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REVIEW

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Mechanisms of antigen-dependent resistance to chimeric antigen receptor (CAR)-T cell therapies

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Abstract

Cancer immunotherapy has reshaped the landscape of cancer treatment over the past decades. Genetic manipulation of T cells to express synthetic receptors, known as chimeric antigen receptors (CAR), has led to the creation of tremendous commercial and therapeutic success for the treatment of certain hematologic malignancies. However, since the engagement of CAR-T cells with their respective antigens is solely what triggers their cytotoxic reactions against target cells, the slightest changes to the availability and/or structure of the target antigen often result in the incapacitation of CAR-T cells to enforce tumoricidal responses. This results in the resistance of tumor cells to a particular CAR-T cell therapy that requires meticulous heeding to sustain remissions in cancer patients. In this review, we highlight the antigen-dependent resistance mechanisms by which tumor cells dodge being recognized and targeted by CAR-T cells. Moreover, since substituting the target antigen is the most potent strategy for overcoming antigen-dependent disease relapse, we tend to highlight the current status of some target antigens that might be considered suitable alternatives to the currently available antigens in various cancers. We also propose target antigens whose targeting might reduce the off-tumor adverse events of CAR-T cells in certain malignancies.

Keywords Chimeric antigen receptor, Resistance, Antigen loss, Antigen downregulation, Solid tumors, Toxicity

Introduction

The way targeted cancer therapies substantially minimized the side effects and toxicities of cancer treatment methods can be a story of excellence. The revolution that started with the application of monoclonal antibodies (mAbs) to target and eliminate distinct tumor cells continued with the development and clinical success of various other treatment modalities that can be considered as mAb derivatives. Antibody–drug conjugates (ADCs), bispecific T-cell engagers (BiTEs®), immunotoxins, and chimeric antigen receptor (CAR) T cells and natural killer (NK) cells can all be named as targeted cancer treatment approaches whose advent has changed the face of the fight against cancer. Of the mentioned novel approaches, CAR-T cells are the most novel ones. To this date, CAR-T cell therapies have received the green light

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from the United States Food and Drug Administration (US FDA) for the treatment of six different malignancies that include relapsed/refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), Primary mediastinal large B-cell lymphoma, and multiple myeloma (MM) [1–7]. As of today, five CAR-T cell products (*tisagenlecleucel*, *axicabtagene ciloleucel*, *brexucabtagene autoleucel*, *lisocabtagene maraleucel*, and *obecabtagene autoleucel*) target CD19 as the target antigen whereas two other (*idecabtagene vicleucel* and *ciltacabtagene autoleucel*) target B-cell maturation antigen (BCMA) [1–7].

The intelligence behind selecting such antigens corresponds to the favorable qualities that represent them as suitable target antigens for cancer immunotherapy [8, 9]. It may not come as a surprise that all of the mentioned CAR-T cell therapies have been US FDA-approved for the treatment of certain patients with certain hematologic malignancies [1–7]. One of the reasons for the absence of CAR-T cell therapies for the treatment of solid tumors is the lack of tumor-specific antigens (TSA) or the poor therapeutic suitability of tumor-associated antigens (TAA). In sharp contrast with CD19 and BCMA, since most of the TAAs targeted by different CAR-T cells with the intention to treat solid tumor patients are also expressed by normal cells of healthy tissues, their targeting by CAR-T cells leads to life-threatening and irreversible toxicities to the vital organs of the respective patients, an occurrence referred to as "*on-target off-tumor*" toxicities (as opposed to B-cell aplasia which occurs in the case of hematologic malignancies and is manageable via immunoglobulin replacement) [10–13]. On-target off-tumor toxicities in CAR-T cell therapy refer to the unintended CAR-T cell-mediated targeting of healthy cells and tissues that express the same target antigens as the tumor cells however at physiological levels as opposed to overexpressing them. In other words, on-target off-tumor toxicities occur when the targeted antigen is a TAA rather than a TSA [10, 11]. In solid tumors, such as breast cancer or ovarian cancer, this toxicity may occur because the TAAs are not exclusively expressed by tumor cells but are also expressed on the surface of normal cells, such as endothelial cells or organs [14, 15]. In hematologic malignancies such as acute myeloid leukemia (AML), targeting antigens like CD33, which is expressed on both leukemia cells and normal hematopoietic cells, can lead to myelosuppression, resulting in reduced blood cell counts and increased infection risks [16, 17]. These toxicities limit the effectiveness of CAR-T cell therapies and pose challenges for patient safety. This fact highlights the sheer need for the discovery of suitable target antigens that besides being universally applicable for the treatment of a particular cancer type in a broad population of patients, their physiological expression in normal cells is negligible or they are not expressed by normal cells.

Moreover, the need for the discovery of novel immunotherapy target antigens is not satisfied even after the development and approval of a CAR-T cell product that targets a favorable antigen, as tumor cells evade immune recognition by a variety of complicated trickeries [18, 19]. Some of such escape mechanisms are antigen-dependent [18, 19]. Antigen loss is a significant challenge in CAR-T cell therapy for both solid tumors and hematologic malignancies. In this scenario, tumor cells undergo genetic mutations or downregulation of the targeted antigen, leading to the escape of tumor cells from CAR-T cell recognition [20]. In solid tumors, antigen heterogeneity within the tumor can result in subpopulations of cells that no longer express the targeted antigen, therefore, they are not targeted or eliminated by CAR-T cells [20]. Similarly, in hematologic malignancies, antigen loss can occur through clonal evolution, with some leukemia cells losing the targeted marker, such as CD19 in B-cell malignancies [20]. This antigen loss reduces the efficacy of CAR-T cell therapy, limiting long-term treatment success. While such mechanisms are undertaken by the tumor cells of a patient, a particular CAR-T cell treatment whose antigen has been the subject of tumor evasion can no longer be utilized for the treatment of that patient. Such patients should now undergo CAR-T cell treatments that target a different suitable antigen (for example, in the case of a B-ALL patient with CD19-negative disease relapse, CD22- or CD123-redirected CAR-T cells might be reliable therapeutic options) [8]. In this review, we detail the antigen-dependent mechanisms that tumor cells undertake to evade the tumoricidal force of a particular antigen-dependent treatment modality. Moreover, we highlight the possible applicability of some of the most novel antigens that have been targeted by CAR-T cells for more precise antitumor purposes by minimizing the off-tumor toxicities or as alternative target antigens in times of antigen-negative disease relapse.

CAR-T cell therapy basics and manufacturing

Introduced in the late 1980s, CAR-T cells came into the spotlight as genetically modified T cells engineered to express a single-chain variable fragment (scFv), derived from a mAb, recombinantly fused to the CD3ζ chain of the T cell receptor (TCR)/CD3 complex [21–23]. Of note, our research team has used single-domain antibodies (also known as nanobodies or VHH) as the antigen-recognition domain of CAR-T cells and has demonstrated that nanobody-based CAR-T cells are also functionally comparable to their scFv-based counterparts [24, 25]. Since then, more than five generations of CAR-T

cells have been developed by scientists to address the limitations of this novel type of cancer treatment. Anatomically, a conventional CAR construct is composed of a targeting moiety, fused to a spacer fragment that connects it to the hydrophobic membrane-spanning domain, all of which are followed by the intracellular domains consisting of one or two costimulatory domains and an activation domain (which are responsible for the activation and tumoricidal signaling of CAR-T cells following target antigen encounter) [9]. To this date, researchers have chosen different components for the construction of their desired CAR molecule due to the substantial impacts each of the domains could have on the persistence and antitumor efficacy of the resultant CAR-T cells. The early-day CAR-T cells (dubbed first-generation CARs) expressed synthetic receptors that only harbored an activation domain as their signaling domain. As time passed, researchers reported poor CAR-T cell activation and tumoricidal efficacy, to address which one or two costimulatory domains were introduced into the construct of the first-generation CARs only to develop novel generations of these synthetic receptors (named second- and third-generation CARs, respectively) [26]. Recently, fourth- and fifth-generation CAR-T cells have also been devised by investigators to amplify the tumoricidal capacity of CAR-T cells, especially in the context of solid tumors [26, 27]. As slightly modified adaptations of the second-generation CARs, fourth-generation CAR-T cells (also known as *armored CAR-T cells*) are designed with a built-in domain that can initiate the expression of a cytokine of interest upon the antigen-dependent activation of the CAR-T cell whereas fifth-generation CARs contain a truncated fragment of an intracellular domain of a certain cytokine that capacitates fifth-generation CAR-T cells to initiate the downstream signaling of the mentioned cytokine following CAR-T cell activation [26, 27].

CAR-T cell manufacturing begins with the process of leukapheresis to isolate T cells from the patient's peripheral blood. CAR-T cells produced using the mentioned procedure (which are known as autologous CAR-T cells, as is in the case of FDA-approved products) manage to tackle the limitations of graft-versus-host disease (GvHD) or graft rejection which might occur in the case of allogeneic CAR-T cells (CAR-T cells produced from T cells obtained from healthy third-party donors) [28]. However, some clinical trials have investigated the safety and therapeutic efficacy of allogeneic CAR-T cells reasoning that this platform benefits from several advantages such as allogenic CAR-T cells are less time-consuming to produce or the end-product might be more affordable [28]. After leukapheresis, the ex vivo activation of the obtained T cells is carried out using CD3/CD28-specific mAb-coated beads or artificial antigen-presenting cells that surface-express cognate ligands for CD3 and CD28, as the T cells are supplemented with a variety of important cytokines (such as IL-2 or IL-7/15) [29, 30]. Following this step, T cells are genetically manipulated to express the desired CAR constructs on their surface. This step is carried out using viral approaches (such as using retroviral or lentiviral vectors) or non-viral techniques (such as using mRNA electroporation or transposon-based gene delivery methods) [31–33]. Of note, even though the mentioned viral vectors insert the CAR construct-encoding DNA fragment into the genome of the transduced T cells in a random fashion, they have been regarded as a safe approach, so far [34]. Furthermore, since mRNA-based CAR-T cells transiently express CAR molecules, they might be a more sensible platform for CAR-T cell therapies entwined with severe off-tumor toxicities [31]. mRNA-based CAR-T cells are also less expensive to produce, in comparison with CAR-T cells generated using viral methods [31]. After genetic manipulation, the T cells are cultured until they reach the desired population required for an effective and safe CAR-T cell therapy [32]. Ultimately, the resultant CAR-T cells are cryopreserved and shipped to the desired medical center for infusion into the desired patients, a process which is normally carried out as a single round of administration or sequential administrations (over the course of 2 to 3 days).

CAR-T cell manufacturing faces several challenges, including the time-consuming and costly process of extracting, engineering, and expanding T cells ex vivo. These steps can lead to variability in product quality and delays in treatment, especially for patients with aggressive cancers. Additionally, complex infrastructure and skilled personnel are required for production. To address these issues, scientists are investigating in vivo CAR-T cell generation, where CAR transgenes are directly delivered to a patient's T cells in the body. This approach could streamline production, reduce costs, enhance scalability, and provide faster, more accessible therapies, potentially overcoming the limitations of traditional methods.

