].

Overall, considering the aforementioned preclinical results, CD72 CAR-T cells are very promising candidates to be tested in clinical trials as a second-line treatment in patients relapsing after CD19-targeted immunotherapy.

Other targets

Other B cell-specific antigens such as CD23 [55], CD79b [56], CD37 [57], BAFFR [58], and BCMA [59] are currently being tested as CAR targets in malignancies derived from mature B cells, such as chronic lymphocytic leukemia,

non-Hodgkin lymphoma, and multiple myeloma. Most of these molecules are not abundantly present on normal immature B cells or in B-ALL cells [60–62]. BAFFR was shown to be expressed in some B-ALL subtypes, mainly E2A-PBX rearranged cells, but the levels were usually low to moderate [63].

Alternative targets in MLLr B-ALL

Particular efforts to identify targets alternative to CD19 have been made in MLLr B-ALL, the extremely poor prognosis subtype. The susceptibility of this subtype to undergo lineage switch and to lose B cell-specific antigens has prompted efforts to identify alternative, B cell-unrelated, targets. One such protein, chondroitin sulfate proteoglycan (CSPG4), also known as neuron-glial antigen 2 (NG2), has been already tested as a putative CAR target in MLLr B-ALL cell line KOPN8 in a proof-of-concept study [64]. NG2 is a diagnostic marker of MLLr B-ALL which is associated with leukemia invasiveness, central nervous system infiltration, and poor patient survival [65]. It was also found that NG2 is important for MLLr B-ALL engraftment to NSG mice and that blockage of NG2 with mAbs leads to relocation of leukemic blasts from the bone marrow to peripheral blood, increasing sensitivity to chemotherapy [66]. However, NG2 is present in only about 50% of leukemic blasts [66] and is also expressed in normal tissues [67], which are significant drawbacks limiting the utility of the antigen as a clinically relevant immunotherapy target.

Another approach proposed in MLLr B-ALL is the simultaneous targeting of two antigens, CD19 and CD133 [68]. CD133, also known as prominin 1 (PROM1), is a stem cell marker and a target gene of the MLL-AF4 oncoprotein [69]. CD133 is abundantly expressed in MLLr B-ALL and is maintained in CD19-negative leukemic cells. Tandem CARs targeting CD19 and CD133 killed leukemic cells expressing only one of the antigens, but the CARs' cytotoxic activity was superior when both antigens were present simultaneously, both *in vitro* and *in vivo*. However, as CD133 is also present on normal hematopoietic stem and progenitor cells (HSPC) at similar levels, the occurrence of on-target off-tumor cytotoxicity has already been reported [70]. Further studies are needed to address the safety issue of these tandem CAR-T cells.

Summary, perspectives, concluding remarks

The success of CD19- and other B cell antigen-targeted therapies in B-ALL has already proved that cell type-specific antigens are appropriate targets for immunotherapy. However, as antigens currently applied in clinics are dispensable for B-ALL cell survival, antigen loss is the major drawback and limitation of B-ALL immunotherapies. This can result from clonal heterogeneity of leukemic population and the selection of antigen-negative subclones or from treatment-related downregulation of target antigens [71]. In CAR-T cell therapy, the emerging approach to overcome this limitation is the use of bi- and tri-specific CARs [72]. Combinatorial targeting has already presented superior efficacy in preclinical models [68, 73] and dual-specificity CAR-T cells directed to CD19 and CD20/CD22 are already being assessed in clinical trials for the treatment of B cell malignancies. In this context, better characterization of clonal heterogeneity, predominantly with respect to target antigens expression, may translate into rational design of combinatorial immunotherapies and may diminish the risk of relapse.

Finally, novel surface antigens crucial for malignant cell survival should be identified and tested as potential alternative immunotherapy targets. This is particularly needed in subtypes derived from B cell precursors at early stages of B cell development and displaying phenotypic plasticity, such as MLLr B-ALL or TCF3-ZNF384 fusion. The broader range of possible immunotherapy targets may pave the way towards more effective and durable immunotherapies.

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Author's contributions

MF — sole author.

Conflicts of interest

The author declares no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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