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# Mechanisms of resistance to chimeric antigen receptor-T cells in haematological malignancies

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## Abstract

Chimeric antigen receptor (CAR)-T cells have recently emerged as a powerful therapeutic approach for the treatment of patients with chemotherapy-refractory or relapsed blood cancers, including acute lymphoblastic leukaemia, diffuse large B cell lymphoma, follicular lymphoma, mantle cell lymphoma and multiple myeloma. Nevertheless, resistance to CAR-T cell therapies occurs in most patients. In this Review, we summarize the resistance mechanisms to CAR-T cell immunotherapy by analysing CAR-T cell dysfunction, intrinsic tumour resistance and the immunosuppressive tumour microenvironment. We discuss current research strategies to overcome multiple resistance mechanisms, including optimization of the CAR design, improvement of in vivo T cell function and persistence, modulation of the immunosuppressive tumour microenvironment and synergistic combination strategies.

## Introduction

Chimeric antigen receptor (CAR)-T cells have revolutionized the field of cancer therapy and are considered one of the major medical breakthroughs of the past few decades. So far, the FDA has approved six CAR-T cell products for haematological malignancies, including products targeting the CD19 antigen for B cell leukaemia and non-Hodgkin lymphoma

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Author contributions

All authors researched data for the article, contributed substantially to discussion of the content, wrote the article, and reviewed and/or edited the manuscript before submission.

Competing interests

M.R. holds patents related to chimeric antigen receptor-T cells that are managed by the University of Pennsylvania, and some of them are licensed to Novartis, Tmunity and viTToria Biotherapeutics. M.R. has served as a consultant for BMS, GSK, Scailyte, Bayer, GLG and AbClon. M.R. receives research funding from viTToria Biotherapeutics, ONI, Beckman Coulter and AbClon. M.R. is the scientific founder of viTToria Biotherapeutics. M.V.M. is an inventor on patents related to chimeric antigen receptor-T cell therapies held by the University of Pennsylvania (some licensed to Novartis) and Massachusetts General Hospital (some licensed to Promab and Satellite Bio). M.V.M. holds equity in 2Seventy Bio, TCR2, Century Therapeutics, Oncternal and Neximmune, and has served as a consultant for multiple companies involved in cell therapies. M.V.M. serves on the Board of Directors of 2Seventy Bio. F.K. and P.P. declare no competing interests.

(NHL), and B cell maturation antigen (BCMA) for multiple myeloma[1-8]. These novel therapies were initially approved based on impressive results in single-arm phase II studies that were later confirmed by real-world data analyses[9-14].

Despite these compelling results, most patients treated with CAR-T cells ultimately have progressive disease. This can be due to an initial lack of response or primary resistance, which is the case in 10–30% of B cell acute lymphoblastic leukaemia (B-ALL), roughly 50% of diffuse large B cell lymphoma (DLBCL), around 10% of mantle cell lymphoma, 5–15% of follicular lymphoma and 20% of multiple myeloma. Alternatively, disease progression can be due to relapse after an initial, transient response, also known as secondary resistance, which is the case of roughly 40–50% of B-ALL and DLBCL, 40% of mantle cell lymphoma, 30% of follicular lymphoma and 30% of multiple myeloma[1-3,7,8,15-18]. Several mechanisms of resistance to CAR-T cell immunotherapy have been identified (Fig. 1), namely, CAR-T cell dysfunction (Fig. 2), tumour-intrinsic mechanisms (Fig. 3) and the immunosuppressive tumour microenvironment (TME) (Fig. 4). These mechanisms must be studied to develop more effective strategies to achieve durable responses in patients. This Review summarizes the most recent findings on the clinical and correlative resistance mechanisms to CAR-T cell immunotherapy and discusses the most promising approaches to overcome resistance.

## Clinical resistance to CAR-T cells

Primary and secondary resistance features after CAR-T cell therapy differ by product and disease. In the registration studies for DLBCL (JULIET and ZUMA-1 trials), rates of complete remission ranged from 39% to 58%[4,13,19]. The registration trials for each product had differences in design and patient populations and cannot be directly compared. However, notably, primary resistance was observed in 42% of patients treated with axicabtagene ciloleucel (axi-cel), in 61% of patients treated with tisagenlecleucel (tisa-cel) and in 47% of patients treated with lisocabtagene maraleucel (liso-cel). These results demonstrate that, overall, primary resistance of lymphoma to CD19-directed CAR-T cells occurs in approximately half of the treated patients.

By contrast, B-ALL seems to be more responsive to CAR-T cell therapy, with 81% of paediatric patients with B-ALL achieving complete remission with tisa-cel in the ELIANA study and adults having a complete remission rate of 53–60% with brexucabtagene autoleucel in the ZUMA-3 study[3,6,20]. In the ZUMA-2 trial, 68% of patients with mantle cell lymphoma treated with brexucabtagene autoleucel had complete remission, whereas patients with follicular lymphoma who received axi-cel in the ZUMA-5 study had a 74% complete remission rate[16,17]. Finally, in multiple myeloma, a complete remission to therapy was achieved in 67–79% across the two approved BCMA-targeted products: idecabtagene vicleucel and ciltacabtagene autoleucel[7,8]. In larger real-world studies, complete remission rates of 53–65% were reported for axi-cel and 40–42% for tisa-cel, which were comparable to results of ZUMA-1 and JULIET trials, respectively[10,21-23]. Therefore, a significant need exists to reduce the incidence of primary resistance, particularly in DLBCL and adult B-ALL.

Secondary resistance, by contrast, is observed when a patient relapses after achieving a complete remission. The time of relapse varies according to the disease treated and the product used. At a median follow-up of 40 months, patients who received tisa-cel for DLBCL achieved an overall survival of 11.1 months and median progression-free survival (PFS) of 2.9 months[13]. A follow-up analysis of axi-cel from the ZUMA-1 study, with a median follow-up of 63.1 months, displayed a 5-year overall survival rate of 43% and median PFS of 5.9 months[24,25]. For liso-cel, with a median follow-up of 12.3 months, the median overall survival was not reached[26]. Updated results of tisa-cel in B-ALL displayed a 24-month relapse-free survival rate of 62% and 18-month overall survival rate of 70%[27]. Real-world results in the same setting reported comparable results with an overall survival rate of 77.2% (median follow-up of 13.4 months)[23]. Patients with mantle cell lymphoma within the ZUMA-2 study had a duration of response, PFS and overall survival of 28.2, 25.8 and 46.6 months, respectively[16]. For multiple myeloma, 2-year follow-ups for idecabtagene vicleucel and ciltacabtagene autoleucel achieved median durations of response of 11.1 and 21.8 months (median PFS of 9.8 months and 24-month PFS of 54.9–60.5%), respectively[28-30].

The identification of the determinants, mechanisms and prognostic factors driving primary resistance and secondary resistance (or relapse) is an area of very active research[31]. Several studies have investigated clinical and laboratory parameters that are routinely evaluated in patients and correlate with inferior outcomes. For instance, lower disease burden correlates with lower toxicity and increased efficacy, a principle that applies across multiple treatment modalities and types of disease in oncology[32]. In addition, a higher number of CAR-T cells at peak or area under the curve levels of CAR-positive T cells in blood correlate with a longer durability of response[33]. Moreover, in both tisa-cel and axi-cel for DLBCL, risk factors for disease relapse included worse performance status (`Eastern Cooperative Oncology Group` grade ≥2), higher stage disease, ≥2 extranodal sites, elevated laboratory parameters of lactate dehydrogenase (LDH) and/or C-reactive protein (CRP) and high `International Prognostic Index`[34]. High ferritin pretreatment levels have been associated with lower CAR-T cell expansion[34,35]. In addition, lower prelymphodepletion levels of LDH as well as a higher number of platelets were independent factors associated with better event-free survival[36]. Patients with active disease who require ‘bridging chemotherapy’ (anticancer treatment administered between `apheresis` and lymphodepleting chemotherapy with the aim of preventing the progression of the disease during this interval) had worse overall survival[10]. Thus, despite the overall success of CAR-T cell therapies in haematological malignancies, primary and especially secondary resistance are important clinical problems.

The next sections explore the biologic mechanisms of resistance to CAR-T cells, which are divided into T cell-related mechanisms (such as failure in CAR-T cell manufacturing or T cell exhaustion) (Fig. 2), tumour-intrinsic mechanisms (such as the loss of target antigen expression, expression of immune checkpoint molecules or resistance to apoptosis) (Fig. 3) and immunosuppressive TME-related mechanisms (such as barrier to CAR-T cell infiltration, presence of immunosuppressive cells and cytokines or lack of nutrients or hypoxia) (Fig. 4).

## CAR-T cell dysfunction

The role of T cell differentiation in CAR-T cell persistence is key for understanding the long-term effectiveness of CAR-T cell therapy (reviewed recently[37]). T cell differentiation refers to the process by which T cells transition from a naive state to an effector or memory state with distinct functional characteristics. In CAR-T cell therapy, the persistence of CAR-T cells within the body of the patient is closely linked to their ability to maintain their effector functions targeting cancer cells over an extended period. Less differentiated T cell subsets, such as central memory T cells and stem cell memory T cells, tend to have superior persistence than more differentiated effector memory T cells and terminally differentiated effector cells. Central memory T cell and stem cell memory T cell subsets have self-renewal capabilities and can proliferate on re-encountering the antigen, thereby sustaining CAR-T cell populations over time. By contrast, the presence of highly differentiated subsets, such as terminally differentiated effector cells, can lead to a decrease in CAR-T cell persistence due to their limited proliferative potential and increased susceptibility to exhaustion or apoptosis. Dysfunctional T cells may develop owing to multiple causes including prolonged antigen stimulation, previous lymphotoxic therapies, immunodeficiencies or other undetermined factors[38]. Dysfunctional T cells can be exhausted or senescent (reviewed elsewhere[39]) and show lower expansion capacity, reduced cytotoxic function and higher expression of inhibitory receptors[38,39]. Methods to reduce exhaustion and senescence represent a strategy to enhance CAR-T cell functionality, and several approaches are being studied. Table 1 provides an overview of clinical trials evaluating new strategies to overcome CAR-T cell dysfunction, in particular, studies regarding blockade of programmed cell death 1 (PD1) and its ligand, programmed cell death 1 ligand 1 (PDL1); blockade of PD1 and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT); the combination of CAR-T cells with signal transduction inhibition via BTK-dependent and independent mechanisms; and the use of natural killer (NK) cells instead of T cells.

Exhausted T cells were initially described in chronic viral infection and are characterized by the co-expression of several inhibitory receptors, including PD1, cytotoxic T lymphocyte antigen 4 (CTLA4), T cell immunoglobulin domain and mucin domain 3 (TIM3), lymphocyte activation gene 3 (LAG3), TIGIT and others[38,40-42]. The number and abundance of expression of inhibitory receptors correlate with the severity of T cell dysfunction. In addition, exhausted T cells show impaired cytokine secretion (IFNγ, IL-2 and tumour necrosis factor (TNF)) and limited cytotoxicity. Exhausted T cells also express transcription factors associated with effector and memory cells, such as nuclear factor of activated T cell (NFAT) and the downstream effector NR4A, the nuclear thymus high-mobility group box protein (TOX), T-bet or eomesodermin, and adopt a unique epigenetic landscape[43], resulting in the induction of an exhaustion programme that prevents T cell overstimulation and reduces the response of T cells to tumour.