In a 2020 study by Agarwal and colleagues, the investigators introduced a groundbreaking method for generating CAR-T cells directly in the body, focusing on CD4+ T cells [35]. Researchers employed a CD4-specific lentiviral vector (CD4-LV) to deliver the CD19-CAR transgene exclusively to CD4+ lymphocytes [35]. In a preclinical model using NSG mice with human peripheral blood mononuclear cells, treatment with CD4-LV led to the successful expression of the CAR in 40–60% of CD4+ cells, while sparing CD8+ T cells [35]. These manufactured CAR-T cells exhibited a Th1/Th2 phenotype and demonstrated the ability to effectively target

and eliminate CD19+ B cells [35]. In tumor-bearing mice, CD4-LV treatment resulted in more rapid and effective tumor clearance compared to treatments involving CD8-LV or combinations [35]. The study highlighted the applicability of in vivo CAR-T cell generation made up of a specific cell pool of CD4+ cells [35]. In a 2022 investigation by Agarwalla and colleagues, the researchers explored an innovative approach for the in vivo generation of CAR-T cells using bioinstructive scaffolds [36]. The researchers designed implantable scaffolds that provide a controlled environment for the localized expansion and activation of T cells, which were genetically engineered to express CARs [36]. These retroviral particle-supplied scaffolds, when implanted into mice, facilitated the rapid in vivo production of CAR-T cells without requiring ex vivo manipulation [36]. The scaffolds were engineered to release cytokines and other signals that promoted T cell activation and proliferation, leading to a substantial number of CAR-T cells in the local tissue [36]. These CAR-T cells were subsequently released into the bloodstream, where they exhibited significant tumoricidal activity against distal tumors [36]. The study demonstrated that these bioinstructive scaffolds enabled both the efficient generation and controlled release of CAR-T cells, offering a promising alternative to traditional cell-based therapies and potentially overcoming limitations such as delayed production [36]. In a recent study by Billingsley and colleagues, the investigators presented a novel approach for generating CAR-T cells in vivo using an mRNA technology to minimize manufacturing costs and adverse effects associated with permanent CAR expression [37]. The researchers developed antibody-equipped ionizable lipid nanoparticles (LNPs) designed to deliver mRNA encoding a CAR directly into T cells by targeting pan-T cell markers. These LNPs were engineered to have extrahepatic tropism, thus enabling localized CAR-T cell generation [37]. Upon administration, the LNPs successfully delivered the mRNA to peripheral T cells, inducing their transformation into CAR-T cells without requiring ex vivo cell manipulation [37]. The engineered CAR-T cells showed effective antigen recognition and cytotoxic activity against tumor cells in mouse models [37]. Conclusively, the authors asserted that this technique offered a highly efficient and minimally invasive method allowing for rapid in vivo generation of CAR-T cells with potentially reduced side effects and cost [37]. Moreover, In a study by Jiang et al., the researchers evaluated the safety and efficacy of an innovative dual-targeted CAR-T cell therapy in patients with relapsed/refractory MM [38]. This CAR-T cell product targeted both BCMA and CD19, two antigens commonly expressed by myeloma cells and B cells, respectively [38]. The platform named *FasT CAR* , designed for rapid production, involved engineering T cells to express CARs targeting these two antigens, allowing for broader and more potent tumoricidal reactions (38). In this multicenter trial, the therapy demonstrated promising clinical outcomes, with a significant proportion of patients achieving CR (56.3%) or partial responses while the treatment was well-tolerated with manageable adverse events [38]. These results highlighted the potential of dual-targeted CAR-T cell therapy as an effective treatment option for patients with relapsed/refractory MM, offering an expedited path to therapy delivery [38].

Antigen-dependent mechanisms of resistance

All of the CAR-T cell therapies available in the market have been FDA-approved for the treatment of R/R hematologic malignancy patients who have failed previous lines of treatment. This relapse yet somehow manages to stay in the picture, even sometimes after successful CAR-T cell treatments. As cases of relapse undergo other rounds of CAR-T cell treatments, some patients are non-respondent and become resistant to the treatment which necessitates other medical interventions. Over the past years, scientists focused on the identification of the resistance mechanisms by which malignant cells escape immunosurveillance in patients undergoing CAR-T cell treatments and found that target antigen loss, antigen downregulation, lineage switching, epitope loss, CAR expression by leukemic cells, antigen heterogeneity, and antigen shedding are all among antigen-dependent resistance mechanism. In this section, we will discuss these resistance mechanisms in more detail.

Antigen loss

Since the principal signaling cascades driving the activation of CAR-T cells are achieved through CAR constructs following binding to the specific epitope on the target antigen, the slightest changes in the presence and/or structural conformation of the target antigen would render CAR-T cells susceptible to incapacitation. Various clinical trials investigating different CAR-T cell products have more or less struggled with relapse cases in which antigen loss has been documented. According to a study by Orlando and colleagues in which the relationship between disease relapse and CD19 loss was investigated in R/R B-ALL participants (both pediatric and adult) from two Phase II trials investigating *Tisagenlecleucel*, it was elucidated that 12 out of 17 patients (approximately 70%) had CD19-negative disease relapse [39]. In detail, a large population of tumor cells was found to be associated with mutations that result in a truncated and impaired CD19 antigen molecule [39]. Moreover, all of the patients with CD19-negative disease relapse had mutations in exons 2–5 of the CD19 gene [39]. This loss of antigen expression was further investigated and it was

found to be exclusively CD19-associated (as other B-cell antigen genes, such as CD22 and CD20, showed no evidence of mutations) [39]. Such occurrences leave room for targeting other B-ALL-associated antigens (by other CAR-T cell products) whose expression is still maintained by tumor cells in the respective patients. Furthermore, another example can be based on a clinical trial (NCT03185494) conducted by Dai and co-investigators in which CD19/CD22 bispecific CAR-T cells were used for the treatment of 6 R/R B-ALL participants [40, 41]. Five months following the treatment, diseased relapse was reported in one of the participants (all of whom had achieved minimal residual disease (MDR)-negative complete remission (CR) following CAR-T cell treatment) in whom leukemia blasts had lost CD19 expression and also diminished CD22 expression (40, 41). Dai and co-investigators' report points out two potential mechanisms of resistance (antigen loss and antigen downregulation) to CAR-T cell therapies (or any other antigen-dependent immune-based treatment modality) which are regularly undertaken by malignant cells to offset immune recognition [40, 41].

Over the past years, researchers have put a remarkable deal of effort into identifying the mechanism of antigen loss. So far, genetic alterations and epigenetic modifications have been identified as potential antigen loss mechanisms [39, 42]. Regarding genetic alterations (which include mutations, deletions, or gene rearrangements) that occur during CAR-T cell treatment as a result of selective pressure or spontaneously, such occurrences enable malignant cells to escape CAR-T cell-mediated immunosurveillance [39, 42]. In a study by Cortés-López and colleagues, these researchers investigated the mechanisms of CD19 loss following CAR-T cell treatment and identified approximately 200 single-point mutations capable of disrupting CD19 splicing [43]. Moreover, they also uncovered nearly 100 previously unreported splice isoforms arising from "*cryptic splice sites*", which likely produce non-functional CD19 molecules [43]. Cortés-López and colleagues also identified important cis-regulatory elements and trans-acting RNA-binding proteins (namely, PTBP1 and SF3B4), that regulate CD19 splicing, loss of which was shown to induce CD19 mis-splicing, contributing to antigen loss [43]. These findings highlight the complexity of CD19 splicing regulation and its implications for resistance to CD19-based treatment modalities such as CAR-T cells and antibody-based therapeutics [43]. Moreover, such findings accentuate the importance of understanding CD19 splicing dynamics to devise counterstrategies against treatment resistance through interventions targeting splicing mechanisms. Epigenetic modifications also contribute to antigen loss as malignant cells within the tumor milieu undergo epigenetic alterations (such as DNA methylation and histone modifications) that result in silencing or reduced expression of the target antigen through changes in chromatin structure and transcriptional regulation [44, 45

Antigen downregulation and trogocytosis

Antigen downregulation can be considered as one of the most potential mechanisms that nullify the therapeutic effects of a particular CAR-T cell treatment. In 2013, Huang et al. developed a meticulous single-molecule imaging approach to investigate the sensitivity of T cells to antigen peptides presented to them in major histocompatibility complexes (MHC) [46]. In detail, it was elucidated that naïve and memory CD4-positive T cells can be activated by the stimulation of a single peptide-bound MHC (pMHC) molecule and that excessive pMHC molecules could not increase the cytokine production profile of the activated T cells [46]. According to a study conducted by Purbhoo and colleagues, with the help of a single-molecule labeling technique, it was demonstrated that cytotoxic T cells require interactions with only three pMHC molecules to initiate their cytotoxic reactions (47). This scenario is slightly different in the context of CAR-T cells as they require the engagement of a high number of their CARs with target antigens only to initiate the satisfactory signals that govern their cytotoxicity against tumor cells [48]. In this regard, any decrease in the density of tumor surface antigens might be deemed a misfortune for a given CAR-T cell treatment, as it would be expected to result in poor clinical responses. Additionally, other mechanisms have also been known to contribute to a decline in the density of tumor antigens. In a process known as *trogocytosis*, T cells remove target antigens from particular tumor cells and present those antigens at their surface [49]. This occurrence negatively impacts the success rate of CAR-T cell therapy in various aspects (as the low level of target antigens on tumor cells confers them immunity and the expression of tumor antigens by T cells results in their exhaustion and consequent fratricide-mediated elimination) [49, 50]. Combinatorial antigen targeting has been proposed as a potential countertactic in this regard since it can orchestrate more pronounced tumoricidal responses [49].

Lineage switching

Lineage switching has also been regarded as one of the most important resistance mechanisms in patients undergoing CAR-T cell therapies. Substantial evidence reports the appearance of myeloid subtypes in B-ALL patients with mixed-lineage leukemia (MLL) rearrangement (referred to as B-ALL^{MLL}) under the pressure of CD19-based immunotherapeutics [18]. In 2016, Gardner reported the successful treatment of 7 B-ALL^{MLL} patients

with CD19-redirected CAR-T cells, a month after which, two of the patients exhibited the emergence of AML with the loss of CD19 expression (51). According to a report by Jacoby and colleagues, subpopulations of B-ALL cells possess intrinsic plasticity to escape immune recognition through the establishment of myeloid lineages [52]. Whether this phenomenon is restricted only to leukemia malignancies or not needs to be deciphered. Nevertheless, leukemia switching to a myeloid phenotype with the loss of the most important B-cell marker, CD19, can be recognized as a potential resistance mechanism to CD19-based immunotherapeutics, inclusive of CAR-T cell therapies, which might be overcome by leveraging other alternative target antigens.

