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Dual targeting CAR-T cells for B-cell acute lymphoblastic leukaemia and B-cell non-Hodgkin lymphoma

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Abstract:
Relapse after CD19-directed chimeric antigen receptor (CAR)-T cell therapy remains a major challenge in B-cell acute lymphoblastic leukaemia (ALL) and B-cell non-Hodgkin lymphoma (B-NHL). One of the main strategies to avoid CD19-negative relapse has been the development of dual CAR-T cells targeting CD19 and an additional target, such as CD22 or CD20. Different methods have been used to achieve this, including co-administration of two products targeting one single antigen, co-transduction of autologous T-cells, use of a bicistronic vector or the development of bivalent CARs. Phase 1 and 2 trials across all manufacturing strategies have shown this to be a safe approach with equivalent remission rates and initial product expansion. CAR-T cell persistence remains a significant issue, with a majority of antigen-positive relapses after CAR-T cell infusion. Further, despite adding a second antigen, antigen-negative relapses have not yet been eliminated. This review will summarise the state-of-the-art with dual targeting CAR-T cells for B-cell ALL and B-NHL, challenges encountered, and possible next steps to overcome them.

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Abbreviation key:

ALL	Acute lymphoblastic leukaemia
Axi-cel	Axicabtagene ciloleucel
B-LLy	B-cell lymphoblastic lymphoma
CAR	Chimeric antigen receptor
CLL	Chronic Lymphocytic leukaemia
CR	Complete Response
CRS	Cytokine release syndrome
DH HGBL	Double-hit high-grade lymphoma
DLBCL	Diffuse large B-cell lymphoma
FL	Follicular lymphoma
GCB	Germinal-centre B-cell like
HLH	Haemophagocytic lymphohistiocytosis
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplant
ICANS	Immune effector cell-associated neurotoxicity syndrome
ILBCL	Intravascular large B-cell lymphoma
NOS	Not otherwise specified
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD	Progressive Disease
PFS	Progression-free survival
PM LBCL	Primary mediastinal large B-cell lymphoma
RFSD	Relapse-free survival
r/r	Refractory/relapsed
scFv	Single-chain fragment variable
Tisa-cel	Tisagenlecleucel
tFL	Transformed follicular lymphoma
VL	Variable light chain
VH	Variable heavy chain

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Abstract

Relapse after CD19-directed chimeric antigen receptor (CAR)-T cell therapy remains a major challenge in B-cell acute lymphoblastic leukaemia (ALL) and B-cell non-Hodgkin lymphoma (B-NHL). One of the main strategies to avoid CD19-negative relapse has been the development of dual CAR-T cells targeting CD19 and an additional target, such as CD22 or CD20. Different methods have been used to achieve this, including co-administration of two products targeting one single antigen, co-transduction of autologous T-cells, use of a bicistronic vector or the development of bivalent CARs. Phase 1 and 2 trials across all manufacturing strategies have shown this to be a safe approach with equivalent remission rates and initial product expansion. CAR-T cell persistence remains a significant issue, with a majority of antigen-positive relapses after CAR-T cell infusion. Further, despite adding a second antigen, antigen-negative relapses have not yet been eliminated. This review will summarise the state-of-the-art with dual targeting CAR-T cells for B-cell ALL and B-NHL, challenges encountered, and possible next steps to overcome them.

Main text

1. Introduction

In B-cell acute lymphoblastic leukaemia (ALL), the first trials using CD19-directed CAR-T cells[1-5] showed response rates of around 80 – 90% in a patient population that was previously unsalvageable with conventional therapies (table 1). This led to licensing of tisagenlecleucel (Kymriah®) for patients 25 years or under with B-cell ALL in 2018 and brexucabtagene autoleucel (Tecartus® or KTE-X19) for patients over the age of 18 in 2021. Since then, both trial and real-world data have shown that 40 – 50% of patients who respond to CAR-T cells are cured without further therapy[6,7]. Whilst most patients respond initially, around 50% relapse after CAR-T cell therapy these patients and have a poor prognosis. In B-cell non-Hodgkin lymphomas (NHL), the first multicentre trials targeting CD19[8-13] showed complete response (CR) rates ranging from 40 to 74%, a practice-changing breakthrough in this highly chemo-refractory population (table 2). For large B-cell lymphoma (LBCL), 30% to 40% of patients have sustained responses with CAR-T cells as a standalone therapy and median progression-free survival (PFS) ranges from 3 – 55 months[14,15]. Paediatric real-world data in B-NHL show best sustained responses in B-cell lymphoblastic lymphoma (B-LLy) histology[16]. A detailed overview of the licensed products including axicabtagene ciloleucel (axi-cel), brexucabtagene autoleucel, tisagenlecleucel (tisa-cel), and lisocabtagene maraleucel has recently been published[17].