Specifically, in CAR-T cell therapy, preclinical evidence shows that exhausted $CD8^+$ CAR-T cells and tumour-infiltrating lymphocytes isolated from a mouse model carrying a $CD19^+$ B16-OVA melanoma xenograft showed overexpression of PD1 and TIM3 and a similar enrichment for consensus binding motifs for NR4A and NFAT transcription factors[44,45]. In patients with chronic lymphocytic leukaemia (CLL), transcriptomic analysis after tisa-cel

infusion showed that T cells from non-responders had upregulated pathways associated with T cell exhaustion, effector differentiation, glycolysis and apoptosis compared with T cells from responders. Co-expression of PD1, LAG3 and TIM3 was also upregulated on these CAR-T cells[31]. A highly significant association was seen between the likelihood of responding to therapy and the infusion of high doses of $CD27^{+}PD1^{-}CD8^{+}$ CAR-T cells. Similarly, durable remission was associated with a higher frequency of memory-like T cells ($CD27^{+}CD45RO^{-}CD8^{+}$) and a higher frequency of IL-6 and STAT3-related gene signatures at apheresis. In the JULIET study that led to the approval of tisa-cel for DLBCL and transformed follicular lymphoma, patients with the highest PD1–PDL1 interaction scores and high proportions of $LAG3^{+}$ T cells either did not respond to tisa-cel or relapsed shortly after[46]. A multicentre retrospective study reported that among 76 patients with B cell lymphoma (BCL), all three patients with $PDL1^{+}$ tumour had primary resistance to axi-cel therapy. In addition, recent studies have shown how $PDL1^{+}$ DLBCL is refractory to anti-$CD19^{-}$ CAR-T cell treatment but that administration of the PD1-blocking antibody pembrolizumab resulted in an expansion of CAR-T cells and tumour regression[47].

One challenge of autologous CAR-T cell therapy is that T cells come directly from the patient, and patients with haematological malignancies, especially those patients who have had multiple lines of cytotoxic chemotherapy, have more senescent T cells and fewer of the memory-like $CD8^{+}$ cells that are associated with durable remissions after CAR-T therapy[31,48]. Therefore, the status of T cells at infusion influences the response to CAR-T cell therapy, and monitoring CAR-T cell polyfunctionality preinfusion has proven to be a key attribute in predicting clinical response. A single-cell analysis of the pre-infusion axi-cel product from patients with NHL[49] showed that CAR products contain polyfunctional T cell subsets capable of deploying multiple immune programmes (cytokines and chemokines). A prespecified T cell polyfunctionality strength index applied to a pre-infusion axi-cel product was significantly associated with clinical response. Combined with CAR-T cell expansion or pretreatment serum IL-15 levels, polyfunctionality strength index confers additional significance (NCT00924326)[49].

Other work has shown promise by using a preselection of T cells during manufacturing, as it allows the removal of potentially dysfunctional T cells[50]. Preselected naive/stem memory T cells provided higher antitumour activity and displayed less susceptibility to toxicity induced by CAR-T cells[51].

## Strategies to overcome CAR-T cell dysfunction

### Optimization of the CAR-T cell product

Many previous reviews have discussed at length the various CAR construct designs, particularly the binder, hinge domain and co-stimulation domains that can increase CAR-T cell antigen recognition, signalling and persistence[52,53]. Here, we focus on other aspects beyond CAR design that can influence the CAR-T cell product.

In most patients with haematological malignancies, T cells are exhausted and terminally differentiated owing to the presence of the tumour and previous cytotoxic treatments. Despite ex vivo activation and engineering, CAR-T cells derived from these patients perform

somewhat poorly relative to T cells derived from young, healthy donors. A potential strategy to decrease the fraction of dysfunctional T cells is to start with less-differentiated, marrow-infiltrating T cells instead of circulating peripheral blood T cells, but this requires a more invasive procedure[54]. Alternatively, T cells harvested early in the disease course and, thus with less exposure to lymphotoxic therapies, could retain better fitness and proliferative capacity than those collected after relapse[55]. Although, the presence of disease at any stage may affect T cell function and be present in the final product[56]. The efficacy and safety of axi-cel as a first-line therapy in patients with high-risk large BCL (LBCL) was tested in ZUMA-12 (NCT03761056)[57]. The trial identified that a higher proportion of naive-like T cells ($CCR7^+CD45RA^+$) is associated with greater expansion of CAR-T cells than ZUMA-1, which suggests that the inclusion of CAR-T cells in earlier lines enhances T cell function and, therefore, clinical efficacy.

Shortening the culture period of CAR-T cells also favours a more cytotoxic and less-differentiated cellular product. Highly functional CAR-T cells can be generated within a day by leveraging the unique ability of lentiviral vectors to enter and integrate into the genome of non-dividing cells. Minimizing ex vivo manipulation preserved less-exhausted T cells and could also be of particular benefit to patients with rapidly progressive disease, who may otherwise be unable to receive the therapy[58]. Of course, it still needs to be determined whether the delivery time to the patient will be significantly shortened, owing to the time needed to complete testing before release for the patient.

To better understand the mechanisms leading to exhaustion, Lynn et al.[59] recently developed a cellular model of exhaustion by introducing a tonic signalling CAR (HA-28z CAR) into T cells. Chromatin accessibility paired with gene expression analysis revealed increased accessibility of the transcription factor, activator protein 1 (AP-1) motif and preferential binding of IRF4 and BATF over JUN because those transcription factors compete for the same sites. The forced overexpression of JUN prevented the exhaustion by promoting direct transcriptional activation of AP-1 target genes as well as by indirectly disrupting immunoregulatory AP-1–IRF transcriptional complexes that drive exhaustion-associated gene expression. These results showed how epigenetics and transcriptional reprogramming could revert CAR-T cell exhaustion.

A positive balance in favour of CAR-T cell proliferation over exhaustion can be stimulated with cytokines. Increased antitumour activity has been documented in CAR-T cells expressing a constitutively active form of the IL-7 receptor along with the CAR or insertion of the IL-15 receptor within the CAR construct[60,61]. Additional transgenes can also be incorporated into the same vector constructs to enhance CAR-T cell persistence, such as BCL-2 family members[62,63]. Avoiding cell death could also be accomplished with dominant-negative FAS overexpression[64] or FAS–41BB switch receptors[65]. Emerging data from genome-wide CRISPR screens in CAR-T cells also indicate that deletion of other genes (such as *RASA2*) may enhance CAR-T cell manufacturing, persistence or function[66,67]. One challenge for the field is that the number of potential edits, transgenes and knockouts that can be hypothesized and tested in preclinical models far exceeds the number of potential products that could feasibly be clinically developed for patients.

### Immune checkpoint blockade

PD1 blockade might overcome PDL1$^+$ tumour immunosuppression, thereby considerably enhancing the anticancer activity of human CAR-T cells against haematological and potentially solid tumours[68-71]. PD1 blockade by using antibodies has demonstrated remarkable clinical efficacy in various cancer types by reinvigorating anticancer T cell immunity; however, at the same time, immune-related adverse events have been observed, in particular, autoimmunity.

A study focusing on adding PD1 blockade to CD19 CAR-T cell therapy in 14 children (13 of whom were receiving pembrolizumab and 1 receiving nivolumab) with heavily pretreated, refractory B-ALL resulted in an improvement in the persistence of CAR-T cells, and 50% (7 of 14 patients) maintained either a complete or a partial response[72]. In addition, three of the patients treated with a PD1 inhibitor developed recurrent B cell aplasia, suggesting sustained CAR-T cell function.

Targeted modulation of PD1 in CAR-T cell products by gene editing presents the advantage of protecting CAR-T cells from PD1-induced exhaustion while sparing bystander T cells, thereby reducing the risk of autoimmune toxicities[73,74]. Another strategy to block this pathway is delivering a PD1 inhibitor by an ‘armoured’ CAR-T cell that secrets an immune checkpoint blockade single-chain variable fragment (scFv)[75-77]. This strategy showed excellent results in preclinical syngeneic and xenogeneic mouse models of PDL1$^+$ haematological malignancies[77]. Other elegant methods to inhibit PD1, such as introducing a dominant-negative PD1 extracellular domain or a novel PD1–CD28 chimeric switch receptor, have been developed in the context of immunotherapies for solid tumours[69]. The PD1–CD28 chimeric switch receptor containing the extracellular domain of PD1 fused to the intracellular co-stimulatory molecule, CD28, serves two purposes: binding its ligand PDL1 and transmitting an activating signal instead of the inhibitory signal[78]. T cells carrying this construct showed superior activity against PDL1$^+$ lymphoma cells ex vivo and in vivo than second-generation CD19 CAR-T cells. Of note, the authors describe a durable clinical response in one patient treated with this approach, without significant toxic effects[79].

Finally, Lee et al.[80] recently used a lentiviral two-in-one CAR construct. Two checkpoint receptors are downregulated simultaneously by a dual short hairpin RNA cassette integrated into a CAR vector. Using this system, the authors evaluated CD19-targeting CAR-T cells with four checkpoint combinations – PD1–TIM3, PD1–LAG3, PD1–CTLA4 and PD1–TIGIT – and found that downregulation of PD1 and TIGIT had unique synergistic antitumour effects. In the study, downregulation of PD1 enhanced the short-term effector function of CAR-T cells, whereas downregulation of TIGIT was responsible for maintaining a less-differentiated/exhausted state, providing a potential synergy. The efficacy and safety of PD1–TIGIT-downregulated CD19-targeting CAR-T cells are currently being evaluated in adult patients with relapsed or refractory LBCL (NCT04836507). Many ongoing clinical trials have assessed the effects of immune checkpoint blockade in combination with CD19 CAR-T cells (such as NCT04163302). The optimal timing and combination of checkpoint inhibition remain to be determined and could be surprising; for example, genetic knockout of PD1 in a clinical study of T cell receptor (TCR)-transduced T cells seemed to confer a disadvantage rather than increased persistence[81].

### Combination with small molecules

A possible strategy to reduce T cell dysfunction is to combine CAR-T cells with small molecules that leverage T cell biology to enhance T cell activity. For instance, tyrosine kinase inhibitors such as ibrutinib can enhance antitumour activity when combined with CD19 CAR-T cells[82]. Ibrutinib inhibits Bruton's tyrosine kinase (BTK), a kinase downstream of the B cell receptor (BCR)[83,84], and also targets IL-2-inducible T cell kinase (ITK), a tyrosine kinase in T cells involved in modulating phospholipase Cγ, which is downstream of TCR signal transduction and is critical for T helper 2-type effector responses. Deletion or inhibition of ITK drives more T helper 1-type responses, and ibrutinib enhances CAR-T cell viability, proliferation, cytokine production and CAR-T cell engraftment in preclinical and clinical studies for patients with CLL[85]. In addition, patients with CLL who had been previously treated with ibrutinib for ≥6 months at the time of T cell collection showed improved ex vivo and in vivo CAR-T cell expansion, comparable with the T cell expansion of a healthy young donor, and this finding correlated positively with clinical response[86]. In addition, when administered concurrently, ibrutinib exposure improved CAR-T cell engraftment, tumour clearance and survival in mouse models of resistant ALL and CLL xenografts[86]. Similarly, results were obtained in preclinical models of CAR-T cells in mantle cell lymphoma[87]. This approach was translated to the clinic by several groups. Gill et al.[88] demonstrated superior T cell proliferation during the manufacturing of autologous CAR-T cells (targeted towards CD19 with a humanized binder) in patients with CLL who had received ibrutinib for >6 months but had persistent disease. In this prospective single-centre study (NCT02640209), the authors reported the results of 18 patients with at least 15 months of follow-up[89]. Of note, three of these patients were previously treated with murine scFv-bearing CD19 CAR-T cells but had progressed. At 12 months, 72% of patients tested had no minimal residual disease and the estimated overall and PFS at 48 months was 84% and 70%, respectively. Cytokine release syndrome (CRS) was frequent but mild–moderate and did not require therapy[89], and improved ex vivo T cell expansion was observed. This observation was in line with data from a clinical trial run at Memorial Sloane Kettering Cancer Center, which showed significant T cell proliferation in 5 of 16 patients with CLL who were receiving ibrutinib at the time of T cell collection and/or CAR-T cell administration[88,90]. Finally, Gauthier et al.[91] showed that ibrutinib was well tolerated when initiated at 420 mg per day at least 2 weeks before leukapheresis and continued for at least 3 months after CAR-T cell infusion. Patients who received concurrent ibrutinib had a higher in vivo CAR-T cell number and lower CRS severity than patients who did not receive it (median CRS grade 1 versus 2, respectively; $P = 0.04$). The authors found a higher proliferation index and a higher percentage of polyfunctional CAR-T cells (expressing ≥2 cytokines) and higher rates of patients with no minimal residual disease in the bone marrow as measured by multiparameter flow cytometry (72%) and by immunoglobulin heavy chain sequencing (85%) in patients receiving ibrutinib. It is not clear whether similar clinical results will be obtained with second-generation BTK inhibitors that are more specific for BTK and do not target ITK.