Epitope loss

Epitope loss has been reported to be present in almost 10–20% of pediatric cases of B-ALL patients; therefore, it has also been recognized as a frequent mechanism of resistance to CAR-T cell therapy [53]. In detail, Sotillo and colleagues demonstrated that the alternative splicing of CD19 mRNAs (those with the absence of exon 2) in leukemia blasts manages to give rise to the expression of a truncated isoform of the antigen that overcomes the mutations of exon 2 (which are known to be associated with antigen loss) [53]. Such truncated CD19 isoforms can no longer be recognized by a particular CAR-T cell product due to the loss of the specific epitope. However, the substitution of the CAR targeting moiety can be undertaken as a counterstrategy in such cases. For instance, Gu and co-investigators conducted a Phase I trial (NCT02975687) to evaluate the tolerability and feasibility of novel CD19-redirected CAR-T cells armed with an scFv that binds a distinct CD19 epitope (rather than the one that FMC63, which is a well-known murine scFv employed as the targeting moiety of particular FDA-approved CAR-T cell products, interacts with) in pediatric and adult R/R B-ALL patients [54]. In detail, with 20 out of 22 patients achieving CR or CR with incomplete count recovery (CRi), the CR/CRi was calculated to be more than 80% [54]. Such clinical success can highlight the feasibility of using CAR-T cell products with novel targeting moieties for tackling the issue of epitope loss.

CAR expression by malignant cells

Recently, it has been demonstrated that some resistant leukemic cells are proficient in the expression of CAR molecules on their surface. This expression results from an inadvertent error in the process of CAR-T cell production. According to a case report by Ruella and colleagues, a patient who had received CD19-redirected CAR-T cells relapsed 9 months following the treatment [55]. Later, it was elucidated that a single leukemic B cell was accidentally genetically manipulated during the process of CAR-T cell production which led to the expression of CD19-specific CARs on its surface [55]. Since the expressed CAR molecules would bind to CD19 on the surface of the leukemic cells and render the antigen inaccessible to CD19-redirected CAR-T cells, CAR-positive leukemic cells could no longer be eliminated by CD19-redirected CAR-T cells [55]. Eventually, Ruella and colleagues devised and developed CAR-T cells specific for the mentioned CD19-specific CAR as a strategy for the eradication of both CD19-specific CAR-positive leukemic cells and CD19-redirected CAR-T cells and reported encouraging in vitro and in vivo results [55]. Furthermore, the investigators demonstrated that such CAR-T cells could be used for minimizing the unfavorable side effects of the prolonged persistence of CD19-redirected CAR-T cells, such as B-cell aplasia [56]. All of the mentioned findings and clinical trials accentuate the fact that regardless of the initial clinical success of a particular CAR-T cell treatment in a patient, there might still be the possibility of disease relapse. To address the issue in certain cases, CAR-T cells should target novel antigens whose expression is still maintained by tumor cells.

Antigen heterogeneity

Antigen heterogeneity in solid tumors refers to the uneven expression of target antigens within different regions or subpopulations of tumor cells [57, 58]. This variability poses a significant challenge to CAR-T cell therapy and represents a major mechanism of resistance. For example, in glioblastoma, the antigen EGFRvIII is a common target for CAR-T cell therapy, but it is expressed in only a subset of tumor cells, leaving EGFRvIII-negative cells to survive and repopulate the tumor [57, 58]. Similarly, in breast cancer, targeting HER2 with CAR-T cells can be effective against HER2-overexpressing cells, but many tumor cells may express low or undetectable levels of HER2, leading to therapeutic failure [59, 60].

This heterogeneity can arise from genetic mutations, epigenetic modifications, or selective pressure exerted by CAR-T cell therapy itself [61]. CAR-T cells effectively eliminate antigen-positive tumor cells, leaving behind antigen-negative or low-antigen-expressing cells, which then proliferate, a process known as antigen escape. For instance, in mesothelioma, targeting mesothelin can fail if some tumor cells lack mesothelin expression. Moreover, solid tumors often contain diverse cancer cell subclones and a suppressive tumor microenvironment, further complicating CAR-T cell efficacy. Addressing this issue requires strategies like targeting multiple antigens (e.g., mesothelin and EGFR), engineering CAR-T cells with broader specificity, or combining CAR-T cell

therapy with other treatments to overcome antigen heterogeneity and reduce resistance [9, 61, 62].

CAR-T cell therapy of hematologic malignancies has also been associated with antigen heterogeneity as a potential mechanism of resistance. Unlike solid tumors, hematologic cancers such as leukemias and lymphomas often exhibit more uniform antigen expression initially. However, heterogeneity arises due to clonal evolution, selective pressure, or genetic alterations during disease progression or after CAR-T cell treatment [63–65]. For example, in B-cell malignancies such as B-ALL or non-Hodgkin lymphoma, CAR-T cells targeting CD19 have shown remarkable success. However, some malignant cells may downregulate or lose CD19 expression entirely, either due to splice variants or genetic mutations. This phenomenon, known as antigen escape, allows CD19-negative subpopulations to evade CAR-T cell-mediated elimination, leading to disease relapse. Similarly, in AML, targeting antigens like CD33 or CD123 is complicated by heterogeneity, as these antigens are variably expressed across different leukemic cell populations [66, 67]. Some subclones may lack these markers, enabling their survival and expansion [67]. Furthermore, antigen expression may vary across different stages of the disease, such as between immature blasts and more differentiated cells, making it challenging to select a single, universally expressed target. Addressing antigen heterogeneity in hematologic malignancies requires strategies like dual-antigen targeting (e.g., CD19 and CD22 in B-cell ALL), bispecific CAR-T cells, or combination therapies to overcome resistance and improve treatment durability.

Antigen shedding

Antigen shedding is a biological process in which tumor cells release surface antigens into their surrounding environment, typically through proteolytic cleavage or other mechanisms [68–70]. This phenomenon can have significant implications in cancer immunotherapy, particularly in CAR-T-cell therapy [69, 70]. Proteolytic cleavage, exocytosis, and tumor microenvironment factors are known as the main contributors of the antigen shedding process [69, 70]. Antigen shedding can result in therapeutic resistance, immune modulation, and tumor immune escape [68–70]. So far, various antigens have been shown to be secreted from tumors, for instance, Glypican-3 (GPC3). GPC3, a cell surface glycoprotein overexpressed in hepatocellular carcinoma (HCC), has emerged as a promising target for immunotherapy. However, serum GPC3 (sGPC3), resulting from the shedding of cell surface GPC3, is frequently elevated in HCC patients [71]. This shed GPC3 is known to inhibit the function of membrane-bound GPC3, potentially impacting the effectiveness of CAR-T cell therapies targeting this antigen. In 2021, Sun et al. investigated the influence of sGPC3 on GPC3-targeted CAR-T cells. Briefly, these researchers developed two CAR-T cell constructs, targeting distinct GPC3 epitopes, and tested them in vitro and in vivo [71]. Both CAR-T cell types exhibited strong, GPC3-specific antitumor activity in models with cell-surface GPC3. However, sGPC3 presence significantly reduced cytokine release and CAR-T cell cytotoxicity in vitro [71]. In animal models with Hep3B xenograft tumors expressing sGPC3, CAR-T cell efficacy was markedly diminished under both low and high tumor burdens [71]. Mechanistically, sGPC3 bound to CAR-T cells without effectively activating them, acting as a dominant negative regulator by competing with cell-surface GPC3 for CAR binding [71]. The study highlighted sGPC3 as a novel mechanism of immune escape in HCC, providing critical insights for patient selection and optimizing GPC3-targeted CAR-T cell therapies in future clinical trials [71].

Alternative antigens

In this section, we propose some target antigens that might someday be suitable for lessening the off-tumor toxicities of CAR-T cells in certain malignancies or be potential alternatives to the currently available target antigens against which CAR-T cells have been developed and investigated in various hematologic malignancies and/or solid tumors (Table 1 and 2).

CLL-1

Most patients with AML suffer from poor prognosis, and treatment options have not been renovated over the past decades [72]. So far, multiple target antigens have been leveraged for the immunotherapy of patients with AML, which include TIM3, FLT3, CD33, CD47, CD70, and CD123 [8]. However, the need for the availability of other substitute antigens with high expression levels in AML is still sensed. CLL-1 (alternatively known as CD371) might be a suitable candidate for an immunotherapy target antigen because of its high expression level in AML patient samples (90%) and that healthy hematopoietic stem cells (HSCs) and non-hematopoietic tissues have been reported to be CLL-1-deficient (despite the fact that HSCs express CD33 and CD123) [73, 74]. In this regard, targeting CLL-1 for therapeutic purposes enables hematopoietic recovery in the respective AML patients [72, 74]. In 2017, Leong et al. developed CD3/CCL-1-bispecific antibodies with low- or high-affinity CD3-specific arms and reported that only the low-affinity panel of the bispecific antibodies exerted cytotoxic effects against target cells as they were well-tolerated in preclinical monkey models [72]. In 2018, De Togni et al. developed CLL-1-redirected CAR-T cells and reported that a particular panel of these CAR-T cells exhibited dose-dependent

Table 1 A summary of CAR-T cells redirected against different antigens for the treatment of hematologic malignancies

Clinical trial identifier	Antigen	Indication	Estimated enrollment	Start date	Estimated Completion Date	Source	Conditioning regimen	Phase	Location
NCT04257175	CD19	Acute myeloid leukemia	10	February 18, 2020	December 1, 2023	–	Flu/Cy	II/III	–
NCT04181827	BCMA	Relapsed and Lenalidomide-refractory multiple myeloma	400	June 12, 2020	April 10, 2026	Autologous	–	III	Multiple locations
NCT04287660		Multiple myeloma	30	October 19, 2017	January 31, 2024	Autologous	Clarithromycin, lenalidomide, and dexamethasone	III	China
NCT04340167	CD22	R/R acute lymphoblastic leukemia	100	May 1, 2020	October 1, 2022	Autologous	Flu/Cy	II	China
NCT04689659	CD7	T-cell leukemia	50	February 1, 2021	February 1, 2023	Allogeneic	–	II	China
NCT04351022	CD38	R/R acute myeloid leukemia	20	July 1, 2017	December 31, 2023	–	–	I/II	China
NCT02259556	CD30	Hodgkin's lymphoma/ non-Hodgkin's lymphoma	30	October 2014	October 2029	Autologous	Flu/Cy	I/II	China
NCT03277729	CD20	Hematologic malignancies	35	December 5, 2017	November 16, 2037	Autologous	Flu/Cy	I/II	US
NCT04499339	SLAMF7	Multiple myeloma	38	July 22, 2020	March 2024	Autologous	–	I/II	Germany
NCT02958397	CD33	Myeloid malignancies	45	October 2016	October 2020	–	–	I/II	China
NCT04230265	CD123	Acute myeloid leukemia and B-cell acute lymphoblastic leukemia	45	January 28, 2020	April 2022	–	Flu/Cy	I	Germany
NCT04219163	CLL-1	Acute myeloid leukemia	18	July 9, 2020	July 31, 2038	Autologous	–	I	US
NCT04712864	CD4	R/R T-cell lymphoma	50	May 2021	September 2025	Autologous	–	I	US
NCT02706392	ROR1	Lymphoma and leukemia	60	March 16, 2016	December 1, 2036	Autologous	Flu/Cy	I	US
NCT04690595	BAFF-R	R/R B-cell acute lymphoblastic leukemia	21	April 20, 2021	April 20, 2024	Autologous	–	I	US
NCT03672318	CD138	Multiple myeloma	33	January 14, 2019	October 2032	Autologous	Flu/Cy	I	US
NCT03125577	CD70	B-cell malignancies	100	July 15, 2017	December 2021	Autologous	Flu/Cy	I/II	China
NCT04429438	CD79b and CD13	B-cell lymphoma	11	June 1, 2020	December 31, 2023	–	–	I/II	China
NCT04555551	GPRC5D	Multiple myeloma	36	August 19, 2020	August 2023	Autologous	–	I	US
NCT02541370	CD133	Acute myeloid and lymphoid leukemias	20	June 2015	June 2019	Autologous	–	I/II	China