Relapse after CAR-T cell therapy follows 2 main patterns: CD19-positive relapse (CD19+), usually due to poor CAR-T cell persistence, and CD19-negative (CD19-) relapse, because of antigen escape or lineage switch[18,19], though other mechanisms have been described[20]. The ELIANA study for B-cell ALL reports predominant CD19- relapses (48%) with very few CD19+ relapses (6%)[4,6]. In contrast, real-world studies have shown higher rates of CD19+ relapses versus CD19- relapses (i.e. 60% vs 30% in a UK national study[7] or 58% vs 42% in data from the Real-World Pediatric CAR Consortium (PRWCC)[21]). Pre- and post-relapse sample analysis on the ZUMA-1 study in B-NHL showed a higher proportion of CD19+ relapses as well (around 64%)[8].

To infer persistence, B-cell aplasia in peripheral blood is most commonly used as a surrogate marker[3,4,22]. Data from studies with tisa-cel suggest that recovery of B-cells before 6 months from infusion is associated with a higher risk of relapse and warrants therapeutic intervention[19,23]. Early loss of CAR-T cell persistence may reflect either intrinsic factors making CAR T cells less "fit" (including CAR design, the memory phenotype of the starting material, and production methodology)[24,25], CAR-T cell exhaustion *in vivo*[26,27], or immune-mediated rejection[5,25,28]. Currently it is not known which of these is the dominant cause of early loss of CAR T cells in patients with ALL.

Resistance to CD19-targeted CAR-T cells may also be due to loss or down-regulation of CD19 surface antigen expression due to selection of acquired mutations or splice site variations[29,30]. Incorporating an additional target represents a logical strategy to overcome this challenge on the basis that a single leukaemic stem cell is unlikely to lose or down-regulate 2 antigens simultaneously.

In this manuscript we will focus on the different strategies used to deliver dual targeting CAR-T cells to patients, and will review the published data on construct design, toxicity, expansion, response rates, relapse incidence, and outcomes following dual-targeting CAR T-cells for B-cell ALL and B-NHL.

2. Dual antigen targeting CAR-T cells

2.1. Potential targets

CD19 is almost universally expressed with high antigen densities on B-cell ALL blasts[31,32]. Its expression is more variable in B-NHL however. Certain types of lymphoma, such as diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL) can show diminished surface levels of CD19 and significant interpatient variability[33,34].

CD22 is also almost always expressed in B-cell ALL with the exception of a proportion of patients with infant ALL[35]. In B-cell ALL, treatment with CD22 CAR-T cells alone have shown good expansion and complete remission rates[36-39] but high rates of relapse were observed due to down-regulation of CD22 expression unless used as a bridge to allogeneic hematopoietic stem cell transplant (allo-HSCT)[37,39]. This suggests that the ability of CD22 CAR-T cells to recognise targets with low-antigen density may be critical. In the B-NHL patient population, single targeting CD22 CAR-T cells have also been explored[40], however CD22 expression seems to be more variable in the range of 60 – 85% CD22-positive cases depending on histology[41] and this could potentially impact on efficacy.

CD20 is another possible target which is expressed on most B-NHL, approximately 40 – 50% of B-cell ALL, and CAR-T cells for B-NHL have been developed[42-44]. Importantly, though CD20-targeted therapy (Rituximab) is used throughout B-NHL therapy, malignant cells rarely seem to lose or downregulate CD20[45].

Several trials are underway using different manufacturing methods with CAR-T cells targeting CD19 and CD22, or CD19 and CD20, which are reviewed here[46,47]. Indeed, some groups are exploring targeting all three antigens and pre-clinical xenografted leukaemia and lymphoma models have shown superior activity with this trispecific approach[48].

2.2. Strategies for delivery of dual-targeting CAR-T cells

There are currently four main strategies to deliver dual targeting CAR-T cells to patients (figure 1): co-administration, co-transduction, use of bicistronic vectors, and bivalent tandem CARs. Each has different advantages and disadvantages, summarised in table 3.