Another tyrosine kinase inhibitor, dasatinib, has been used with a dual purpose in combination with CAR-T cell therapy. Dasatinib interferes with lymphocyte-specific protein tyrosine kinase (LCK), preventing the phosphorylation of CD3ζ and ZAP70 downstream

of the TCR. This mechanism can increase the safety index of CAR-T cells by disrupting the downstream signalling cascade in CAR-T constructs harbouring either CD28–CD3ζ or 4-1BB–CD3ζ activation modules[92]. At a clinically achievable concentration (100 nM), dasatinib can switch off activated CAR-T cells to prevent cytotoxicity without compromising their viability; this ‘CAR-T paused state’ is reversible, and discontinuation of dasatinib restores CAR-T cell cytolytic activity, cytokine secretion and proliferation[93]. In mice engrafted with Nalm6 leukaemia cells engineered to express GD2, the introduction of dasatinib during treatment with GD2-targeting CAR-T cells (GD2.28ζ.FKBP) promoted a transient cessation of CAR signalling, mitigated exhaustion, promoted T cell memory during ex vivo expansion and enhanced CAR-T cell antitumour functionality in vivo[94].

The PI3K–AKT pathway has a crucial role downstream of the TCR, co-stimulatory molecules and cytokines receptors[95]. Inhibitors of PI3K–AKT enhance T cell central memory phenotype, reduce terminal differentiation and increase proliferation. Therefore, the use of AKT inhibitors has been explored to prolong CAR-T cell persistence. Preclinical studies showed that inhibition of AKT signalling during the CD19 CAR-T cell production resulted in T cells with superior antitumour activity in vitro and in vivo[96]. This approach was also effective during the manufacturing of anti-BCMA CAR-T cells. The use of IL-2 in combination with the PI3K inhibitor bb007 resulted in a CAR-T cell product (bb21217) enriched in memory-like T cells, reduced differentiation and improved expansion[97,98]. These data suggest that therapeutic modulation of PI3K–AKT might be a strategy to augment the antitumour efficacy of CAR-T cell therapy, which could transition to the clinic once AKT inhibitors are approved for clinical use.

Overall, small molecules represent important tools for enhancing CAR-T function; however, the dosing, schedule and long-term effects require further testing in the clinic.

## Allogeneic T cells

CAR-T cells can be manufactured using T cells from healthy donors, offering several potential advantages over patient-derived T cells. First, healthy donors may provide a more functional T cell product and avoid T cell dysfunction[31,99]. In addition, this approach limits the risk of manufacturing failures, an issue occurring in 10–20% of CAR-T cell approval studies[1,3], and reduces the time to infusion with banked and available off-the-shelf CAR-T cells. However, as with any allogeneic therapy (such as stem cell transplantation) graft-versus-host disease (GVHD) and CAR-T cell rejection are major hurdles to overcome. To avoid these complications, different strategies are in development. Gene editing, selection of specific subsets of T cells (γδ T cells or virus-specific T cells) or NK cells from healthy donors could evade GVHD[100,101]. In addition, allogeneic αβ T cells can be knocked out for the TCR α-chain constant gene (*TRAC*)[102-104]. Additional gene editing or stealth strategies can be used to avoid host rejection of the therapeutic product. Examples include the use of CRISPR–Cas9 or other editing technologies to modify loci associated with the major histocompatibility class I complex (MHC-I). However, despite some promising preliminary data, overall results to date have not achieved the same level of efficacy as autologous products[104,105].

Gene-edited allogeneic CAR-T cells targeting CD19, such as 'UCART19'[106], have shown some efficacy[105]. Seven children and 14 adults were treated as part of UCART19. Fourteen (67%) of the 21 patients had a complete response or a complete response with incomplete haematological recovery 28 days post-infusion. However, the patients who did not receive alemtuzumab (four patients) did not show UCART19 expansion. Three patients (14%) had grade 3–4 CRS. Additional adverse events included grade I acute skin graft disease in two patients (10%) and prolonged grade IV cytopenia in six patients (32%). Two treatment-related deaths were recorded; one patient developed neutropenic sepsis with concomitant CRS, and another patient developed pulmonary haemorrhage with persistent cytopenia.

Thus, although the development of alloreactions such as GVHD can be largely prevented, rejection of the allo-CAR-T is still a major barrier to achieving durable remissions. However, UCART19 may serve as a potential bridge-to-transplantation option.

Avoiding the rejection of allogeneic therapeutic cell products requires preventing both T and NK cell responses that eliminate cells identified as foreign. A synthetic receptor, alloimmune defence receptor (ADR), which selectively recognizes 4-1BB expressed in activated T cells, has been developed[107]. ADR-expressing T cells target alloreactive lymphocytes while sparing quiescent lymphocytes, thereby resisting rejection. CAR-T cells engineered to express ADR demonstrated persistent tumour eradication in both liquid and solid in vivo tumour models, thus offering an off-the-shelf approach to generating rejection-resistant allogeneic T cell products. Other groups aim at rendering healthy donor T cells resistant to lymphodepleting agents, as intensified lymphodepletion may enable allogeneic CAR-T cells to increase expansion and antitumour activity before host immune recovery[108-110]. Umbilical cord blood-derived T cells might serve as another T cell source aside from healthy donor T cells, reducing GVHD frequency owing to lower levels of released pro-inflammatory cytokines in patients receiving allogeneic stem cell transplantation[111-113]. Other options such as immune effector cells derived from induced pluripotent stem cells are also in early developmental stages[114].

## Non-T cell immune effector cells

Building on the success of T cell therapies, adoptive cell therapy strategies have begun to include other immune effector cells or T cell subsets, such as NK cells, macrophages, γδ T cells and invariant natural killer T (iNKT) cells.

**NK cells.—**NK cells are cytotoxic cells with essential roles in the innate immune response against virus-infected or bacterium-infected and injured cells. Owing to their intrinsic properties, they offer potential as allogenic cellular immunotherapies because they do not recognize allogeneic antigens and do not cause GVHD. However, they are still susceptible to allogeneic rejection by the host immune system owing to the expression of MHC[115]. Activation of NK cells is regulated by a group of diverse transmembrane receptors, including activating, inhibitory, cytokine and chemokine receptors[115]. MHC downregulation, a common feature of tumour cells, provides an activation mechanism for NK cells that kills via the 'missing-self' mechanism. In addition, NK cells possess activating receptors such as NKG2D and activating killer immunoglobulin-like receptors

(KIRs) capable of recognizing ‘induced-self’ ligands preferentially expressed by cancer cells. Another potential advantage of NK cells relies on their killing mechanism that could be boosted by transduction with a CAR. CAR-NK cells can eliminate target cancer cells via CAR-dependent or CAR-independent mechanisms. Most CAR constructs used for NK cells share the same structure used in CAR-T cells, with a CD3ζ intracellular domain and 4-1BB co-stimulatory domain to improve CAR-NK activation and cytokine production (such as IFNγ, granulocyte–macrophage colony-stimulating factor (GM-CSF))[115,116]. Other constructs have been designed using NK cell-specific co-stimulatory domains (DAP12 and 2B4) and may be superior to 4-1BB in terms of cytotoxicity and in vivo antitumour effect[117].

The theoretical advantages of CAR-NK cells have led to considerable enthusiasm, with many clinical studies ongoing. As of this writing, at least 32 clinical trials have been planned or activated with CAR-NK cells, including 11 targeting acute leukaemias and 10 targeting lymphomas, and one product against multiple myeloma. Target antigens are CD19, CD22, CD19/CD22, CD33, BCMA and CD7 and are described in recent review articles[118,119].

NK cells derived from adult peripheral blood mononuclear cells are difficult to transduce, but NK cells are more amenable to gene engineering and can be obtained from umbilical cord blood, adult bone marrow $CD34^+$ haematopoietic stem cells or inducible pluripotent stem cells. A phase I/II clinical trial in patients with NHL or CLL showed a complete response and sustained remission in patients treated with NK cells from cord blood transduced with anti-CD19 CAR and engineered to express IL-15 for improved persistence (the rimiducid-inducible caspase 9 suicide gene was included as a safety measure to trigger apoptosis in case of adverse events but was not triggered)[120,121]. Of the 11 patients, 8 patients responded to the therapy, 7 patients had complete remission and 1 patient had remission of the Richter’s transformation component but persistent CLL. Interestingly, NK cells persisted approximately 2 weeks after infusion, which has the potential advantage of limiting cytotoxicity in the case of on-target off-tumour effects. However, multiple infusions of CAR-NK cells are needed to achieve tumour clearance. It is important to note the limited number of patients in this trial, the fact that most responding patients received additional treatments after CAR-NK cells and the absence of long follow-up.

Several differentiation protocols are now available to produce NK cells from inducible pluripotent stem cells[122], a renewable source of NK cells that can be used as a platform for cloning and genome editing. They may lead to reduced manufacturing costs because they can be cryopreserved and administered to patients at different timings.

Although CAR-NK cell therapy has some advantages over CAR-T cell therapy, important limitations also exist. NK cells are effective cytotoxic cells, but they can also become functionally exhausted in the TME, mainly because of hypoxia. Nonetheless, hypoxia can be used as an advantage when constructing CAR-NK cells. Juillerat et al.[123] demonstrated that the incorporation of a hypoxia-inducible factor 1α (HIF1α) domain in the CAR construct could generate a system in which the expression of CAR is stimulated by low oxygen in the TME. In addition, CRISPR–Cas9 can be used to alter pathways that are involved

in NK cell exhaustion or function, or counteract transforming growth factor-β (TGFβ) immunosuppression[124,125].

**Macrophages.—**Macrophages are a cellular component of the innate immune system. Macrophage cells can traffic to tumour cells, modulate the TME and present various antigens. Overcoming the immunosuppressive TME and negative immunomodulatory signals is one of the main challenges faced by CAR-T cells[126]. CAR-macrophages have thus emerged as a possible alternative tool, especially against solid tumours, owing to their ability to infiltrate the TME and modulate the immune response[127]. A crucial challenge when generating macrophage-based immunotherapies is enabling their pro-inflammatory effects[127]. CAR-macrophages designed to recognize CD19, HER2 or mesothelin with a CD8 transmembrane and CD3ζ intracellular domain efficiently redirected macrophages towards an M1 phenotype, stimulating antigen-dependent phagocytosis, cytokine release and antitumour activity[128]. Morrissey et al.[129] engineered a family of CARs for phagocytosis that direct macrophages to engulf specific antigen-coated particles (CD19 and CD22) and tested a panel of intracellular domains. Because macrophages evolved to detect and respond to exogenous nucleic acids and present limited proliferative capacity, the delivery of the CAR construct is challenging. Klichinsky et al.[128] used the elegant replication-deficient chimeric adenoviral vector Ad5f35 to deliver the CAR. This system transduced the macrophages with high efficiency and reproducibility and resulted in M1 differentiation, thus creating a pro-inflammatory TME and synergizing with the CAR activity.

Several clinical trials are ongoing evaluating CAR-macrophages for solid cancers (NCT04660929).

## Tumour-intrinsic resistance

### Antigen escape

Antigen-negative escape has been observed since the early clinical trials of second-generation CAR-T cells. Extensive data on the incidence of CD19 loss are available and more data are emerging for BCMA and CD22 loss. Antigen loss can be a consequence of several mechanisms, including lineage switching, alternative splicing, frameshift or gene deletions, abnormal processing or trafficking of the antigen, or epitope masking. The altered clone is probably a pre-existing clone that has a survival advantage under the strong selective pressure of CAR-T cells.

**CD19.—**The loss of expression of the CD19 protein on cancer cells can occur as a mechanism of immune escape to CD19-targeted therapies. Antigen-negative disease relapse following CD19 CAR-T cell therapy has been described primarily in patients with B-ALL, with some recent data confirming it in NHL cases. The incidence of CD19 loss after CAR-T cell therapy varies between B-ALL and NHL and between paediatric versus adult cases.