Table 1 (continued)

Clinical trial identifier	Antigen	Indication	Estimated enrollment	Start date	Estimated Completion Date	Source	Conditioning regimen	Phase	Location
NCT04803929	ILT3	R/R acute myeloid leukemia	25	March 3, 2021	March 1, 2026	Autologous	–	Early I	China
NCT02958384	Lewis Y	Myeloid malignancies	45	October 2016	October 2020	Autologous	–	I/II	China
NCT03710421	CS1	Multiple myeloma	30	February 13, 2019	December 10, 2021	Autologous	Flu/Cy	I/II	US
NCT03018405	NKG2D	Acute myeloid leukemia, multiple myeloma	146	December 2016	August 2021	Autologous	–	I/II	US and Belgium
NCT03473457	CD56	R/R acute myeloid leukemia	50	April 1, 2018	December 31, 2022	–	–	NA	China
NCT03291444	CD117	Acute lymphoblastic leukemia, acute myeloid leukemia	30	September 23, 2017	March 1, 2022	–	–	I	China
NCT04429438	CD70	B-cell lymphoma	11	June 1, 2020	December 31, 2023	–	–	I/II	China
NCT00881920	Kappa light chain	Lymphoma, myeloma, and leukemia	54	July 2009	July 2034	Autologous	Flu/Cy	I	US

Abbreviations: Flu, fludarabine; Cy, cyclophosphamide

cytolytic activities against the CLL-1-positive cell line U937 [73]. Moreover, as in vivo experiments further confirmed, CLL-1-redirected CAR-T cells inhibited tumor progression and prolonged the survival of mouse models established with the CLL-1-positive cell line THP-1 [73]. Additionally, Wang and colleagues shed more light on the applicability of CLL-1 as a possible cancer immunotherapy antigen as they generated CLL1-redirected CAR-T cells that, besides favorable tumoricidal capacity towards certain CLL-1-positive cell lines and primary AML patient malignant cells, induced pronounced antileukemic responses in preclinical animal models [75]. In detail, CLL-1-redirected CAR-T cells were tumoricidal against both progenitor and mature myeloid cells as they managed to spare CLL-1-negative HSCs [75]. In 2020, Ataca Atilla and colleagues attempted to adjust the tumoricidal capacity of CLL-1-redirected CAR-T cells both in vitro and in vivo by incorporating different components in their CAR constructs and also by engineering their CAR-T cells to secret IL-15 (hereafter referred to as 15.CLL-1.CAR-T cells) [76]. Aside from showing stronger expansion and fewer signs of differentiation as compared to CAR-T cells not co-expressing transgenic IL-15 (with the CD28-CD3ζ-CD8 construct) in vitro, 15.CLL-1.CAR-T cells also showed better expansion profiles in AML patient-derived xenograft (PDX) models and those established with AML cell lines [76]. However, the investigators reported lung and liver inflammation attributed to 15.CLL-1.CAR-T cell administration accompanied by elevated levels of IL-2, IL-15, and TNF-α for the management of which antibody-dependent blockade of TNF-α and at-will depletion of 15.CLL-1.CAR-T cells achieved through the implementation and activation of a small molecule-induced caspase 9 switch (iCasp9) were taken into account [76]. The combinatorial management countertactic was reported to amplify the tumoricidal capacity of 15.CLL-1.CAR-T cells while being capable of minimizing the mentioned toxicities [76]. According to another study, Lin et al. generated CLL-1-redirected CAR-T cells deficient in the expression of PD-1 (achieved through shRNA-induced silencing) and reported that this strategy augmented the antitumor activity of CLL-1-redirected CAR-T cells against CLL-1-positive AML cells [77]. Moreover, according to a recent case report by Zhang et al., CLL-1-redirected CAR-T cells were capable of inducing morphological and complete molecular remission (CMR) in a patient with secondary AML (which was sustained for more than 10 months) [78]. Also, according to the results of an ongoing Phase I/II clinical trial, Zhang et al. reported encouraging results in four pediatric R/R AML patients following the administration of autologous iCasp9-equipped CLL-1-redirected

Table 2 A summary of CAR-T cells redirected against different antigens for the treatment of patients with solid tumors

Clinical trial identifier	Antigen	Indication	Estimated enrollment	Start date	Estimated Completion Date	Source	Conditioning regimen	Phase	Location
NCT03373097	GD2	Neuroblastoma	42	January 5, 2018	December 2027	Autologous	–	I/II	Italy
NCT02744287	PSCA	Pancreatic cancer, prostate cancer	151	November 2016	February 2024	–	–	I/II	US
NCT03941626	DR5	Hepatoma	50	September 1, 2019	December 1, 2021	Autologous	Flu/Cy	I/II	China
NCT04429451	PSMA	Various solid tumors	100	January 1, 2020	December 31, 2024	Autologous	–	I/II	China
NCT02541370	CD133	Advanced solid tumors	20	June 2015	June 2019	Autologous	–	I/II	China
NCT04107142	NKG2DL	R/R solid tumor	10	December 1, 2019	March 1, 2021	Allogeneic	–	I	Malaysia
NCT03874897	Claudin18.2	Advanced solid tumor	50	March 26, 2019	March 20, 2022	Autologous	Flu/Cy	I	China
NCT02442297	HER2	Brain tumor	28	February 2016	January 2036	Autologous	–	I	US
NCT04153799	EGFR	Non-small cell lung cancer	11	November 1, 2019	December 2022	Autologous	–	I	China
NCT04214392	MMP2 (Matrix Metallopeptidase 2)	Glioblastoma	36	February 26, 2020	February 6, 2023	Autologous	–	I	US
NCT02905188	Glypican 3	Hepatocellular carcinoma	14	March 28, 2019	October 2036	Autologous	Flu/Cy	I	US
NCT04003649	IL-13Rα2	R/R glioblastoma	60	September 26, 2019	December 1, 2022	Autologous	–	I	US
NCT03851146	Lewis Y	Advanced solid tumors	21	November 24, 2016	December 31, 2024	Autologous	Flu/Cy	I	Australia
NCT04025216	TnMUC1	Advanced solid tumors	112	October 10, 2019	October 31, 2036	Autologous	Flu/Cy	I	US
NCT04185038	B7-H3	Central nervous system tumor	70	December 11, 2019	May 2041	Autologous	–	I	US
NCT02915445	EpCAM	Nasopharyngeal carcinoma and breast cancer	30	July 2016	July 2022	Autologous	Cy	I	China
NCT02706392	ROR1	Non-small cell lung cancer and breast cancer	60	March 16, 2016	December 1, 2036	Autologous	Flu/Cy	I	US
NCT04020575	cleaved form of MUC1 (MUC1*)	Breast cancer	69	January 15, 2020	January 15, 2035	Autologous	–	I	US
NCT04513431	CEA	Colorectal cancer	18	August 30, 2020	August 30, 2023	–	––	Early I	China
NCT03323944	Mesothelin	Pancreatic cancer	18	September 15, 2017	September 2021	–	Cy	I	US
NCT03993743	CD147	Advanced hepatocellular carcinoma	34	May 27, 2019	May 27, 2022	Autologous	–	I	China
NCT03907527	MUC16	Ovarian cancer	71	April 30, 2019	April 1, 2026	Autologous	–	I	US
NCT03283631	EGFRvIII	Glioblastoma	2	May 30, 2018	June 30, 2020	Autologous	–	I	US
NCT02617134	MUC1	Glioblastoma, gastric and colorectal cancer	20	November 2015	November 2018	Autologous	–	I/II	China
NCT03672305	c-Met	Hepatocellular carcinoma	50	October 1, 2018	October 30, 2019	–	–	Early I	China
NCT01218867	VEGFR2	Renal cancer	24	November 10, 2010	December 15, 2015	Autologous	Flu/Cy	I/II	US

Table 2 (continued)

Flu, fludarabine; Cy, cyclophosphamide

CAR-T cells as all of the reported patients experienced mild and manageable toxicities [79]. In detail, three of the patients (75%) experienced MRD-negative CR which highlights the therapeutic applicability and the favorable tolerability index of CLL-1-redirected CAR-T cells for the treatment of R/R AML, despite the fact that more meticulous clinical investigations are warranted [79].

CD13

CD13 expression has been detected in a significant proportion (73.9 to 82%, according to a report by Thalhammer-Scherrer) of AML patients [80]. In 2018, Lee et al. generated CAR-T cells targeting CD13 and CD33, as two AML antigens [81]. They reported that these CAR-T cells efficaciously targeted CD13- and CD33-expressing cell lines in culture [81]. Moreover, the researchers proposed that targeting more than one AML antigen using combinatorial CAR-T cells may diminish off-tumor toxicities that occur following the administration of such CAR-T cells in preclinical animal models of AML [81]. In 2020, He et al. investigated bispecific split CAR-T cells targeting CD13 and TIM3 [82]. First, these researchers generated nanobodies targeting CD13 and demonstrated that these nanobodies precisely target CD13-expressing AML cells in vitro [82]. Next, they incorporated these nanobodies into CAR constructs and developed CD13-redirected CAR-T cells [82]. In vivo assessments in NSG mice transplanted with THP-1 cells also demonstrated that treatment with CD13-redirected CAR-T cells effectually results in tumor outgrowth repression in these animals [82]. He et al. also investigated the potential of CD13-redirected CAR-T cells in PDX NSG mouse models signifying that these CAR-T cells are capable of specific tumor cell eradication [82]. Moving forward, He and colleagues generated bispecific split CAR-T cells targeting CD13 and TIM3 and evaluated their tumoricidal activity in AML xenograft and AML PDX models [82]. The CAR molecules of these CAR-T cells were generated from a CD13-specific nanobody joined to the CD3ζ domain and a TIM3-specific scFv joined to CD28 and 4-1BB co-stimulatory domains [82]. Once these CAR-T cells encounter CD13 (present on HSCs and AML stem cells), this interaction would result in their low activation [82]. On the other hand, upon encountering both CD13 and TIM3 (only present on AML stem cells), these CAR-T cells become fully activated and tumoricidal [82]. Conclusively, He et al. claimed that this stratagem may lead to diminished levels of off-tumor toxicities to normal HSCs in preclinical models of AML [82].