2.2.1. Co-administration

Two separate single antigen targeting CAR-T cell products are generated and infused into patients. Two different vectors are used (one encoding a CD19, the other a CD22 or CD20 CAR) and transduced into T-cells separately. Then, the two products can be pooled together[49], infused separately on the same day[50], on sequential days[51-53], or more than 1 month apart[54-56].

2.2.2. Co-transduction

T cells are transduced with two different vectors at the same time generating a mixed population of single- and dual-targeting CAR-T cells.

2.2.3. Bicistronic vector

T cells are transduced with one single bicistronic vector with antigen-binding domains for both antigens. This results in a homogeneous population of CAR-T cells with two separate CARs expressed at an equimolar concentration on their surface.

2.2.4. Bivalent tandem CAR

In this case, T cells are transduced with a bivalent vector that generates one single CAR on the surface of the cell. It has two binding domains, and the variable light (VL) and heavy (VH) chains of the single-chain fragment variable (scFv) can be set up in a sequential or loop design[47].

3. Review of current trials using dual targeting for relapsed/refractory B-cell ALL

The major studies are summarised in table 4.

3.1. CAR constructs and manufacture

Multiple CAR designs and strategies have been applied for B-cell ALL. For example, Wang et al[52] applied third generation CARs with both 41BB and CD28 as co-stimulatory molecules and Cordoba et al[57] used humanised scFvs in their bicistronic vector. Ghorashian et al[58] used the previously reported[5] CAT CAR backbone with lower affinity to the CD19 antigen in combination with a novel CD22 CAR based on the 9A8 binder which recognises target's expression of CD22 at low antigen densities[59]. Tandem CARs have generally utilised the murine anti-CD19 FMC63 scFv and the human anti-CD22 m971 scFv, however varying in disposition of the light and heavy chain arrangements. Because of these differences in CAR design, it is difficult to generalise observed differences in outcomes between the varying dual targeting strategies above or to attribute these specifically to the approach used.

CAR-T cells were manufactured using both closed[57,60,61] (such as the CliniMACS Prodigy® system) and open[49,56,58] processing procedures, variable sources of activation beads (CD3/CD28 dynabeads or TransAct™), variable cytokines (for example Cordoba et al.[57] adding Il-7 and IL-15 and Ghorashian et al adding no cytokines[58]) and durations of manufacture. These variables may impact on the phenotype of the final CAR-T product which may in turn affect persistence (see section 5 below).

3.2. Toxicity

Toxicity observed in trials in B-cell ALL is summarised in table 5. In general, the published data do not suggest increased toxicity with the addition of a CD22-targeting construct. CAR-related toxicities were mild-moderate (grade 1-2) in most patients. The rate of grade 3/4 cytokine release syndrome (CRS) ranged from 0 to 28.4% and from 0 to 17.6% for neurotoxicity (ICANS), which is comparable to

single targeting. Previously reported[62] immune effector cell-associated haemophagocytic lymphohistiocytosis-like syndrome (IEC-HS) after single antigen targeted CD22 has not been widely seen except in the series of Spiegel et al[61] where 2 cases of IEC-HS were observed using a tandem construct.

3.3. Expansion of CAR-T cells

Regardless of strategy, most clinically tested dual-targeting CAR-T cell products have shown broadly similar initial expansion kinetics and peak levels to tisa-cel[63,64]. A 2022 study from Shanghai[49] pooled 2 different CAR-T cell populations together at a 1:1 ratio and saw an earlier and higher peak expansion of CD19 CAR-T cells compared to CD22 CAR-T cells.

With the co-transduction method, expansion of different CAR-T cell populations can vary widely. During manufacture T cells are exposed to two lentiviral vectors and therefore have different transduction efficiencies. Products can therefore be balanced or skewed towards a certain CAR component. Ghorashian et al[58] reports a product composition with predominantly CD19/22-CAR expressing cells (median 54,4%) with lower, but balanced CD19-CAR (13%) and CD22-CAR (11,6%) components. After infusion, early in vivo expansion reflected the phenotype of the product with predominant engraftment of CD19/22 double transduced T-cells and balanced but lower engraftment of CD19 and CD22 single positive populations. In contrast however, early reports from the PLAT05 study showed a skewed in vivo expansion of the CD19-CAR component using the CAR19x22v1 product[65]. In view of this, the manufacturing methodology was altered to favour the CD22 CAR-T cells in the product. However, when this was infused, in vivo expansion was then skewed towards the CD22-CAR component.[66].