Clinical data from paediatric trials described 18–24% of cases (13 out of 55 patients) with initial complete responses developing $CD19^{-}$ relapse[130-132]. In patients with axi-cel and tisa-cel, CD19 escape was seen in 9–25% in B-ALL and 27–35% in DLBCL[15,133-135]. Longer CAR-T cell persistence is typically associated with a decreased overall risk of

relapse but an increased rate of antigen loss as a mechanism of relapse. When anti-CD19 CAR-T cell therapy was used as a 'bridge to allo-HSCT' in B-ALL cases, none of the patients who received a transplant developed antigen escape relapse, whereas antigen-negative B-ALL was observed in cases that were not eligible for transplant[136]. In NHL, in a combined analysis of phase I and phase II trials of axi-cel, 3 out of 11 patients (27%) who progressed after a response had CD19 loss as determined by immunohistochemistry (IHC)[2]. A study from Spiegel et al.[137] evaluated resistance to CD19 CAR-T cell therapy in NHL and its association with low levels or loss of CD19 at relapse, using the semiquantitative IHC H-score to measure CD19 expression at baseline and at disease progression in patients treated with axi-cel. Among patients with paired pre-therapy and post-therapy H-scores, 60% (9 patients out of 15) who expressed CD19 showed an expression loss of CD19 at relapse. Still, a pretreatment semiquantitative IHC measurement of CD19 expression did not identify patients at risk of relapse. In the ZUMA-2 trial, Wang and colleagues[138] reported 23% relapses among 60 patients with mantle cell lymphoma, with only one case (7%) having undetectable CD19 (ref. 5). In the ZUMA-5 trial for patients with follicular lymphoma, none of the patients had a loss of CD19 expression at disease progression. Overall, CD19 loss seems to occur more frequently when the tumour arises from more immature B cells.

CD19 loss can occur through various mechanisms, including genetic alterations, such as CD19 gene mutations or deletions, or epigenetic modifications that lead to silencing of the *CD19* gene expression. Sotillo and colleagues[139] at The Children's Hospital of Philadelphia were, to our knowledge, the first to describe CD19 mutations and splice variants in B-ALL paediatric cases that had relapsed after CD19 CAR-T cell therapy. The study compared four samples before and after CD19 CAR-T cell treatment and detected CD19 splice variants involving exon 2 (Aexon2) or exons 5–6 skipping in relapsed leukaemias[139]. A more recent analysis with a different algorithm also highlighted intron 2 retention, which would cause either nonsense-mediated decay of the transcript or a truncated CD19 protein. Splice variants involving Δexon2 variants have also been reported by Fischer and colleagues[140], and this analysis also confirmed the existence of alternative CD19 spliced isoforms at the disease diagnosis. Δexon2 variants are unable to fold correctly and traffic to the cell surface and are retained by the endoplasmic reticulum[141]. Under selective CD19 CAR-T cell pressure, cells carrying those variants could evolve as a dominant clone. Single-cell RNA sequencing has confirmed that $CD19^-$ B-ALL cells can exist before CAR-T cell therapy[142].

An analysis of specimens collected at enrolment and at the time of clinical relapse from two phase II CD19 CAR-T cell trials of paediatric and young adult patients showed that 12 out of 17 patients analysed had $CD19^-$ relapse. Single-cell RNA sequencing identified frameshift mutations throughout exons 2–5 of *CD19* in all 12 specimens, predictive of a truncated protein lacking membrane anchorage[134]. In a recent report, three patients with $CD19^-$ relapsed DLBCL after CD19 CAR-T therapy carried mutations in CD19 exon 3, highlighting the first exons of *CD19* as a possible mutational hotspot[143]. Braig et al.[144] observed that one out of four patients with B-ALL who developed $CD19^-$ relapse after treatment with the CD19–CD3 bispecific T cell engager (BiTE) blinatumomab had no mutations in *CD19* and expressed full-length *CD19* mRNA but had no expression of CD81. CD81 associates with CD19 in the endoplasmic reticulum and is critical for the transport of CD19 to the cell surface. Therefore, $CD19^-$ immune escape had resulted from a lack

of CD81 expression and subsequent defective transport and/or maturation of CD19 in the endoplasmic reticulum and/or Golgi[144]. Moreover, the expression of the Golgi-resident intramembrane protease signal peptide peptidase-like 3 (SPPL3) in malignant B cells has recently been identified as a potent regulator of resistance to CAR-T cell therapy[145]. A loss of SPPL3 leads to hyperglycosylation of CD19, suppressing CAR-T cell effector function and cytotoxicity, whereas overexpression of SPPL3 induces a loss of the CD19 protein and thus resistance to CAR-T cells. Another mechanism appearing as antigen loss is lineage switching, particularly in tumours originating from highly immature cells, such as pre-B cell ALL or mixed-lineage-rearranged leukaemia. The phenomenon was described initially in paediatric patients with B-ALL who relapsed after chemotherapy, and it was recently observed after CD19 CAR-T cell therapy or after blinatumomab treatment. Anti-CD19 CAR-T cell therapy imposes a selective pressure on B-ALL leukaemia expressing CD19 and leads to an outgrowth of tumours expressing markers of a different lineage (from lymphoid to myeloid blasts), including a loss of the target antigen CD19. Gardner et al.[131] found that two out of seven patients with B-ALL with a rearrangement in histone-lysine *N*-methyltransferase 2A (KMT2A) developed acute myeloid leukaemia at the time of relapse after anti-CD19 CAR-T cell therapy. Jacoby et al.[146] showed a similar interesting result, in which an infant with KMT2A-rearrangement ALL treated with CD19 CAR-T cells showed initial clearance of blasts followed by disease relapse with the expression of CD34, CD11b and CD33, of which all are myeloid markers. The TME may also play a part in lineage determination, suggesting that a combination of the oncogenic driver with environmental cues can contribute to pressuring lineage switch[147]. Finally, Ruella and colleagues[56] described a case of epitope masking in a paediatric patient with ALL who relapsed 9 months after CD19 CAR-T cell therapy. Although it initially seemed to be $CD19^-$ by flow cytometry, a deeper analysis of the leukaemic blasts showed that CD19 was in fact expressed but bound in *cis* to the CD19 CAR that was aberrantly expressed in leukaemic blasts. The aberrant expression of the CAR in the leukaemic blasts was due to the accidental transduction of a single leukaemic cell during manufacturing[56].

**CD22.—**CD22 is a cell-surface molecule that, like CD19, is broadly and uniquely expressed by B-lineage cells[148,149]. Interestingly, downregulation of CD22 seems to be the major mechanism of escape following anti-CD22 CAR-T cell therapy, probably related to the relative lower efficacy of CD22 CAR-T and the tendency of CD22 to be internalized. CD22-directed CAR-T cells have been investigated in paediatric and adult patients with relapse or refractory (R/R) B-ALL as well as in adult NHL after failure of CD19 CAR-T cell therapy[150,151]. In a single-institution phase I dose-escalation trial, three patients with LBCL and six patients with ALL received CD22 CAR-T cell therapy after a median of six previous lines of treatment, including previous CD19-directed CAR-T cell therapy. All patients with LBCL achieved complete response with a median follow-up of 8.4 months, and all patients with ALL achieved a complete response with a median of 5.1 months[151]. The LBCL cohort was recently updated, with 36 patients (median follow-up 15.7 months) achieving objective and complete response rates of 72% and 53%, respectively[152]. Shah et al.[148] conducted a single-centre, phase I trial in 58 patients with R/R $CD22^+$ malignancies, including 56 B-ALL and one DLBCL. In this cohort, 87.9% of patients had received previous CD19-directed therapy. The complete remission rate was 70%. The median overall survival was

13.4 months (95% CI of 7.7–20.3 months). Among those patients who achieved a complete response, the median relapse-free survival was 6.0 months (95% CI of 4.1–6.5 months). Of note, 86.2% experienced CRS, but only 10% were grade 3 or above. Interestingly, approximately 32.8% of participants exhibited haemophagocytic `lymphohistiocytosis`-like manifestations requiring the use of anakinra (cytokine blockade targeting IL-1) and high-dose steroids. This clinical trial highlighted the importance of antigen density in response to CD22 CAR-T cells. In another study, treatment with CD22-targeted CAR-T cells in 21 children and adults, including 17 patients previously treated with CD19-directed immunotherapy, showed dose-dependent antileukaemic activity, with complete remission obtained in 73% (11 of 15) of patients, including all patients with $CD19^{low}$ or $CD19^{-}$ B-ALL[149]. In this study, relapses were associated with diminished CD22 density, without gene mutations or alteration in the mRNA levels, indicating a post-translational mechanism of CD22 expression to the cell surface. Lower CD22 protein levels can enable tumour cell escape from CD22 CAR-T-mediated killing. To our knowledge, this was the first study in which a direct correlation between CD22 levels and CAR-T cell-mediated killing was proven in vivo and in vitro by generating leukaemia lines with variable CD22 expression levels and directly demonstrating that tumour control in xenograft models was dependent on surface expression levels of CD22. The inability of CAR molecules to bind to low levels of antigens might derive from differences between the endogenous TCR and the CAR and/or differences at the `immunological synapse`. Natural TCRs evolved to recognize antigens at low density and contain several intracellular signalling domains. CAR molecules typically incorporate only the gene encoding the TCRζ chain and, during the course of antigen recognition, they form an immunological synapse that is differently organized compared with that of a natural TCR[153,154]. Results from a different phase Ib trial showed that CD22-targeting CAR-T cells have a good safety profile and resulted in high rates of complete response and no minimal residual disease in 18 adults and children, independent of previous treatment with CD19 CAR-T cells, blinatumomab or inotuzumab[155].

**BCMA.—**Antigen escape has been documented in patients with multiple myeloma treated with CAR-T cells targeting BCMA. Two studies have shown that BCMA can be lost or significatively reduced at relapse. Ali et al.[156] reported that 8% (1 in 12 patients) of targeted-treated patients exhibited a $BCMA^{-}$ biopsy. In another cohort, 67% of patients (12 of 18) with evaluable biopsy pretreatment and post-treatment with BCMA CAR-T cells showed a reduction in BCMA intensity on myeloma cells, including 4 out of 9 non-responders[157]. A total of 1 out of 16 patients with an evaluable bone marrow sample at relapse experienced antigen loss in the idecabtagene vicleucel approval study[158]. Further isolated cases of downregulation or loss of BCMA have been observed in patients who relapsed after CAR-T cell therapy[159-161]. Overall, however, antigen escape associated with anti-BCMA therapies does not seem to be as common as with CD19-targeting CAR-T cells, probably because BCMA is essential for plasma cell survival[158].

## Antigen-positive tumour escape

Outside B-ALL, most of the relapses after CAR-T cell therapy are characterized by the continued presence of the target antigen. Typically, these relapses occur earlier than the antigen-negative ones and are thought to be linked to poor CAR-T cell persistence or

function. Recent data in B-ALL revealed that decreased expression of death receptor signalling molecules, such as components of the FAS pathway, or pro-apoptotic mediators of the extrinsic pathway of apoptosis constitute a novel mechanism of primary resistance to CAR-T cell therapy[162]. Through an unbiased, genome-wide CRISPR–Cas9 loss-of-function screen, the authors identified progressive impairment in CAR-T cell function initially driven by downregulation of the FAS-associated protein with death domain (FADD). Together, these results suggested that disruption of the death receptor signalling pathway leads to decreased tumour killing and persistent antigen exposure that, in turn, results in global transcriptomic and epigenetic reprogramming of CAR-T cells, adversely affecting their function. The findings were further validated using samples from two clinical trials in which the leukaemic cells in non-responders had a significantly lower death receptor signature expression than responders. A more recent report added that hypermethylation of DNA, a stem cell-like phenotype and inherent plasticity, and decreased antigen presentation are features of leukaemia cells that do not respond to CAR-T cell therapy[163].

A better understanding of how T cells eliminate tumours is essential to unravel resistance mechanisms. Using a CRISPR–Cas9 screen, one group found that activation of FAS mediated `bystander killing` of antigen-negative tumour cells, thereby increasing the clearance of antigen-heterogeneous tumours[164]. In addition, in a large clinical study, FAS expression in tumour cells was able to predict the survival of patients treated with CAR-T cells (NCT02348216) and can thus contribute to further understanding of CAR-T cell response[164]. Other clinical studies focused on antigen-positive escaped are displayed in Table 2.