LILRB4

Acute monocytic leukemia (FAB M5) is a subtype of AML occurring in 1 out of 5 AML cases in children [83]. It is also observed in more than 50% of infants with AML [83]. Extramedullary relapse of this AML subtype after allogeneic hematopoietic stem cell transplantation (HSCT) is very common which highlights the necessity for developing more effective treatment modalities [84]. CD33, CD123, and FLT3 are all among the popular AML target antigens against which CAR-T cells have shown tumoricidal activity both in vitro and in vivo [8, 85–87]. However, these antigens are also expressed by normal HSCs [86, 88, 89]. This non-exclusive expression of such antigens renders HSCs susceptible to CAR-T cell-mediated target cell eradication resulting in on-target off-tumor toxicities in preclinical models [86, 88, 89]. In this regard, a target antigen with monocytic AML cell-restricted expression may be an ideal CAR-T cell target unlikely to mediate such toxicities.

AML cells express the leukocyte immunoglobulin-like receptor B (LILRB) family members [83, 90]. Among several members, LILRB4 is highly expressed on monocytic AML cells allowing for the selective targeting of this subtype with significant discrimination from non-monocytic ones [83]. In 2018, John et al. developed LILRB4-redirected CAR-T cells capable of inducing antitumor effects against LILRB4-positive monocytic AML cells [83]. This study also added that LILRB4-redirected CAR-T cells do not mediate cytotoxicity against normal CD34-positive umbilical cord blood cells, as demonstrated in in vitro and in vivo assessments [83]. The researchers also reported that these CAR-T cells mediated significant tumoricidal activity specifically toward monocytic AML cells while sparing normal HSCs [83]. Overall, this rationale might pave the way for such antigens whose tumor-restricted expression can minimize off-tumor toxicities and disease relapse possibilities and elevate the overall survival rate of patients with this subtype of AML receiving CAR-T cell therapy. However, more in-depth assessments and preclinical studies are required for further validation of such conclusions.

CEACAM7

Pancreatic ductal adenocarcinoma (PDAC) is regarded as one of the deadliest solid malignancies that is commonly associated with high rates of metastasis [91, 92]. Moreover, clinical trials that investigated the therapeutic efficacy of mesothelin- or HER2-redirected CAR-T cells (NCT01935843) in PDAC patients have reported limited

outcomes [31, 93, 94]. In this regard, it is necessary to discover suitable target antigens and develop potential CAR-T cells for improving the overall survival of PDAC patients. CEACAM7 (alternatively known as CGM2), like other members of the CEA protein family, undergoes an altered expression profile following tumorigenesis [95]. However, the CEACAM7 expression profile is somehow distinctive from that of the other CEA family members, as it is limited to the apical membrane of the ductal cells of the pancreas and adult colon epithelium (with no documented expression in the small intestine and stomach) [96–98]. Fortunately, no CEACAM7 expression has been reported in tissues whose target antigen expression has often contributed to the emergence of off-tumor CAR-T cell toxicities (such as the biliary tract and lungs) [96, 97]. Based on these favorable characteristics, Raj et al. generated CEACAM7-redirected CAR-T cells to further investigate the applicability of this antigen in vitro and in vivo [98]. In detail, Raj et al. reported CEACAM7 expression in particular populations of PDAC tumors alongside negligible expression levels in healthy tissues [98]. Moreover, their CEACAM7-redirected CAR-T cells induced remission in PDX tumor models in an antigen-dependent fashion which might highlight the applicability of this antigen for further meticulous studies [98].

αvβ6

αvβ6 is an integrin specific to the epithelium which exhibits an indiscernible or low-rate expression profile in healthy tissues [99, 100]. The upregulation of αvβ6 is observed in various conditions including liver or kidney fibrosis and lung injury as well as in wound healing [99, 100]. Abnormal expression of αvβ6 has been documented in various solid malignancies such as lung, colon, breast, cervical, and pancreatic cancers correlating with unfavorable prognosis and poor survival rates [101]. These facts highlight the critical roles of αvβ6 in tumor cell migration and aggression as well as in the onset of epithelial-mesenchymal transition (EMT) [102, 103].

In 2017, for the first time, Whilding et al. developed second-generation CAR-T cells targeting αvβ6 [104]. The targeting domain of these CAR-T cells consisted of a virus-derived peptide that mediated a highly selective αvβ6 targeting ability [104]. The antitumor activity of these CAR-T cells was confirmed in xenograft models of pancreatic, breast, and ovarian cancers which had varying more-than-normal αvβ6 expression levels [104]. However, despite the highly selective tumoricidal activity of these cells, mild and manageable toxicities were observed in the preclinical models, but only when the CAR-T cell administration dose exceeded the normal therapeutic level [104]. In 2019, Whilding and colleagues co-expressed the IL-8 receptors CXCR1 or CXCR2 in αvβ6-targeting CAR-T cells to exploit tumor-derived IL-8 as a helping hand for enhancing the tumor-site trafficking capability of CAR-T cells in solid tumors [105]. IL-8 plays numerous roles in tumor angiogenesis, cancer stem cell survival, etc. and IL-8 level in the circulation has a direct relationship with malignancy severity and progression in various types of solid tumors [105]. Whilding et al. reported that αvβ6-redirected CAR-T cells co-expressing the IL-8 receptors CXCR1 or CXCR2 (with those CAR-T cells co-expressing the latter IL-8 receptor being superior) effectively migrated towards IL-8-secreting tumor sites [105]. Moreover, administration of αvβ6-redirected CAR-T cells co-expressing CXCR2 resulted in enhanced tumoricidal activity in αvβ6-positive ovarian or pancreatic cancer xenograft models [105]. In 2021, Phanthaphol et al. reported upregulated expression of αvβ6 in the tissue samples of patients with Cholangiocarcinoma (CCA) (in 23 out of 30 patients; almost 73%) correlating with poor prognosis [106]. These researchers developed second- and fourth-generation αvβ6-targeting CAR-T cells and reported that both of these CAR-T cells mediate effective antitumor activity against αvβ6-expressing CCA cells and spheroids [106]. They also added that the fourth-generation αvβ6-redirected CAR-T cells exhibit lower IFN-γ secretion levels and higher expansion capacity upon target antigen engagement in comparison to their second-generation counterparts [106]. Moreover, Phanthaphol et al. proposed that fourth-generation αvβ6-redirected CAR-T cells might be a superior choice for targeting αvβ6-expressing CCA cells [106]. Such data might introduce αvβ6 as a CAR-T cell therapy target in a range of solid tumors. However, more preclinical data can pave the way for evaluating this target antigen in clinical trials.

CD37

CD37 is a cell surface-expressed protein belonging to the transmembrane 4 superfamily possessing roles in lymphocyte adhesion, proliferation, migration, and survival [107–109]. It has an expression pattern restricted to cells of the immune system including B lymphocytes [110, 111]. CD37 overexpression is common in B-cell malignancies including MCL, FL, DLBCL, Burkitt's lymphoma, and chronic lymphocytic leukemia (CLL) [110]. Recently, CD37 expression in several T-cell lymphomas has also been reported to correlate with unresponsiveness to common therapies and poor prognosis [109, 112]. So far, clinical trials have been conducted using mAbs and ADCs to assess the validity of CD37 as a target antigen for the treatment of B-cell and T-cell lymphomas [109, 113, 114].

Scarfò et al. developed CD37-redirected CAR-T cells and reported that these cells exhibited target

antigen-dependent effector function against B-cell and T-cell lymphoma models both in vitro and in vivo [109]. Stepping further, these researchers also generated bispecific CAR-T cells targeting CD19 and CD37 and reported that these bispecific CAR-T cells orchestrate effector function, similar to that of CD37-redirected CAR-T cells, in response to engagement with one or both of the target antigens [109]. It is worth mentioning that the results also indicated that CD37-redirected CAR-T cells mediate considerable tumoricidal activity against B-cell and T-cell lymphomas without signs of substantial fratricide [109].

In 2021, Golubovskaya et al. reported the results of a study investigating the antitumor activity of CD37-redirected and bispecific CD37/CD19-redirected CAR-T cells [115]. In their CAR construct, they used a unique mouse mAb-derived fragment that targets human CD37 [115]. These mAbs possessed significant affinity against CD37 and were capable of exclusively binding the cell surface-expressed CD37 in lymphoma cells [115]. Golubovskaya et al. reported that their mouse and humanized (possessing the humanized version of the CD37-specific scFv) CD37-redirected CAR-T cells and bispecific humanized CD37/CD19-redirected CAR-T cells exhibited specific antitumor effects against various CD37-positive and CD19-positive (in the case of bispecific CAR-T cells) cells in vitro [115]. Moreover, the bispecific CD37/CD19-redirected CAR-T cells suppressed tumor progression in Raji xenograft mouse models [115]. Such data can open a window to more evaluations regarding the application, safety index, and validity of targeting CD37, as a single target antigen or in combination with other blood-based TAAs, in future clinical trials. Considering the expression profile of CD37 in the mentioned blood-based malignancies, it may serve as an alternative CAR-T cell therapy target antigen in the cases of disease relapse with the loss of the primary antigen, however, after taking the safety evaluation steps.