The use of bicistronic vectors does not seem to impact early expansion, with Cordoba et al[57] reporting similar expansion to that of tisa-cel[63,64]. However, in tandem CAR data presented by studies

from NCI and Stanford[60,61], limited expansion and shorter persistence of their tandem CD19/22 CARs were observed when compared to their single antigen targeted CD22 CAR.

3.4 Response

All studies showed MRD-negative CR or CR with incomplete recovery (CRi) rates above 80%, mirroring the clinical experience with CD19-directed CAR-T cell therapy so far. The only study with lower rates of reported response (57%) was the first product tested in the PLAT-05 study using a co-transduction approach[65]. Co-administration strategies showed particularly good responses, with CR rates above 90%. Given that bridging chemotherapy is generally used before lymphodepletion we cannot attribute responses to CAR-T cells alone. However, given the refractory nature of the durability of responses in many such patients, it is unlikely that bridging therapy contributes significantly to response rates.

3.5. Relapse incidence and phenotype

Regardless of the strategy used, antigen-positive relapse has been the predominant cause of treatment failure observed following dual-targeting CAR-T cell therapy, reflecting poor persistence across a substantial number of dual-targeting CAR products[49,56-58,60,61].

Antigen-negative relapse has still been observed in most studies of dual-targeting CAR-T cells in B-cell ALL (Table 4: “Relapse phenotype” column). CD19-negative relapse with ongoing CD22 positivity is the main phenotype, perhaps reflecting the poor performance of the CD22 CAR across the different strategies, shorter persistence in co-administration[49], and stronger selective pressure on the CD19 compared to the CD22 target in bicistronic and tandem CARs[57,61]. Consequently, CD22 negativity is rarely seen. It is important to highlight that since prolonged selective pressure is needed for outgrowth of antigen-negative clones, poor persistence may limit our ability to assess the real prevalence of antigen-negative relapse.

3.6. Outcomes

Clinical outcomes with dual-targeting CAR-T cells in B-cell ALL have generally been equivalent to those reported with the single-targeting CD19 CAR[4,5,7,23].

The most encouraging results have been achieved with co-administration of CD19 and CD22 CAR-T cells. One of the two largest studies[49] of this approach reports a 12-month EFS of 74%. They used a short manufacture time (7 days) and infused a fresh, 1:1 pooled product of CD19 and CD22 CAR-T cells to 225 patients. While these results appear superior to data on tisa-cel reported in the ELIANA trial[6] and real-world data[7,67], it should be noted that the patient characteristics in this study were more favourable with 32% of patients being MRD-negative before infusion. Pan et al.[56] have also shown impressive outcomes with an 18-month EFS of 79%. In this study, CD19 CAR-T cells were infused first, followed 30 days later by a CD22 CAR-T cell infusion for patients in complete remission and without ongoing toxicities. Interestingly, CD22 CAR-T cells expanded and persisted despite eradication of disease with the previous CD19 CAR-T cells. Further, disease surveillance presumably relied on the CD22 CAR-T cells since many patients lost their CD19 CAR-T cells after receiving a second cycle of lymphodepleting chemotherapy.

Using a co-transduction approach, the CARPALL cohort 3 study by Ghorashian et al[58] reports a 12-month EFS of 60%. Whilst data need to be interpreted with caution because of small sample size, antigen-negative relapse was not observed. This may in part reflect the use of CD22 CAR based on the 9A8 binder, which effectively targets tumour cells at low CD22 antigen density. Initial and sustained response was seen in 2 out of 3 patients who had CD19 negative disease on enrolment, demonstrating effective CD22 CAR activity. Additionally, single antigen targeted CD22 CAR-T cells were detectable in blood for longer (median of 7 months vs 5 months) than their single CD19 and double CD19/CD22 targeting CAR-T cell counterparts.