## Strategies to overcome antigen-negative escape

### Co-administration of receptors

Several strategies are being developed to overcome antigen-negative escape, including novel CAR-T cell designs and combination therapies[53,77,135,165,166], such as co-administration of two CAR-T products, co-transduction or bicistronic CAR vectors, or tandem/loop CAR-T cells.

**Co-administration of two CAR-T cell products.—**With this approach, two distinct CAR-T cell products targeting different antigens are generated and administered together or sequentially. In a recent study, adult patients with R/R ALL received two autologous CAR-T cell products (one targeting CD19 (huCAR T19) and the other targeting CD22 (CAR T22-65s)). All evaluable patients ($n = 11$) achieved a complete response with no minimal residual disease, despite previous treatment with CD19-targeting and/or CD22-targeting immunotherapy[167]. Similar approaches have been conducted by other groups[168,169]. The limitations of this approach are the cost and effort to produce two different vectors and two different products and the related regulatory challenges.

**Co-transduction or bicistronic CAR vectors.—**This approach uses a single T cell product to express two CARs that recognize different antigens. This can be achieved by different approaches. T cells can be co-transduced with two different vectors to create a T cell product with a mixed population, in which some of the cells are transduced with one or

both vectors. An alternative strategy uses bicistronic vectors that enable the co-expression of two different CAR molecules on the same T cell. A recent study documented dual targeting of CD19 and CD22 against B-ALL by using a novel anti-CD22 CAR construct able to recognize cells with low CD22 density and lacking tonic signalling[170]. CAR-T cells in the study were co-transduced with two separate vectors: anti-CD22 CAR (9A8-41BBζ) and anti-CD19 CAR (CAT-41BBζ). The obtained CAR-T cell product (CAT/9A8) eliminated single-positive and double-positive target cells in vitro and in vivo. CAT/9A8 CAR-T cells are now being tested in a phase I clinical study (NCT02443831)[170]. In addition, Cordoba et al.[171] conducted a phase I trial in paediatric and young adult patients with R/R B-ALL to test AUTO3 (autologous transduced T cells expressing both anti-CD19 and anti-CD22 CARs with a bicistronic vector encoding both CARs) (Table 3). In the trial, 13 of 15 treated patients responded to the treatment, regardless of disease burden, cytogenetic risk factors or the number of previous lines of therapy, including one case with $CD19^{-}$ $CD22^{+}$ blasts before lymphodepletion, reinforcing the importance of dual targeting. Strategies to optimize the persistence of this dual-targeting product are needed because nine patients ultimately relapsed with low CAR-T cell persistence. The reason for reduced persistence is not fully understood, but it was also observed in other trials with a mixed anti-CD19 and anti-CD22 infusion product or dual CAR-T cells[172,173]. Roddie at al.[174] evaluated dual targeting by using AUTO3 CAR-T cells in combination with PD1 blockade (pembrolizomab) in R/R LBLC and reported it to be safe and suitable for outpatient administration. This treatment approach resulted in lasting remissions in 54.4% of patients who achieved a complete response, accompanied by strong expansion of CAR-T cells. However, both the dual-targeting CAR-T therapy and pembrolizumab failed to prevent relapse in a significant number of patients. Fousek et al. recently reported CD19/20/22-targeting CAR-T cells by co-expressing individual CAR molecules on a single T cell by using one tricistronic transgene. CD19/20/22 CAR-T cells killed $CD19^{-}$ blasts from patients who relapsed after anti-CD19 CAR-T cell therapy. These triple-targeting CAR-T cells formed dense immune synapses with target cells, mediated effective cytolytic complex formation and were as efficacious as CD19 CAR-T cells against primary $CD19^{+}$ disease[175].

**Tandem/loop CAR-T cells.—**These CAR-T cell products contain one receptor molecule with two binding domains able to recognize two targets on the cognate tumour cell. On the basis of clear evidence that both CD19 antigen loss following CD19-directed immunotherapy and diminished CD22 cell-surface expression contribute to relapse, the group of Fry[176] designed a bispecific anti-CD19–CD22 CAR (CD19-22. BB.z-CAR) that simultaneously targets both antigens and preserved bifunctionality against both CD19 and CD22. This loop CD19–CD22 CAR showed strong preclinical data of efficient killing of $CD19^{+}CD22^{+}$, $CD19^{+}CD22^{-}$ and $CD19^{-}$ $CD22^{+}$ B-ALL tumours in xenograft models[176]. This bispecific CAR has progressed to phase I clinical trials for both children and adults with R/R B-ALL (NCT03241940) and R/R $CD19^{+}$ DLBCL (NCT03233854) and children and young adult patients with R/R $CD19^{+}CD22^{+}$ B cell malignancies (NCT03448393)[137,177]. Spiegel et al.[137] used CD19-22.BB.z-CAR in a phase I clinical trial (NCT03233854) of adults with R/R B-ALL or LBCL and observed that relapses were $CD19^{-/low}$ in 50% of B-ALL patients and in approximately 30% of patients with LBCL but never associated with CD22 antigen loss or low expression, suggesting that

selective pressure on the CD22 antigen was lower than when CD22 alone was targeted. Further studies are needed to calibrate and tune different CAR potencies when used in combination[137]. The construct has also been used in a dose-escalation phase I clinical trial in treated children and young adults heavily pretreated with B-ALL, showing only limited CAR-T cell expansion and cytokine production and prompting further construct optimization[177]. A tandem bispecific CAR targeting CD19 and CD20 is currently being tested in a phase I trial in patients with R/R B cell malignancies (NHL and CLL; NCT03019055)[178]. This product is manufactured and infused without cryopreservation. The overall response rate to the dose of $2.5 \times 10^6$ cells per kg with non-cryopreserved infusion ($n = 12$) was 100% (complete response of 92%; partial response of 8%). Several clinical trials are underway to test multiple-targeting CAR-T cells and to overcome antigen loss or downregulation (Table 3). One interesting consideration about this trial is that it showed no correlation between initial levels of expression of CD19 and CD20 and clinical response. In addition, the trial reported that only one patient out of the five patients who entered the trial after receiving anti-CD19 CAR-T cell therapy achieved adequate CAR-T cell expansion and a complete response, suggesting that retreatment with a pure based immunotherapy approach remains challenging. Long-term follow-up (NCT03375619) continues. Ruella et al.[179] also tested the novel combination of anti-IL-3 receptor-α chain (CD123) CAR and anti-CD19 CAR in B-ALL models after observing that CD123 was expressed in relapsed CD19 B-ALL disease. Anti-CD123 CAR-T cells targeted antigen-negative blasts and could be co-administered with anti-CD19 CAR-T cells to prevent antigen-loss relapse. A novel tandem CAR construct that combined CD19-mediated and CD123-mediated T cell activation provided superior in vivo activity against B-ALL than single antigen-expressing CAR-T cells or pooled combination CAR-T cells.

### Fourth-generation CAR-T cells

Fourth-generation CAR-T cells are engineered to secrete additional molecules, such as cytokines, to enhance antitumour activity. As a result, in addition to the direct antitumour attack, they can trigger T cells to eliminate antigen-negative cancer cells at the target site. These T cells are also referred to as T cells redirected for universal cytokine-mediated killing (TRUCKs). Some of these TRUCKs secrete IL-12 or IL-18, and their improved antitumour effects have been demonstrated in xenograft and syngeneic mouse models[180,181].

A different approach is the design of CAR-T cells with the secretion of bispecific antibodies. They can target different tumour antigens and also recruit T cells to the TME. Therefore, the combination of these two strategies can reduce antigen escape and heterogeneity, as well as increase overall antitumour activity and CAR persistence. For the treatment of glioblastoma, a recent study incorporated this idea by evaluating a bicistronic construct that expresses a CAR specific for EGFRvIII – a mutant form of EGFR mostly expressed in glioblastoma – and a bispecific T cell engager against EGFR, which is overexpressed in glioblastoma[182]. In contrast to EGFRvIII CAR-T cell monotherapy, the combination CAR and the bispecific T cell engager eliminated the heterogeneous tumour in a glioblastoma mouse model with a good safety profile. Dual application of CAR-T cells and BiTEs separately can also be successful, as shown in a case study that reported increased CAR-T cell expansion of CD22

CAR-T cells after treatment with blinatumomab, in a patient with R/R B-ALL who had relapsed after CAR-T cell monotherapy[183].

### Combination strategies

Combining CAR-T cells with treatments that maintain target expression on the tumour surface is also a potential strategy to prevent antigen downregulation. BCMA is actively cleaved from the tumour cell surface by γ-secretase, resulting in decreased ligand density. Therefore, pharmacological inhibition of γ-secretases can block BCMA cleavage[184,185]. This strategy has proven to be successful when a combination of γ-secretase inhibitors and BCMA CAR-T cells was tested in murine models of multiple myeloma[185]. Studies assessing γ-secretase inhibition with concurrent BCMA CAR-T cell treatment are underway (NCT03502577, NCT04714827 and NCT04162353)[186,187].

An alternative strategy in the setting oflow antigen density is to modify the design of the CAR molecule to tune the threshold for CAR-T cell activation. An exciting strategy explored by Majzner et al.[188] is based on including additional immunoreceptor tyrosine-based activation motifs (ITAMs) in the CAR to improve the recognition of low antigen density. This strategy was further enhanced when combined with the CD28 hinge transmembrane region, proving that the precise design of CARs can tune the threshold for antigen recognition.

## Immunosuppressive TME

The TME has a crucial role in terms of response to cancer treatment, as it can attenuate the effectiveness of the infused CAR-T cells through several mechanisms, including the physical barrier, soluble factors and immunosuppressive cells[189].

### Mechanisms of TME immune suppression

The TME can pose challenges to effective CAR-T cell therapy by reducing trafficking and inducing T cell dysfunction[190,191]. Immune dysregulation in DLBCL treated with axi-cel showed that resistance to the CAR-T cell therapy centred on circulating mononuclear MDSCs and tumour IFN signalling, which led to insufficient axi-cel expansion as well as elevated expression of immune checkpoint ligands[192]. Although acute exposure to IFNγ increases the antitumour function of immune cells, chronic signalling can lead to immune suppression with resistance to therapeutic methods such as immune checkpoint inhibitor therapy. The importance of IFNγ for host immune activation of CD19-targeted CAR-T cells in the TME has been demonstrated in an immunocompetent mouse lymphoma model[193]. Therefore, IFNγ may have reduced immunosuppression in the TME through checkpoint inhibition or cytokine regulation, influencing the effectiveness of CAR-T cells[194,195]. However, recent work indicated that the loss of genes (IFNγ receptor 1 (*IFNGR1*) and Janus kinase 1 (*JAK1*) and *JAK2*) in the IFNγ receptor signalling pathway conferred resistance to CAR-T cell killing only in solid tumours but did not affect lymphoma or leukaemia cell sensitivity to CAR-T cells[196,197]. In addition, a recent study investigating the mechanisms of action of axi-cel discovered that all important aspects of successful CAR application (efficacy and patients survival as well as lack of toxicity) were linked to the pretreatment and

post-treatment tumour immune contexture[198] – a concept that includes a summary of various parameters of the immune system and a more precise characterization and localization of immune cells within a tumour[199]. Also, the study confirmed the important role of the TME as a powerful resistance mechanism alongside T cell dysfunction and antigen escape.

Importantly, the TME can vary in the different disease types targeted by currently approved CAR-T cell therapy (mainly DLBCL, ALL and myeloma) owing to differences in the disease itself as well as the different anatomical sites such as lymph nodes or bone marrow[200-202]. In B cell NHL, the immunosuppressive milieu of the TME can render CAR-T cells unable to sufficiently attack the tumour. In one report, patients achieving complete responses had decreased levels of tumour-associated macrophages (TAMs), regulatory T ($T_{reg}$) cells and MDSCs, whereas chemokines and MDSCs were overexpressed in patients achieving only a partial remission[203]. Additional studies are necessary to provide a deeper understanding of the contribution of the TME towards CAR-T cell-targeted haematological malignancies.