GPRC5D

In 2012, Atamaniuk et al. reported elevated levels of G-protein-coupled receptor family C group 5 member D (GPRC5D) mRNA in MM patients which was considerably associated with poor prognosis and hazardous chromosomal changes [116]. Despite its low-level expression in healthy tissues, GPRC5D could not be exploited for therapeutic or diagnostic purposes in MM patients due to the incapability of the back-then accessible antibodies to recognize GPRC5D [117]. In 2019, Kodama et al. reported the surface expression of GPRC5D on malignant MM cells and stated that normal human hematopoietic cells are GPRC5D-deficient (except for plasma and B cells) [118]. Furthermore, Kodama et al. attempted to leverage this antigen for therapeutic purposes by developing bispecific IgG-based antibodies specific for GPRC5D and CD3 [118]. In detail, besides favorable in vitro findings, the developed GPRC5D/CD3 bispecific antibodies induced tumor suppression in xenograft models through a T-cell-dependent mechanism [118]. Such findings highlighted the potential of GPRC5D as a reliable target antigen for the development of possible treatment modalities against MM. In 2019, Smith and co-investigators reported that GPRC5D expression on bone marrow MM cells (those CD138-proficient) is distributed in a fashion resembling that of BCMA [119]. Further, the investigators developed GPRC5D-redirected CAR-T cells quipped with a human scFv (clone 109) and reported that these cells eliminated MM and induced prolonged survival in preclinical xenograft models, one of which was a BCMA-negative disease relapse model [119]. According to Smith and colleagues, their GPRC5D-redirected CAR-T cells can be as potent as BCMA-redirected CAR-T cells in vivo and since GPRC5D expression is independent of that of BCMA, GPRC5D-redirected CAR-T cells might hold promising potential for the treatment of MM patients regardless of BCMA expression status [119]. In 2020, de Larrea et al. attempted to tackle the issue of BCMA loss-induced relapse of MM patients by simultaneously targeting two potential MM target antigens, BCMA and GPRC5D, in a preclinical model [120]. In detail, the researchers developed three different platforms that enabled the simultaneous targeting of BCMA and GPRC5D [120]. The platforms entailed CAR-T cells whose CAR constructs were equipped with BCMA- and GPRC5D-specific tandem scFvs, CAR-T cells co-expressing both anti-GPRC5D and anti-BCMA CAR molecules (dual CAR-T cells), and a pooled product of two distinct CAR-T cells with one redirected against BCMA and the other against GPRC5D [120]. The pooled CAR-T cell and dual CAR-T cell platforms exhibited the most potential in the case of BCMA loss disease relapse, whereas the dual CAR-T cell platform outperformed the rest in the case of fighting against BCMA- and GPRC5D-positive disease [120]. Moreover, de Larrea et al. attributed the superiorities of the dual CAR-T cell platform to the fact that these CAR-T cells manage to establish more productive immunological synapses with the respective tumor cells [120]. Of note, in 2020, a Phase I dose-escalation trial (NCT04555551) started at Memorial Sloan Kettering Cancer Center (MSKCC) to investigate the safety of autologous GPRC5D-redirected CAR-T cells in 36 MM patients with at least three prior lines of therapy (inclusive of proteasome inhibitors, immunomodulatory agents, CD38-specific mAbs, and high-dose chemotherapy

with autologous stem-cell transplantation). This trial is estimated to be completed in August 2023, which will then elucidate the safety index of GPRC5D-redirected CAR-T cells for MM patients.

Trop2

Human trophoblast cell surface antigen 2 (Trop2) is a 36 kDa surface antigen expressed in various tumors such as breast, pancreatic, and gastric cancers [121–123]. The overexpression level of Trop2 in such malignancies highly correlates with poor prognosis and unfavorable survival due to the vital signaling roles of Trop2 in supporting tumor development, evasion, angiogenesis, and persistence [121–123].

In 2019, researchers from the University of Pennsylvania developed Trop2-redirected CAR-T cells and evaluated their antitumor functionality against Trop2-positive breast, prostate, and pancreatic tumor cells in culture [124]. To evaluate the specific targeting ability of Trop2-redirected CAR-T cells, these researchers utilized genome-edited Trop2-negative prostate cancer cells and demonstrated that no CAR-T cell-redirected cytotoxicity is meditated against these cells [124]. However, the researchers reported that Trop2-redirected CAR-T cells mediate cytotoxicity against Trop2-negative cells when Trop2-positive cells are present in the culture [124]. This phenomenon is believed to be due to the capability of activated CAR-T cells to induce tumor apoptosis through death receptors [124]. Despite the attempts made in the case of CD19-redirected CAR-T cells where obstructing death receptor ligands eliminated the non-specific effector function of these cells, similar efforts did not result in such outcomes in the case of Trop2-redirected CAR-T cells [124].

Furthermore, in 2019, Zhao et al. developed bispecific Trop2/PD-L1-redirected CAR-T cells [125]. These researchers reported that Trop2/PD-L1-redirected CAR-T cells demonstrated exclusive in vitro antitumor activity, which was superior to that of mono-specific CAR-T cells targeting either of these antigens, against a gastric cancer cell line with Trop2 and PD-L1 expression (125). Moreover, the overexpression of the target antigens of these bispecific CAR-T cells correlated with superior IFN-γ and IL-2 production and secretion by the CAR-T cells [125]. In vivo assessments on xenograft models of human gastric cancer confirmed the potential of these CAR-T cells in suppressing tumor progression upon regional delivery [125]. This effector function has been demonstrated to be superior to that of Trop2-redirected CAR-T cells [125]. In a nutshell, this study indicates that bispecific Trop2/PD-L1-redirected CAR-T cells may enhance the effector function of CAR-T cell therapy in preclinical models of solid tumors, especially in gastric cancer [125]. However, broadening the range of preclinical human tumor models and evaluating the functionality of such products in them may give a more accurate overview of the safety and efficacy of these CAR-T cells in preclinical scales.

Strategies beyond alternative target antigens

Advances in single-cell RNA sequencing and CRISPR screening have shed light on molecular pathways underlying antigen loss mechanisms. Strategies to address antigen loss include targeting multiple antigens simultaneously (dual-CAR or tandem CAR constructs) or incorporating "armored" CAR-T cells that secrete cytokines to recruit and activate endogenous immune responses against antigen-negative variants. Moreover, epigenetic modulators are being explored to restore antigen expression by reversing transcriptional silencing. These efforts aim to enhance CAR-T cell persistence and effectiveness, reducing relapse rates due to antigen escape. Moreover, the application of affinity-tuned CAR-T cells and inhibitory CAR (iCAR)-T cells have also been explored by scientists as potential solutions for minimizing the on-target off-tumor effects of CAR-T cells targeting antigens shared by healthy cells.

Restoring antigen expression by reversing transcriptional silencing

Restoring antigen expression by reversing transcriptional silencing in CAR-T cell therapy is a promising strategy to overcome antigen loss, a key obstacle in ensuring the long-term effectiveness of these therapies. Transcriptional silencing refers to the downregulation or complete loss of target antigen expression on tumor cells, which can occur during the course of treatment as a result of genetic and epigenetic changes. This phenomenon is particularly evident in antigen escape, where tumor cells evolve to evade CAR-T cell recognition, often through mechanisms that silence or alter the expression of the target antigen, such as CD19 in B-cell malignancies.

The process of transcriptional silencing typically involves epigenetic modifications, including DNA methylation, histone modification, and chromatin remodeling. DNA methylation of promoter regions, where the transcription machinery assembles, is one of the most common ways that gene expression is suppressed. In cancer cells, the promoter regions of TAAs like CD19 can become hypermethylated, preventing transcription factors from binding and initiating transcription [126, 127]. Similarly, histone modifications, such as the addition of repressive marks like H3K27me3 (trimethylation of histone H3 at lysine 27), can compact chromatin and inhibit gene expression. This results in a silenced or "off" state

for the antigen gene, even though the DNA sequence itself remains intact [126, 127].

To overcome this form of antigen loss, researchers are exploring epigenetic reprogramming as a means of reactivating silenced antigen expression. This involves the use of small molecules or biologics that target the epigenetic regulators responsible for silencing. Some strategies include DNA demethylation, histone deacetylase inhibitors (HDACi), epigenetic editing, and gene editing with small RNAs.

Briefly, DNA methyltransferase inhibitors (e.g., 5-azacytidine or decitabine) can be used to reverse DNA methylation [128, 129]. These drugs inhibit DNA methyltransferases (DNMTs), leading to the re-expression of previously silenced genes [128, 129]. By treating tumor cells with DNMT inhibitors, researchers can restore the expression of target antigens such as CD19 or CD22, making the tumor cells susceptible to CAR-T cell recognition and destruction [128, 129]. On the other hand, HDACi, like vorinostat or romidepsin, work by promoting the acetylation of histones, which opens up the chromatin structure and enhances transcriptional activity [130, 131]. By reversing repressive histone marks, these inhibitors can restore the expression of target antigens on the surface of tumor cells [130, 131].

Regarding epigenetic editing, scientists can specifically target the promoter regions of antigen genes like CD19 using CRISPR-based epigenome editing tools, such as CRISPR-dCas9 (catalytically dead Cas9) fused to epigenetic modifiers [132]. This technique allows for precise modulation of DNA methylation and histone modifications at the genetic loci that control antigen expression. The goal is to reactivate silenced genes directly at the chromatin level without causing double-strand breaks or permanent DNA alterations. Alternatively, short RNA molecules, such as siRNAs (small interfering RNAs) or shRNAs (short hairpin RNAs), can also be utilized to modulate the expression of genes involved in silencing antigen expression. By targeting specific RNA molecules that regulate the expression of epigenetic silencing factors, researchers can indirectly induce the re-expression of antigens that have been previously silenced.

The clinical implementation of these strategies is still in the experimental phase, but there is accumulating evidence that reactivating silenced antigen expression can improve the efficacy of CAR-T cell therapies [128–131]. By re-engaging the CAR-T cells with antigen-positive tumor cells, these epigenetic strategies can potentially overcome the limitations posed by antigen escape, reducing the chances of relapse and extending the durability of the treatment. Additionally, combining these epigenetic interventions with CAR-T cell therapies could enhance the persistence of CAR-T cells themselves. This could be achieved by modifying CAR-T cells to secrete factors that modulate the tumor microenvironment, allowing for better immune cell infiltration and increased antigen presentation on tumor cells, even if they initially underwent silencing [14, 133]. Despite the promising potential of these approaches, there are several challenges. The tumor microenvironment itself can often be a barrier to the effectiveness of epigenetic drugs, as certain modifications can be reversible or may not result in complete re-expression of the antigen. Furthermore, off-target effects and systemic toxicity remain concerns when using epigenetic modifiers. Therefore, a better understanding of the specific epigenetic changes that drive antigen silencing is needed, as well as the development of more precise delivery systems for these therapeutic agents.

Armored CAR-T cells

Armored CAR-T cells are genetically engineered to enhance their antitumor efficacy by overcoming the immunosuppressive tumor microenvironment. One approach involves modifying CAR-T cells to express interleukin-12 (IL-12), which enhances their proliferation and persistence [134]. IL-12-secreting CAR-T cells not only boost their own activity but also activate innate immune cells, leading to the elimination of tumor cells lacking the target antigen [135]. For example, a study demonstrated that IL-12-secreting CAR-T cells eradicated lymphoma in mice by inducing host immunity [135]. In a study by Kuhn and colleagues, the researchers engineered CAR-T cells to constitutively express CD40 ligand (CD40L) [136]. These modified CAR-T cells demonstrated superior antitumor efficacy in mouse models of leukemia and lymphoma. The expression of CD40L enabled the CAR-T cells to directly target tumor cells through CD40/CD40L-mediated cytotoxicity and to activate antigen-presenting cells (APCs) [136]. This activation led to the recruitment and mobilization of endogenous immune effectors, including tumor-recognizing T cells, thereby inducing a sustained antitumor response [136]. Notably, these effects were absent in CD40-deficient mice, highlighting the critical role of CD40/CD40L interactions in the observed antitumor immunity [136]. The findings suggest that incorporating CD40L into CAR-T cell design can enhance their therapeutic potential by engaging the host's own immune system to combat cancer [136].