Cordoba et al[57] reported a lower EFS using CAR-T cells transduced with a bicistronic CD19-22 CAR vector (AUTO 3), with a median EFS of 5 months and 12-month EFS of 32%. They observed a high rate of antigen-positive relapses associated with CAR-T cell loss and short persistence was thought to be the main factor for these poor results. The authors postulate that this replicated a differentiated phenotype of the CAR-T cell product, which in turn may reflect the production methodology used. It is also possible however that signalling through 2 CARs in a single cell may predispose to activation-induced cell death and/or exhaustion[26].

Using a tandem CD19-22 CAR, Spiegel et al.[61] reported a median EFS of 5.8 months and Shalabi et al.[60] a 12-month EFS of 58% in responding patients. These somewhat disappointing outcomes mirror the issues both groups encountered with the functionality of CD22 targeting in the context of a tandem CAR structure. In the adult cohort of Spiegel et al.[61], they showed that CD19/22 tandem CAR-T cells had reduced cytokine polyfunctionality following stimulation with CD22 positive targets than T-cells transduced with a CD22 CAR alone. Shalabi et al.[60] showed suboptimal CD22-targeting activity of the tandem CAR construct both in vitro with reduced cytokine secretion against CD19-CD22+ Nalm6 cell lines and in vivo with poor anti-leukaemic activity in a xenogeneic CD19-negative, CD22-positive model of B-cell ALL. These data indicate decreased functionality of the CD22 CAR moiety when incorporated into a tandem structure.

Cui et al.[68] reported better results in a cohort of 47 patients (24-month EFS of 69%) using a tandem CAR construct, but these results need to be interpreted with caution as 75% of patients underwent consolidative allo-HSCT at 2 months.

4. Review of current trials using dual targeting for relapsed/refractory large B-cell lymphoma

The major studies are summarised in table 6 (CD19/CD20 CARs) and table 7 (CD19/CD22 CARs).

4.1. CAR constructs and manufacture

Constructs used for B-NHL are more homogeneous than those used in B-cell ALL. Tandem CARs targeting CD19 and CD20 used sequences derived from the murine scFv regions Leu-16 for CD20 and FMC63 for CD19[50,69-71]. As for CD19 and CD22, the studies on co-administration from Wuhan[51,52,72] all applied a third generation CAR with 41BB and CD28 as co-stimulatory molecules. Roddie et al[73] used 2 humanised scFv regions in a bicistronic vector: LT22 for CD22 and HD37 for CD19, the same product (AUTO3) Cordoba et al[57] used for B-cell ALL. Tandem CARs targeting CD19 and CD22 use the same scFv as described for B-cell ALL, FMC63 for CD19 and m971 for CD22, in a second-generation backbone[61,74,75].

In terms of manufacturing, as with B-cell ALL, processing procedures varied across studies. Larson et al.[69] specifically enriched the apheresis product for CD62L in order to obtain a higher yield of naïve and memory T-cells. They performed a prolonged expansion period of 12 to 16 days, before cryopreserving the final product. Manufacturing times varied from 8 to 14 days. Whilst a shortened manufacturing methodology such as the T-Charge platform have been used with CD19-directed CAR-T cells[76], this has not so far been applied to dual-targeting CAR-T cells.

4.2. Toxicity

The toxicity profile across the reviewed trials for B-NHL is summarised in table 8. There does not seem to be any increased toxicity when adding CD20 or CD22 antigen-recognition. Grade 3/4 CRS ranged from 0 to 28.5% and grade 3/4 ICANS from 0 to 13.6% across all studies. Larson et al.[69] reported a relatively low incidence of adverse events in their trial. They noted low peak cytokine levels while maintaining clinical efficacy of their CAR-T cell product. This could be explained by the skewed naïve/memory T-cell phenotype achieved during production, or the thorough pre-clinical

construct optimisation[77], leading to increased clinical efficacy and consequently allowing for a lower CAR-T cell dose (median of 55 x 10^6 cells).

4.3. Expansion of CAR-T cells

Despite using more complex constructs, CAR-T cells expand well and peak around 2 weeks, with a tendency towards higher expansion in patients who show a response[70,71,75]. Persistence, however, has been reported to be very short in the B-NHL cohort. CAR-T cells are lost earlier compared to the B-cell ALL population, with most trials reporting 3 – 6 months persistence[73,75,78,79]. As observed with single antigen targeting CAR-T cells, it is not clear if a shorter persistence correlates with relapse in the B-NHL cohort. An early, higher expansion might be more significant for durable remission in lymphoma[78] compared to B-cell ALL.