## Overcoming TME immunosuppression

Altering and modifying the TME through various strategies, such as directly targeting TAMs and other immunosuppressive factors, has potential to improve the clinical efficacy of CAR-T cell therapy and achieve more durable remissions. The immunosuppressive influence of the TME can be reduced in different ways (Table 4). TRUCKs, discussed in the previous section, are one potential strategy. Various cytokines can be incorporated, such as IL-7, which selectively expands tumour-redirected cytotoxic T lymphocytes independently of $T_{reg}$ cells, or IL-15, which increases T cell expansion after antigen recognition and can simultaneously increase the cytotoxicity of tumours[204,205]. Similarly, secretion of IL-12 or IL-18 can induce a pro-inflammatory remodelling of the TME[180,181,206-208].

CAR-T cells can also be combined with BiTE, which can activate $T_{reg}$ cells to degranulate and kill target cells via perforin release. Another possibility is to incorporate synthetic enzyme-armed NK cells with attached inactive prodrugs, programmed to be activated directly at the tumour site and resulting in increased activity against the target tumour[209]. The TME can also be influenced by the co-stimulatory signalling domain of a CAR. The CD28 co-stimulatory domain supports $T_{reg}$ cell persistence through IL-2 secretion. However, the deletion of the LCK-binding moiety of the CD28 endodomain can abolish IL-2 secretion and drastically reduce the number of $T_{reg}$ cells at the tumour site. In addition, removal of this domain does not negatively affect CAR-T cell functionality or IFNγ secretion[210]. Another potential way to circumvent the immunosuppressive nature of the TME is using Toll-like receptor agonists. These compounds can increase the recruitment of leukocytes, particularly both cytotoxic T lymphocytes and T helper cells, to the tumour site and thereby reduce the suppressive TME influence[211,212]. Outside the current B cell disorders for which CAR-T cells are approved, patients with treatment-resistant Hodgkin lymphoma may benefit from CAR-T cell therapy but lack the CD19 antigen, and the TME is very immunosuppressive[213]. In addition, in vitro, Hodgkin lymphoma cells convert macrophages into TAMs, which can severely limit CAR-T cell proliferation. However, anti-CD123 CAR-T cells actively attack TAMs as well as Hodgkin tumour cells and establish long-term immune memory.

Smith et al.[214] published a study on the clinical role of the gut microbiota in CAR-T cells (including both 4-1BB and CD28 products) in patients with R/R B-ALL and LBCL. Exposure to antibiotics, such as piperacillin–tazobactam, imipenem and meropenem, during the 4 weeks before CAR-T cell administration was associated with decreased faecal microbiome diversity and overall survival. Also, a higher rate of `immune effector cell-associated neurotoxicity syndrome` was observed in the lymphoma subcohort when antibiotics were administered before CAR-T cell infusion. Conversely, a high frequency of obligate anaerobes such as *Ruminococcus*, *Bacteroides, Faecalibacterium* and *Akkermansia* was associated with a complete response by day 100. Stein-Thoeringer et al.[215] confirmed these data with a cohort of patients with NHL in the USA and Germany who received axi-cel, tisa-cel or liso-cel infusion. *Bifidobacterium longum* and peptidoglycan biosynthesis highly correlated with long-term survival and response to CAR-T cells, independent of demographic or clinical variables. *Lachnospira pectinoschiza* and *Akkermansia muciniphila* significantly associated with $CD3^+$ and $CD4^+$ T cell counts at the time of T cell apheresis, whereas *Bacteroides*, *Blautia* and *Faecalibacterium prausnitzii* had a negative effect on $CD3^+$ and $CD8^+$ T cell levels. Finally, the authors highlighted that *Akkermansia* may be involved in the better quality and performance of the manufactured T cell product. However, both studies did not report any association between the composition of the gut microbiota and CRS.

Aside from the TME itself, other host factors could probably influence CAR-T cell therapy. A recent study found that changes in the intestinal microbiome are associated with clinical outcomes in patients receiving CD19 CAR-T cells for a B cell malignancy[214]. Further evaluation is necessary to investigate the role of the microbiome with regard to resistance to CAR therapy.

## Future perspectives

In recent years, CAR-T cells have transformed the landscape of treatment for lymphoid malignancies, producing unprecedented response rates in patients with otherwise poor prognosis, and successfully moving up to earlier lines of treatment. However, resistance to CAR-T cells remains a key obstacle to the long-term and far-reaching therapeutic success of this therapy and thus is an important area of laboratory and clinical research[53,133,165]. Unlike most cancer therapeutics, in the case of CAR-T cells, potential mechanisms of resistance include failure of the drug itself (dysfunction, exhaustion and lack of persistence) and susceptibility of the drug to other host factors (TME, microbiome and previous therapies), in addition to tumour-related factors that apply to all therapeutic agents (aggressive disease, target loss and primary resistance to cell death pathways). However, unlike small molecules or protein-based biologics, CAR-T cells offer unique opportunities to engineer the therapeutic agent to enable improved products that overcome multiple potential resistance mechanisms, whether they are product related or tumour related. A thorough understanding of how CAR-T cells kill tumour cells and of the different mechanisms of resistance is essential for the further development of the next generation of immunotherapies. Promising strategies that are being developed include CAR-T cells that can target multiple antigens to reduce antigen escape, armoured CARs that release proteins for additional antitumour efficacy, optimization of CAR signalling to improve clinical efficacy, the combination of

CAR-T with small molecules and in general the use of genetic engineering to enhance CAR-T cell functions.

Challenges for the field include the development of representative preclinical models that can recapitulate primary and secondary resistance, whether they are related to the therapeutic agent, the tumour or other host factors. Current mouse models, for example, usually do not represent the TME well, and if they do, compromises have to be made elsewhere, such as the inability to test human T cells in syngeneic models or the use of allogeneic T cells and/or tumours in humanized mouse models. In addition, clinical development presents its own challenges, such as the time, financial and regulatory burdens necessary to test any new CAR-T cell modifications. Another challenge includes the interpretation of clinical data across small clinical trials in which there are often significant differences in other critical factors, such as the manufacturing process and patient populations.

Finally, when considering the effect of a new treatment, it is essential to consider access to this therapy as a potential cause of failure. Indeed, CAR-T cell therapy is a sophisticated therapy that requires significant expertise, infrastructure and cost. Therefore, socioeconomical and geographical factors have a significant role in limiting access to this treatment. Although political reforms and fair distribution of wealth are certainly the key players in reducing inequity in access to CAR-T cells, scientific developments might also play an important part. Point-of-care manufacturing, non-viral transduction strategies, short manufacturing and off-the-shelf products can all potentially reduce costs and ensure larger access to this life-saving therapy.

We remain optimistic that multiple biologic limitations of the currently approved CAR-T cells can be overcome with creative engineering strategies and combination therapies and that cell-based therapeutics are uniquely poised to enable long-term durable remission in patients with cancer.

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## Glossary

**Apheresis**

A method by which the blood of a person is passed through a device that separates one particular constituent and returns the remainder to circulation.

**Bystander killing**

The activation of surrounding T cells in the absence of chimeric antigen receptor-T cell-induced antigen recognition. This bystander killing is important for the eradication of antigen-negative target cells as well as for overall therapy response.

**CD45RO$^{-}$**
CD45 (also known as lymphocyte common antigen) is a receptor-bound protein tyrosine phosphatase that is expressed on leukocytes and has different isoforms. CD45RA T cells define a ‘naive’ trait, whereas CD45RO T cells are classified as ‘memory’ T cells.

**Eastern Cooperative Oncology Group**
A status scale widely used to assess the functional status of a patient.

**Immune effector cell-associated neurotoxicity syndrome**
Sometimes synonymously referred to as neurotoxicity, a clinical syndrome that can occur in the first days to weeks after chimeric antigen receptor-T cell administration. The symptoms, which in most cases are self-regulating and completely regress, range from mild confusion to life-threatening and sometimes fatal neurological complications, with application of steroids as the current standard of care.

**Immunological synapse**
The space between chimeric antigen receptor-T cells and the antigen-presenting tumour cell, in which the immune response is regulated and triggered by molecular interactions.

**International Prognostic Index**
The most frequently used prognostic factor system for patients with aggressive non-Hodgkin lymphoma.

**Lymphohistiocytosis**
A rare but sometimes fatal condition, caused mainly by infection, in which certain types of white blood cells (histiocytes and lymphocytes) can accumulate in various organs. These cells and other blood cells can be severely damaged or destroyed.

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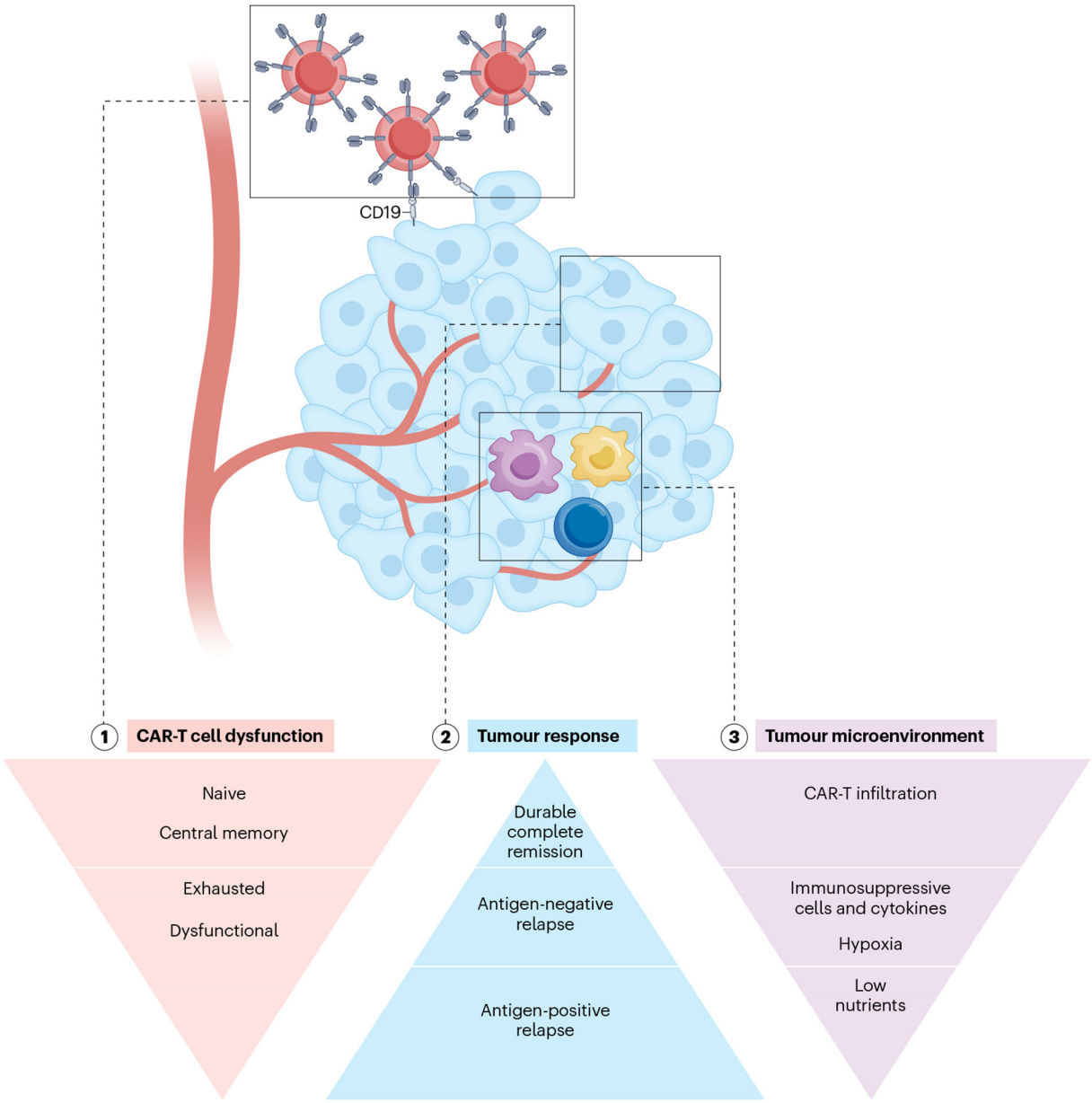


**Fig. 1 |. Overview of CAR-T cell resistance mechanisms.**

Several mechanisms of resistance to chimeric antigen receptor (CAR)-T cell immunotherapy have been identified and can be broadly related to CAR-T cell dysfunction (1), tumour-intrinsic resistance (2) or the surrounding immunosuppressive tumour microenvironment (3). CAR-T cells from responders are characterized by a more naive and central memory phenotype, as opposed to exhausted or dysfunctional CAR-T cells from non-responders. In addition, many tumour-intrinsic resistance mechanisms, beyond antigen-negative relapse, have been characterized. A ‘hot’ tumour microenvironment with high CAR-T cell infiltration, polarization and trafficking is usually predictive of a better response.