Another strategy includes engineering CAR-T cells to resist the suppressive effects of transforming growth factor-beta (TGF-β) by incorporating a dominant-negative TGF-β receptor, thereby improving their function in TGF-β-rich environments [137]. Additionally, arming CAR-T cells with interleukin-10 (IL-10) has been shown to counteract dysfunction and exhaustion, leading to

improved antitumor activity in solid tumors [138]. These advancements demonstrate the potential of armored CAR-T cells in enhancing cancer immunotherapy outcomes.

Dual and tandem CAR-T cells

Other advanced strategies to enhance efficacy and mitigate tumor antigen escape in CAR-T cell therapy include the application of dual-CAR and tandem CAR constructs. In the dual-CAR construct scheme, T cells are engineered to express two separate CARs, each targeting a different TAA [139]. This dual specificity allows CAR-T cells to recognize and eliminate tumor cells expressing either or both antigens, thereby reducing the likelihood of tumor evasion through loss or downregulation of a single antigen [139]. For instance, a study targeting BCMA and GPRC5D demonstrated that dual-targeted CAR-T cells could prevent BCMA escape-driven relapse in MM [139].

Tandem CAR constructs, also known as TanCARs, involve a single CAR molecule engineered with two distinct antigen-binding domains, enabling simultaneous recognition of two different antigens [57]. This design facilitates a more robust activation of CAR-T cells upon engagement with either or both antigens on tumor cells. Research has shown that tandem CAR-T cells targeting both EGFRvIII and IL-13Rα2 exhibited enhanced cytotoxicity against heterogeneous glioblastoma populations, effectively addressing tumor antigen heterogeneity [57].

Both strategies aim to improve CAR-T cell therapy efficacy by targeting multiple antigens, thereby reducing the risk of tumor escape due to antigen loss. However, dual-CAR constructs involve two separate CARs expressed by a particular CAR-T cell product, each specific to a different antigen, whereas tandem CARs incorporate two antigen-binding domains within a single CAR construct [140]. This structural difference can influence the signaling strength and functional outcomes of the CAR-T cell product. Notably, studies suggest that tandem CARs may be more effective than dual CARs in preventing tumor escape in heterogeneous leukemic cells [141]. By employing these multi-targeted approaches, CAR-T cell therapies can better address tumor antigen heterogeneity and minimize the chances of tumor evasion through antigen loss, leading to more durable and comprehensive antitumor responses [57, 139, 141].

Affinity-tuned CAR-T cells

Tuning the affinity of the antigen-recognition domain of CAR-T cells serves as a potential strategy for minimizing the on-target off-tumor effects of CAR-T cells that target TAAs. It has been demonstrated that affinity-tuned CAR-T cells have the ability to discriminate between tumor cells and healthy cells as the former overexpress the antigen while the latter express it at physiological levels, offering an engineered selectivity. In this scheme, scFvs or VHHs that serve as the antigen-recognition domain of CAR-T cells are engineered in a way that their affinity is adjusted to an optimum level, rather than a high or low affinity [142]. There are several methods for engineering affinity-tuned antigen-recognition domains which include directed evolution or mutagenesis, rational design, or the application of alternative binders [143]. Briefly, in the directed evolution method, random mutations are introduced into the sequence of the scFv, VHH, or any other ligand to develop a library of the prospective antigen-recognition domain with a wide range of affinities [143, 144]. Next, the constructed library is screened using display techniques (such as phage display or yeast display) to isolate the most favorable binder with the optimal affinity [143, 144]. In a 2022 study, Butler and colleagues employed directed evolution to enhance the affinity of NKp30 variant-based B7H6-redirected CAR-T cells, and reported improved signaling outputs while retaining ligand recognition advantages over traditional scFvs [143]. Moreover, in silico and computational techniques could also be taken into consideration for affinity adjustment [24]. Briefly, investigating the interactions of a given binder with the target antigen could provide insights into how amino acid substitutions could be introduced to reduce to increase the affinity of the binder as desired [24]. In terms of alternative binders, in a study by Han and colleagues, the researchers applied adnectin (derived from the 10th fibronectin type III domain) as the antigen-recognition domain of EGFR-redirected CAR-T cells, instead of scFvs, and demonstrated that these engineered T cells were as potent as their scFv-based counterparts in vitro and in vivo in terms of mediating tumoricidal effects [145]. Moreover, through precise affinity adjustments, the adnectin-based CAR exhibited enhanced specificity for cells with elevated EGFR expression over those with lower levels [145].

In a study by Liu et al., the researchers engineered CAR-T cells with different target affinities [146]. Briefly, high-affinity CAR-T cells recognized targets across all expression levels, including low levels found in normal cells that flow cytometry could not detect [146]. In contrast, affinity-optimized CAR-T cells displayed strong tumoricidal abilities while sparing normal cells with physiological target antigen expression [146]. This approach highlights the potential of affinity-tuned CARs to expand the applicability of CAR-T cell therapies for solid tumors, even for antigens targeting which could result in off-tumor effects [146]. In a 2023 study, Vander Mause and colleagues developed CD229-redirected CAR-T cells to investigate them as a potential treatment

for MM; however, since healthy lymphocytes are also proficient in CD229 expression, these researchers attempted to increase the selectivity of their CAR-T cells to minimize off-tumor toxicities [147]. Briefly, they developed different variants of antigen-recognition domain differing in their affinity using a single amino acid substitution strategy, and following proper screening procedures, they identified a binder with a μM affinity [147]. Upon CAR construction, it was demonstrated that these CAR-T cells were as potent as their parental counterparts in terms of antitumor effects in vitro and in preclinical models but without off-tumor effects against healthy lymphocytes [147]. Such findings highlight the applicability of affinity-tuned CAR-T cells for minimizing the off-tumor effects of targeting TAAs. Further clinical investigations could shed more light on their translatability in clinics.

iCAR-T cells

iCAR-T cells are a specialized form of CAR-T cells designed to mitigate the risks of on-target off-tumor effects that occur when CAR-T cells target antigens expressed on healthy tissues [148, 149]. Unlike conventional CAR-T cells that activate cytotoxic functions upon antigen recognition, iCAR-T cells are engineered to inhibit T-cell activation when they recognize specific antigens present in normal tissues. These cells employ inhibitory signaling domains, such as those derived from immune checkpoint molecules like PD-1, CTLA-4, or LAG-3, to suppress immune responses selectively [148, 149]. Studies have demonstrated the efficacy and safety of iCAR-T cell designs. For instance, inspired by the natural function of immune inhibitory receptors, researchers have created antigen-specific inhibitory iCARs to control T cell activity in a targeted manner [150]. iCARs incorporating CTLA-4 or PD-1 domains can specifically reduce cytokine production, cytotoxicity, and proliferation induced by the native T cell receptor or an activating chimeric receptor [150]. This inhibitory effect is temporary, enabling T cells to regain functionality upon subsequent encounters with the activating antigen. These researchers concluded that serving as a dynamic and self-regulating safety feature, iCARs are designed to mitigate problems caused by inadequate T-cell specificity before they occur, rather than addressing them retroactively [150]. iCAR-T cells are particularly beneficial in reducing on-target, off-tumor toxicities by ensuring that cytotoxic activity is only triggered in the presence of tumor antigens and suppressed in healthy tissues expressing shared antigens. This dual regulation enhances the specificity and safety of CAR-T cell therapy, making it a promising approach for solid tumors and other malignancies where antigen overlap between normal and tumor tissues poses a significant challenge.

Concluding remarks

Treatment of cancer patients with CAR-T cells is no longer just an idea, but a promising therapeutic option capable of inducing high rates of disease-free survival in individuals with certain hematologic malignancies. However, accumulating evidence suggests that this achieved remission can be volatile in a proportion of patients due to the compromised status of the target antigen and/or the poor persistence of the infused CAR-T cells [140]. For instance, since the antigen-recognition domains of CAR-T cells are often derived from animal antibodies (such as murine or camelid), they can provoke an immune response in patients due to their immunogenicity [151]. Such immune responses culminate in the production of neutralizing antibodies, which reduce CAR-T cell efficacy by blocking their function or clearing them from circulation [151]. Humanization, involving the replacement of animal sequences with human counterparts while preserving antigen-binding specificity, significantly mitigates this issue [24, 151]. By reducing the foreign epitopes recognized by the immune system, humanized CAR-T cells exhibit lower immunogenicity, improved persistence, and enhanced therapeutic outcomes [24, 151].

As discussed throughout this review, tumor cells strive to find ways to tackle the deleterious effects of the immune system. Since numerous of these mechanisms are antigen-dependent, the discovery and meticulous evaluation of novel antigens for finding applicable substitutes for the compromised antigens is a *must*. Alternative target antigens for a particular oncological indication can give patients another chance to undergo another potential antigen-dependent treatment modality after antigen-compromised disease relapse [8]. Nevertheless, alternative antigens might also be beneficial in a more pronounced and/or precise fight against cancer since they enable the development and application of multi-targeting CAR-T cell platforms [140]. For instance, administration of a pooled product of CAR-T cells which entails two or three CAR-T cell products each redirected against a different antigen, CAR-T cells that simultaneously surface-present two or three distinct chimeric receptors that recognize different target antigens, and CAR-T cells that are armed with bispecific synthetic receptors [140]. Moreover, the availability of two (or even more) qualified target antigens for a certain malignancy also allows the generation of CAR-T cells that are specific for one of the antigens but are capable of producing and secreting bispecific T-cell redirecting antibodies [8, 152]. Furthermore, the availability of qualified target antigens can also be leveraged for reducing the on-target off-tumor toxicities of CAR-T cells through the development of CAR-T cells that are only responsive towards

tumor cells that express both of the antigens (rather than healthy cells that express only one) or malignant cells that express only one of the antigen but are deficient in the expression of the other [152].

Aside from CAR-T cell therapies, other territories of immunotherapy can also be expanded based on alternative antigens. For instance, TCR-engineered T-cell therapies represent a cutting-edge immunotherapy that harnesses the specificity of TCRs to recognize intracellular antigens presented on tumor cells by major histocompatibility complex molecules. Unlike CAR-T cells, which are limited to targeting extracellular antigens, TCR-engineered T cells can recognize a broader array of TAAs, including intracellular proteins [153, 154]. The US FDA has approved therapies like tebentafusp-tebn for uveal melanoma, targeting the gp100 peptide, and afamitresgene autoleucel from Adaptimmune Therapeutics, which targets MAGE-A4, a cancer-testis antigen expressed in tumors such as synovial sarcoma and liposarcoma [155, 156]. Additionally, NY-ESO-1-specific TCR-engineered T cells have emerged as a promising therapy, particularly for tumors like sarcomas, melanomas, and MM that express this cancer-testis antigen [153]. NY-ESO-1-specific TCR-T cells have shown significant clinical responses in early-phase trials, with durable remissions in patients with advanced cancers [153, 154]. These therapies address antigen heterogeneity in solid tumors, and advancements in TCR affinity engineering and gene editing technologies enhance their safety and efficacy. By overcoming barriers such as poor T cell infiltration and the immunosuppressive tumor microenvironment, TCR-engineered T cells hold significant potential for improving outcomes in solid tumor patients.