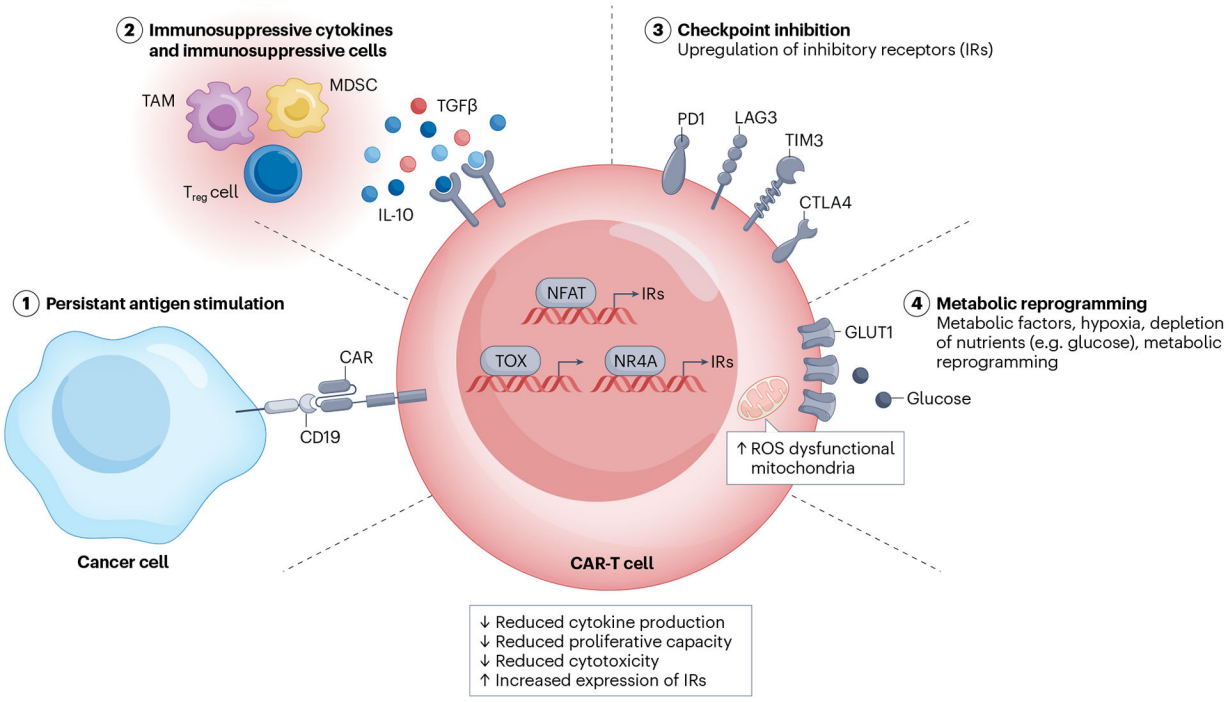


**Fig. 2 |. Mechanisms of CAR-T cell dysfunction.**

Chimeric antigen receptor (CAR)-T cell exhaustion is characterized by the sequential reduction of T cell effector functions, such as their ability to induce tumour lysis, produce cytokines and proliferate. (1) CAR-T cell activation requires binding to antigen-positive tumour cells. Persistent antigen stimulation and/or tonic CAR signalling result in CAR-T cell exhaustion. (2) Other factors contributing to exhaustion are the presence of immunosuppressive cell types in the tumour microenvironment (myeloid-derived suppressor cells (MDSCs), regulatory T ($T_{reg}$) cells and tumour-associated macrophages (TAMs)) and immunosuppressive ligands (IL-10 and transforming growth factor-β (TGFβ)). (3) Gene expression and epigenetic changes consistent with CAR-T cell exhaustion include activation of nuclear factor of activated T cell (NFAT) and the thymus high-mobility group box protein (TOX)–NR4A axis that drive the exhaustion programme of T cells and promote the expression of multiple inhibitory receptors (IRs) such as programmed cell death 1 (PD1), lymphocyte activation gene 3 (LAG3), T cell immunoglobulin domain and mucin domain 3 (TIM3) and cytotoxic T lymphocyte antigen 4 (CTLA4). (4) External factors and signals coming from IRs (such as PD1) compromise CAR-T cell metabolism. Exhausted CAR-T cells have an impaired ability to use aerobic glycolysis and oxidative phosphorylation and upregulate the glucose transporter (GLUT1) to compensate for the lack of glucose. ROS, reactive oxygen species.

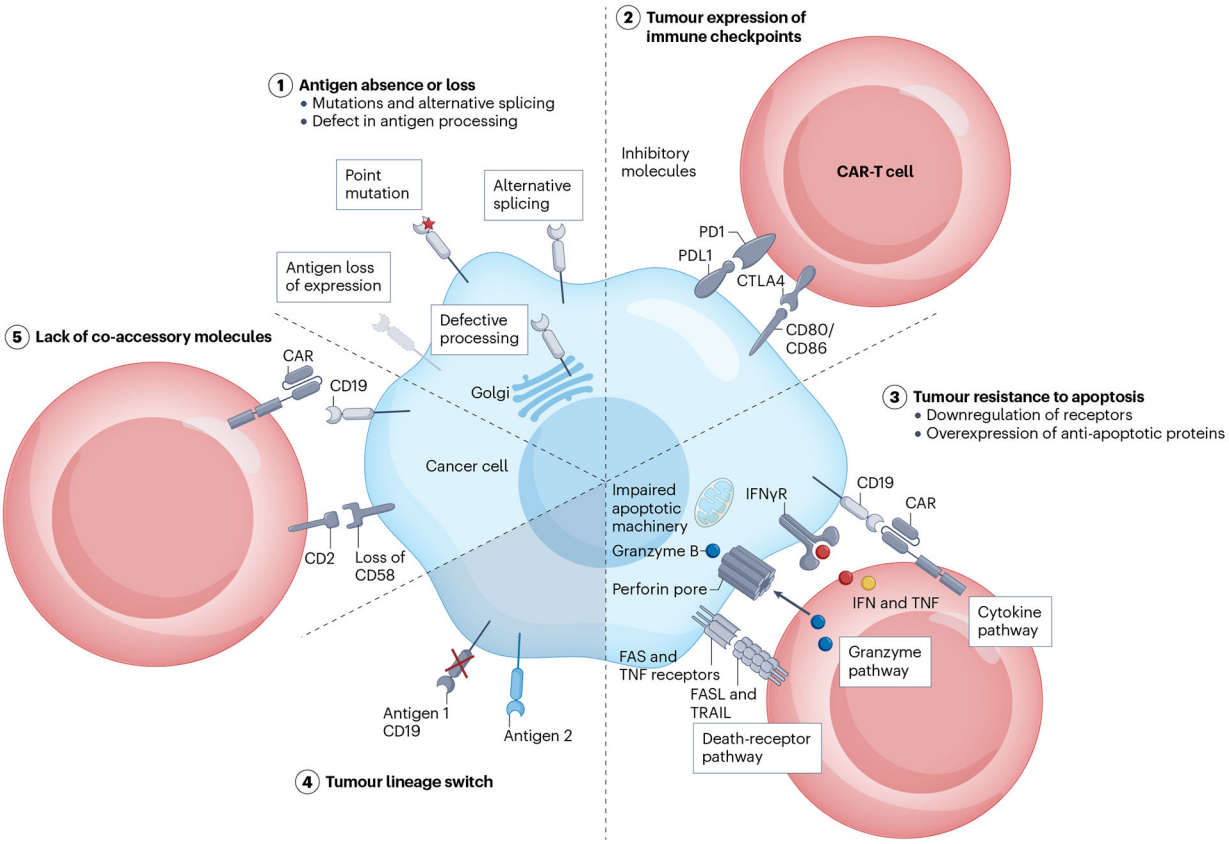


**Fig. 3 | Tumour-intrinsic mechanisms of resistance to CAR-T cells.**
Several tumour-intrinsic mechanisms have been described as factors associated with chimeric antigen receptor (CAR)-T cell failure. Owing to tumour heterogeneity, pre-existing antigen-negative tumour cells can be responsible for resistance to CAR-T cell therapy. (1) For CD19, antigen point mutations or alternative splicing result in a different form of the antigen (such as truncated) that can no longer be recognized by CAR-T cells. Antigen loss might also be due to defects in target antigen maturation and trafficking due to a lack of appropriate chaperons. (2) Tumour expression of inhibitory ligands, such as programmed cell death 1 ligand 1 (PDL1) or CD80, inhibits CAR-T activation and induces exhaustion, independently of antigen-target recognition. (3) Impaired apoptotic machinery in the tumour cells or downregulation of cell death receptors at the tumour level can confer tumour resistance to apoptosis induced by CAR-T cells. (4) Lineage switch may be responsible for complete phenotypic changes, including the loss of the target antigen. (5) CAR sensitivity depends on CAR binding to the antigen and antigen level of expression, but it is also increased when the tumour cells express the correct ligands for CAR co-accessory molecules. Defects in the engagement of co-accessory molecules (such as the loss of CD58 expression on tumour cells) can result from inefficient accessory receptors and CAR-T cell activation. CTLA4, cytotoxic T lymphocyte antigen 4; IFNγR, IFNγ receptor; LAG3, lymphocyte activation gene 3; PD1, programmed cell death 1; TIM3, T cell immunoglobulin domain and mucin domain 3; TNF, tumour necrosis factor.

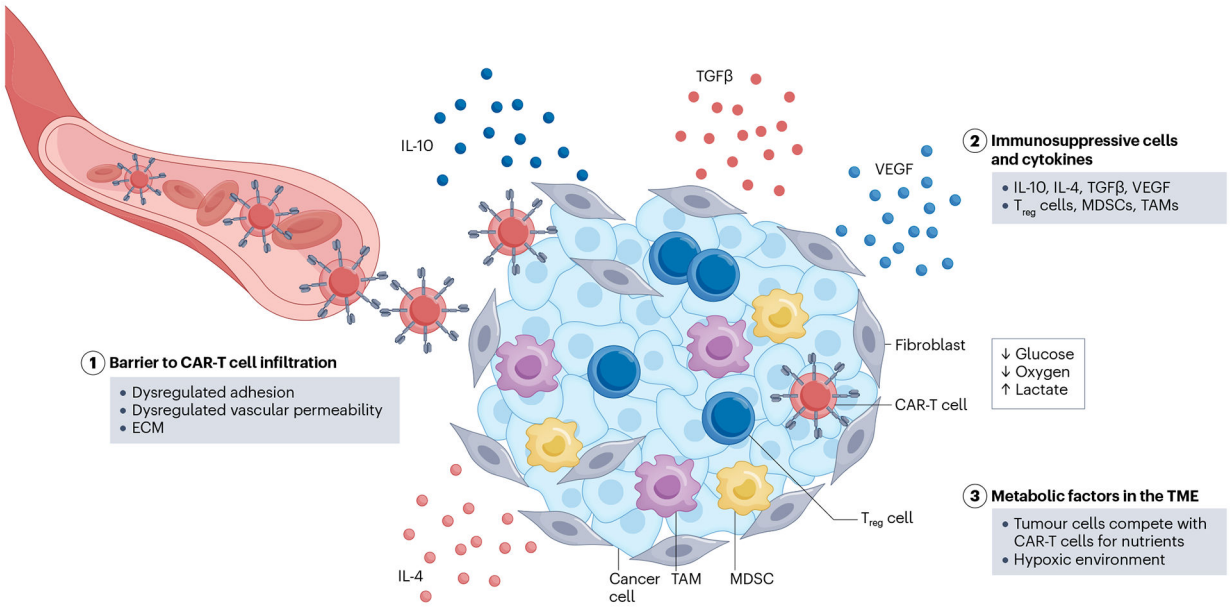


**Fig. 4 | Tumour microenvironment barriers to CAR-T cell therapy.**

Chimeric antigen receptor (CAR)-T cells might encounter environmental barriers that prevent their ability to infiltrate the tumour and activate and kill the target cells. (1) Barriers to CAR-T cell infiltration include tumour vasculature with downregulated expression of endothelial adhesion molecules by endothelial cells, which is necessary for T cell migration from the endothelium into tumours, as well defects in vascular permeability and the deposition of a fibrotic extracellular matrix (ECM) that physically blocks T cell migration and lowers T cell motility. (2) Tumour-associated macrophages (TAMs) and regulatory T ($T_{reg}$) cells contribute to CAR-T cell exhaustion. $T_{reg}$ cells present in the tumour microenvironment (TME), for example, often express the inhibitory ligand programmed cell death 1 ligand 1 (PDL1), which is able to suppress CAR-T cell functionality. In addition, immunosuppressive cells and tumour cells secrete inhibitory cytokines (IL-10, IL-4 and transforming growth factor-β (TGFβ)) that also diminish the effector function of CAR-T cells. (3) Finally, tumour cells compete with CAR-T cells for nutrients (such as glucose and tryptophan) and oxygen. MDSC, myeloid-derived suppressor cell; VEGF, vascular endothelial growth factor.

**Table 1 |**

Clinical trials that address CAR-T cell dysfunction

| ClinicalTrials.gov identifier | Country | Disease | Construct | Summary of results (if available) |
|---|---|---|---|---|
| **PD1–PDL1 blockade** | | | | |
| NCT03044743 | China | EBV-associated malignancies (DLBCL, among others) | EBV cytotoxic lymphocytes | NA |
| NCT04163302 | China | BCL | Anti-CD19 CAR-T cell | NA |
| NCT03298828 | China | B cell malignancies | Anti-CD19 CAR-T cell | NA |
| NCT04539444 | Taiwan | BCL | Anti-CD19/CD22 CAR-T cell | NA |
| NCT03258047 | China | BCL | Anti-CD19 CAR-T cell | CD19–PD1/CD28–CAR-T are safe and effective in $PDL1^{+}$ BCL[79] |
| NCT04381741 | China | DLBCL | Anti-CD19 CAR-T cell | NA |
| **PD1–TIGIT blockade** | | | | |
| NCT04836507 | Republic of Korea | LBCL | Anti-CD19 CAR-T cell | NA |
| **Combining CAR therapy with signal transduction inhibition via BTK-dependent and BTK-independent mechanisms** | | | | |
| NCT01747486 | USA | CLL/SLL | Anti-CD19 CAR-T cell | NA |
| NCT02640209 | USA | CLL/SLL | Anti-CD19 CAR-T cell | Adding autologous anti-CD19 humanized binding domain T cells to ibrutinib in patients with CLL not in complete remission led to deep and durable remissions[87] |
| NCT03331198 | USA | CLL/SLL | Anti-CD19 CAR-T cell | Administration of ibrutinib in combination with CAR-T cells was well tolerated and might decrease the incidence of severe cytokine release syndrome and improve responses[216] |
| **Using NK cells instead of T cells** | | | | |
| NCT03056339 | USA | B cell malignancies | Anti-CD19 CAR NK cell | Patients achieved response to treatment without the development of major toxic effects[120] |
| NCT05020678 | USA/Australia | B cell malignancies | Anti-CD19 CAR NK cell | NA |

BCL, B cell lymphoma; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; EBV, Epstein–Barr virus; NA, not applicable; LBCL, large B cell lymphoma; NCT, National Clinical Trial, NSCLC, non-small-cell lung cancer; PD1, programmed cell death 1; PDL1, programmed cell death 1 ligand 1; SLL, small lymphocytic lymphoma; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains.

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**Table 2 |**

Clinical trials that address antigen-positive escape (excluding T cell dysfunction)

| ClinicalTrials.gov identifier | Country | Disease | Construct |
|---|---|---|---|
| NCT03331198 | USA | R/R CLL or SLL | JCAR017 (lisocabtagene maraleucel) + venetoclax |
| NCT03310619 | USA | Aggressive B cell NHL | JCAR017 (lisocabtagene maraleucel) + ibrutinib |
| NCT04234061 (ref. 217) | Australia | MCL | Tisagenlecleucel and ibrutinib |
| NCT03876028 | USA | R/R DLBCL lymphoma | Tisagenlecleucel in combination with ibrutinib |
| NCT04257578 | USA | BCL | Axicabtagene ciloleucel in combination with acalabrutinib |

BCL, B cell lymphoma; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; MCL, mantle cell lymphoma; NHL, non-Hodgkin lymphoma; R/R, relapsed or refractory; SLL, small lymphocytic lymphoma.

**Table 3 |**

Clinical trials that address antigen-negative escape mechanisms

| ClinicalTrials.gov identifier | Country | Disease | Product | Observations | Refs. |
|---|---|---|---|---|---|
| **CD19/CD20** | | | | | |
| NCT03019055 | USA | R/R B cell malignancies | Anti-CD19 and anti-CD20 tandem receptor | ORR 100%; CR 92%; PR 8% for doses of $2.5 \times 10^6$ cells per kg with non-cryopreserved infusion ($n = 12$) | 178 |
| NCT03097770 | China | R/R BCL | Anti-CD19 and anti-CD20 tandem receptor | Best ORR 78% (95% CI 68–86); CR 70% (95% CI 59–79); PR 8% (95% CI 3–16) ($n = 87$) | 218,219 |
| NCT03207178 | China | R/R BCL | Co-administration of anti-CD19 and anti-CD20 | OS 8.1 month and PFS 5.0 months | 220 |
| NCT04007029 | USA | R/R FL, DLBCL, MCL and CLL/SLL | CD19–CD20 bispecific CAR | CR 7 of 8 of patients, median follow-up of 12 months; median PFS and OS were not reached | 221 |
| **CD19/CD22** | | | | | |
| NCT03620058 | USA | R/R B-ALL | CART22-65s with huCART19 | Relapse rate: 1/11 at a median follow-up of 6 months | 167 |
| NCT03233854 | USA | R/R B cell malignancies | CD19-22.BB.z | LBCL: $n = 21$, best ORR 62% (95% CI 38–82%); CR 29% (95% CI 11–52%) B-ALL: all patients had a response ($n = 17$); 14 with CR (82%) and 3 with partial remission | 137 |
| AMELIA trial, EUDRA CT 2016-004680-39 | UK | Paediatric and young adult patients with relapsed or refractory B-ALL | AUTO3, autologous transduced T cells expressing both anti-CD19 and anti-CD22 CARs | At 1 month after treatment, the remission rate (CR or CR with incomplete bone marrow recovery) was 86% (13 of 15 patients). The 1-year OS and event-free survival rates were 60% and 32%, respectively | 171 |
| NCT03448393 | USA | Children and young adults with B-ALL | Bicistronic CD19–CD22 CAR | CR 60% (12 of 20) in the full cohort and 71.4% (10 of 14) in CAR-naive patients. The 6-month and 12-month RFS in those achieving CR was 80.8% and 57.7%, respectively | 177 |
| **CD19/CD20/CD22** | | | | | |
| NCT05094206 | USA | R/R B cell malignancies | CAR20.19.22 T cells | NA | NA |
| NCT05418088 | USA | R/R B cell malignancies | Anti-CD19/CD20/CD22 CAR-T cells | NA | NA |
| **BCMA** | | | | | |
| NCT03502577 | USA | Relapsed or persistent MM | BCMA-specific CAR-T cells combined with a γ-secretase inhibitor (JSMD194) | CR 44%, median PFS is 11 months | 222 |
| NCT04182581 | China | R/R MM | BCMA–CD19 dual-target CAR-T | ORR in DL1 ($1 \times 10^5$ cells per kg) was 100% (2 of 2), DL2 ($2 \times 10^5$ cells per kg) was 80% (8 of 10), DL3 ($3 \times 10^5$ cells per kg) was 93.8% (15 of 16); BRR in 21 of 28 patients (75.0%) across all dose levels | 223 |
| **CLL1–CD33** | | | | | |
| NCT05467254 | China | R/R AML | CLL1 + CD33 CAR-T | NA | NA |

AML, acute myeloid leukaemia; B-ALL, B cell acute lymphocytic leukaemia; BCL, B cell lymphoma; BCMA, B cell maturation antigen; BRR, best response rate; CAR, chimeric antigen receptor; CR, complete response; CLL, chronic lymphocytic leukaemia; DL, dose level; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; LBCL, large B cell lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NA, not applicable; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PR, partial response; RFS, relapse-free survival; R/R, relapsed or refractory; SLL, small lymphocytic lymphoma.

**Table 4 |**

Clinical trials that address tumour microenvironment resistance mechanisms

| ClinicalTrials.gov identifier | Country | Disease | Product | Results | Ref. |
|---|---|---|---|---|---|
| NCT04684563 | USA | CLL and NHL | huCART19-IL18 | Dose-escalation study aimed to investigate the MTD of the therapy as well as other outcomes such as safety, PK and efficacy | NA |
| NCT03602157 | USA | R/R CD30$^{+}$ HL and CTCL | Autologous T lymphocyte CAR cells targeted against the CD30 antigen with CCR4 (ATLCAR.CD30.CCR4) | 8 Patients with HL have responded, with 6 CR (75%) and 2 PR; median PFS for all 10 evaluable patients is5.2 months at 12.7 months; median OS has not been reached | 224 |
| NCT04099797 | USA | GD2-expressing brain tumours | GD2-specific chimeric antigen and constitutively active IL-7 receptors | NA | NA |
| NCT03089203 | USA | Castrate-resistant prostate cancer | Dual PSMA-specific/TGFβ-resistant CAR-modified autologous T cells (CART-PSMA-TGFβRDN cells) | Preliminary evidence for early antitumour function was observed in this trial, including a PSA decline in approximately 30% of treated patients, although PSA responses were of limited durability | NA |
| NCT04556669 | China | Cervical cancer sarcoma, NSCLC | Autologous anti-PDL1 armoured anti-CD22 CAR-T cells | NA | NA |
| NCT03196830 | China | R/R lymphoma | Anti-CD30 CAR-T cell treatment combined with an PD1 inhibitor | PFS and OS rates were 45% and 70%, respectively; median follow-up of 21.5 months | 225 |
| NCT04844086 | Taiwan | Advanced lymphoid malignancies | Rapid personalized manufacturing of CD19 mbIL-15 CAR-T cells | Phase I, open-label dose-escalation trial, evaluation of safety and tolerability | NA |
| NCT03579927 | USA | BCL and CLL | CD19-CD28ζ-2A-iCasp9-IL15-transduced cord blood natural killer cells when given together with high-dose chemotherapy and stem cell transplant | Of the 11 patients who were treated, 8 (73%) had a response; of these patients, 7 (4 with lymphoma and 3 with CLL) had a complete remission, and 1 had remission of the Richter's transformation component but had persistent CLL | 120 |
| NCT05032820 and NCT03070327 | USA | MM | Anti-BCMA CAR-T cells (bb2121) and lenalidomide | NA | NA |

BCL, B cell lymphoma; BCMA, B cell maturation antigen; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukaemia; CR, complete response; CTCL, cutaneous T cell lymphoma; HL, Hodgkin lymphoma; MM, multiple myeloma; MTD, maximum tolerated dose; NA, not applicable; NHL, non-HL; NSCLC, non-small-cell lung cancer; OS, overall survival; PD1, programmed cell death 1; PDL1, programmed cell death 1 ligand 1; PFS, progression-free survival; PK, pharmacokinetics; PR, partial response; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; R/R, relapsed or refractory; TGFβ, transforming growth factor-β.