Other treatment modalities can also be therapeutically beneficial. As an example, *gemtuzumab ozogamycin* is a CD33-specific ADC approved by the FDA for the treatment of AML [157]. Therefore, the discovery of qualified AML-related alternative target antigens (such as CLL-1 or LILRB-4, aside from FLT3, TIM2, and CD47) can be considered an opportunity for the development of other potential ADCs against AML. Other examples might include *elotuzumab* and *daratumumab* which are humanized SLAMF7- and fully human CD38-specific mAbs, respectively, FDA-approved for the treatment of MM patients [158, 159]. Aside from BCMA, the discovery of novel MM-related antigens, such as CD37, might be a milestone for the development of therapeutic mAbs against MM. In the context of solid tumors, *trastuzumab* is a HER2-specific humanized mAb FDA-approved for the treatment of patients with metastatic breast cancer. In 2013, the FDA approved the medical use of *ado-trastuzumab emtansine* (under the trade name *Kadcyla*®) for the treatment of metastatic breast cancer patients who have previously undergone *trastuzumab* treatment [160]. In detail, *Kadcyla* is an ADC composed of *trastuzumab* conjugated to the cytotoxic agent *emtansine* [160]. Additionally, other ADCs based on *emtansine* have also been developed which include *cantuzumab mertansine* (a MUC1-specific ADC) and *lorvotuzumab mertansine* (a CD56-specific ADC against CD56-positive malignancies such as ovarian cancer) [161, 162]. However, since HER2 is subject to expression loss in triple-negative breast cancer (TNBC) patients, qualified substitute antigens are of paramount therapeutic importance. Out of the alternative antigens discussed in this review (Fig. 1), Trop2 can be a qualified example of a substitute target antigen in this regard. In April 2020, the US FDA gave accelerated approval to *sacituzumab govitecan-hziy* (under the trade name *Trodelvy*®) for the treatment of TNBC patients [163]. Of note, *Trodelvy* is a humanized Trop2-specific mAb conjugated to a highly cytotoxic metabolite of irinotecan, called *SN-38* [163, 164]. Moreover, in April 2021, *Trodelvy* was also FDA-approved for certain patients with metastatic urothelial cancer alongside being granted regular approval for individuals with TNBC [163, 164]. Based on the mentioned information, it could be sensible to recognize Trop2 as a qualified antigen for the treatment of certain patients with solid malignancies. However, Trop2-redirected CAR-T cells have a long way to go (entailing vast preclinical experiments and clinical investigations) before they can set foot in clinics for infusion into cancer patients. Nevertheless, it is encouraging to note that such examples can highlight how the discovery of alternative target antigens can exponentially increase our opportunities for the development of a variety of potential antigen-dependent therapeutics.

Besides antigen-dependent immune evasion mechanisms, CAR-T cells also struggle with resistance hindrances that are independent of target antigen status. Upon tumor infiltration, CAR-T cells encounter the immunosuppressive tumor microenvironments populated with myeloid-derived suppressor cells (MDSCs) and/or regulatory T cells (Tregs) as well as various inhibitory factors such as prostaglandin E2 (PGE_2) and TGF-β [165, 166]. In regards to PGE_2, disruption of the signaling axis of PGE_2/cyclooxygenase-2 (COX2), an enzyme involved in the synthesis of PGE_2, in conjunction with PD-1 blockade might suffice to be useful for restoring the tumoricidal functionality of CAR-T cells [166]. In reference to TGF-β, there have been different solutions for a single problem. While some researchers have used CRISPR/Cas9 for the disruption of the TGF-β receptor II in CAR-T cells and reported prolonged antitumor efficacy, others have combined TGF-β-targeted oncolytic adenoviruses with mesothelin-redirected CAR-T cells and have reported

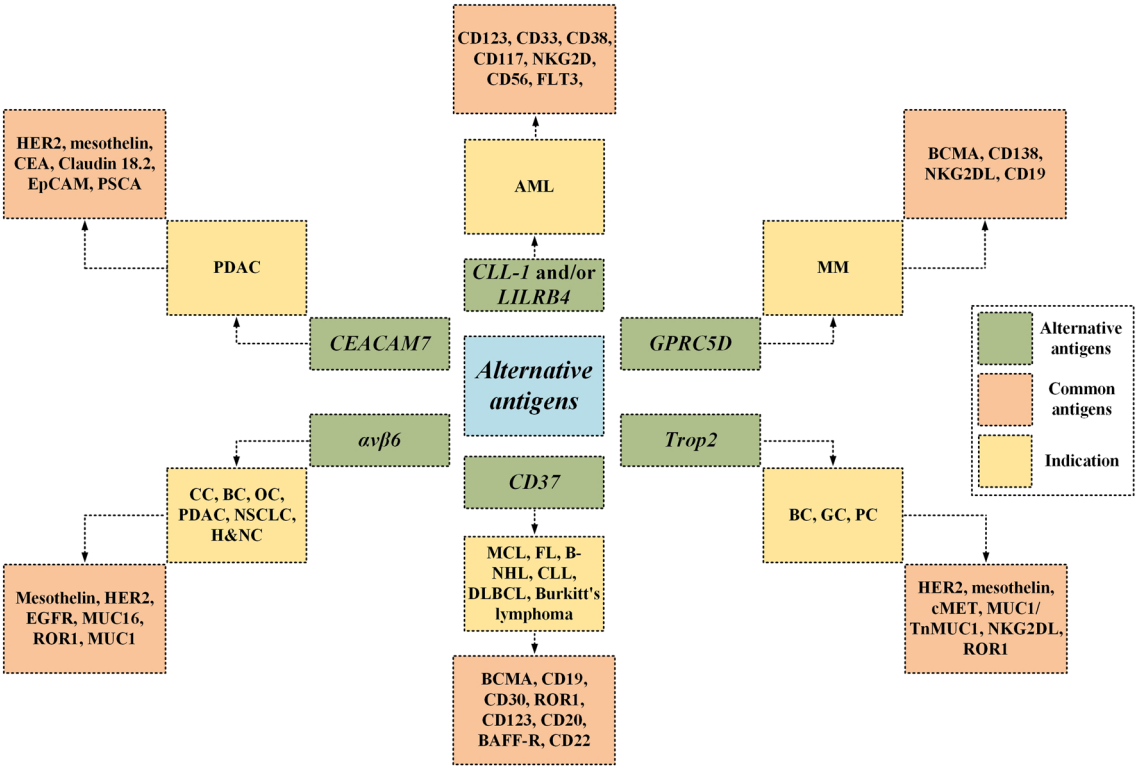

Fig. 1 An overview of the alternative antigens discussed in this review, their involvement in various hematologic malignancies and/or solid tumors, and the currently available target antigens which such alternatives might be suited to substitute in cases of antigen-driven disease relapse. AML, acute myeloid leukemia; BC, breast cancer; B-NHL, B-cell non-Hodgkin's lymphoma; CC, cervical cancer; CLL, chronic lymphoid leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; GC, gastric cancer; H&NC, head and neck cancer; MCL, mantle cell lymphoma; MM, multiple myeloma; NSCLC, non-small cell lung carcinoma; OC, ovarian cancer; PC, pancreatic cancer; PDAC, pancreatic ductal adenocarcinoma

pronounced tumor rejection in breast cancer xenograft models [167, 168]. Moreover, aside from CRISPR-Cas9, other genome-editing techniques such as ZFN and TALEN could also be employed for the development of CAR-T cell insensitive to immunosuppression. Aside from these, the phenotypic characteristics of the T cells from which CAR-T cells are produced (such as those with an effector-like differentiation status) and the disruption of death receptor signaling cascades due to the loss of TRAIL2 and FADD by malignant cells, which renders them resistant to the tumoricidal effects of CAR-T cells, also contribute to the emergence of resistance to CAR-T cell therapies [169, 170].

Such findings alongside the antigen-dependent resistance mechanisms discussed in this review indicate that to maintain and optimize the outcomes of CAR-T cell therapies, it is necessary to keep an eye on all the aspects (even the designing of the CAR construct) of this platform of immunotherapy at once. The discovery of alternative antigens should be a task for scientists to be continued and screening responding and non-responding patients in the related CAR-T cell clinical trials could shed more light on the resistance mechanisms already known or even those that are yet to be deciphered.

Abbreviations

mAbs	Monoclonal antibodies
ADCs	Antibody–drug conjugates
BiTEs	Bispecific T-cell engagers
CAR	Chimeric antigen receptor
NK	Natural killer
US FDA	United States Food and Drug Administration
R/R	Relapsed/refractory
B-ALL	B-cell acute lymphoblastic leukemia
DLBCL	Diffuse large B-cell lymphoma
FL	Follicular lymphoma
MCL	Mantle cell lymphoma
MM	Multiple myeloma
BCMA	B-cell maturation antigen
TSA	Tumor-specific antigens
TAA	Tumor-associated antigens
AML	Acute myeloid leukemia
scFv	single-chain variable fragment
TCR	T-cell receptor
GvHD	Graft-versus-host disease
CD4-LV	CD4-specific lentiviral vector
LNPs	Ionizable lipid nanoparticles
MDR	Minimal residual disease
CR	Complete remission
MHC	Major histocompatibility complexes

pMHC	Peptide-bound MHC
MLL	Mixed-lineage leukemia
GPC3	Glypican-3
HCC	Hepatocellular carcinoma
sGPC3	Serum GPC3
HSCs	Hematopoietic stem cells
PDX	Patient-derived xenograft
iCasp9	Induced caspase 9 switch
CMR	Complete molecular remission
HSCT	Hematopoietic stem cell transplantation
LILRB	Leukocyte immunoglobulin-like receptor B
PDAC	Pancreatic ductal adenocarcinoma
EMT	Epithelial-mesenchymal transition
CCA	Cholangiocarcinoma
CLL	Chronic lymphocytic leukemia
GPRC5D	G-protein-coupled receptor family C group 5 member D
MSKCC	Memorial Sloan Kettering Cancer Center
Trop2	Trophoblast cell surface antigen 2
iCAR	Inhibitory CAR
HDACi	Histone deacetylase inhibitors
DNMTs	DNA methyltransferases
siRNAs	Small interfering RNAs
shRNAs	Short hairpin RNAs
IL-12	Interleukin-12
CD40L	CD40 ligand
APCs	Activate antigen-presenting cells
TGF-β	Transforming growth factor-beta
IL-10	Interleukin-10
MDSCs	Myeloid-derived suppressor cells
Tregs	Regulatory T cells
PGE2	Prostaglandin E2
COX2	Cyclooxygenase-2

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