4.4. Response

Overall response rates range from 60% to 90% across different trials, whereas complete responses range from 29% to 81%. These numbers do not differ significantly from the responses seen with single antigen targeting CAR-T trials[17]. Deep initial responses with dual-targeting CAR-T cells seem to correlate with durable remissions[52], as has also been seen with single antigen targeted CAR-T cell therapy[17]. Shah et al[70] report a trend towards a higher naïve and central memory phenotype in the apheresis products of patients who showed good clinical response. Whilst bridging therapy is frequently used in B-cell ALL, its use in B-NHL has varied historically in pivotal trials and varies across dual-targeting studies as well with some studies not giving any[50,71,74], others permitting its use at each centre's discretion[49,61,72], and some reporting its use on the study[69,70,73]. Roddie et al.[73] comments on the role of effective bridging to debulk disease before CAR-T cell infusion and how low disease burden was a predictor of response to their product, AUTO3. On the other hand, Zurko et al.[78] found inferior survival in patients that required bridging therapy, which may reflect higher disease burden on recruitment.

4.5. Relapse incidence and phenotype

In lymphoma, a biopsy is needed to assess antigen expression on tissues, often with patchy lymphoma involvement, which makes representation of CD19 and CD20/CD22 expression at baseline and relapse more challenging. Modalities to assess pre- and post-relapse antigen expression include the H-score[61,80] and flow-based assessment of fine needle aspiration material[61]. From the available data[61,70,73,80], relapses seem to follow the same phenotype as with B-cell ALL. Most relapses retain expression of CD19 and CD22/CD20, as has been the case with CD19-targeted products. For example, in the ZUMA-1 trial, 1/3 of LBCL relapse cases post-axi-cel were from antigen loss and 2/3 of cases relapsed with ongoing CD19 expression[8]. In most lymphoma patients, CAR-T cells do not persist long-term, and this may account for antigen-positive relapse in some cases. However, other factors may also be contributory. Certainly, T-cell fitness and the functionality/expansion potential of CAR-T in vivo plays an important role in the achievement of clinical response, so antigen-positive relapse is more likely where the CAR-T cell product is intrinsically unfit due to prior chemotherapy. Moreover, endogenous immune and tumour microenvironment-associated factors may impede T-cell function in vivo and contribute to the risk of antigen-positive relapse[20].

Despite dual targeting, there are still some observed cases of suspected clonal escape with downregulation of CD19 and CD20/CD22 antigen expression[73,79]. Given that exhaustion is another of the proposed mechanisms of CAR-T cell treatment failure, some studies have attempted adding checkpoint inhibitors after CAR-T cell infusion. Results are mixed. Roddie et al[73] saw no clear benefit in adding Pembrolizumab on day 14 after dual CAR-T cells, in line with the ZUMA-6 results[81]. Zhang et al[82] however report improved response rates and progression-free survival with addition of the PD-1 inhibitor Tislelizumab on day 1 after infusion.

4.6. Outcomes

Results varied regarding outcomes with some studies reporting lower EFS and others superior EFS compared to the pivotal trials as depicted in tables 4 and 5.

The study by Cao et al.[51] using high-dose therapy with autologous hematopoietic stem cell (HSC) infusion followed by CD19 and CD22-targeted CAR-T cells shows a 24-month EFS and OS of 83%, which is higher than high-dose therapy by itself at around 30%-40%[83] or with any of the CD19-directed studies[13,14,16,84]. It should be noted however that the patient population in this study was predominantly below 50 years (73%) and transplant naïve. Besides, it is a complex approach that requires two apheresis procedures, one with stem cell mobilisation, and includes a toxic myeloablative conditioning.

Roddie et al.[73] used a bicistronic vector towards CD19 and CD22 and they encountered similar issues to those reported in the B-cell ALL cohort with short persistence (perhaps reflecting the differentiated phenotype of the product), leading to a lower EFS of 25% at 12 months. Effective CD22 targeting can however be inferred because 7 out of 13 cases downregulated CD22 at relapse.

With tandem products, Spiegel et al.[61] reported an EFS of 25% at 12 months in their B-NHL cohort and the potential reasons for these poor outcomes have been discussed in the B-cell ALL section. Larson et al.[69] produced CD19-20 tandem CAR-T cells through bead-based enrichment of CD62L expression, generating a final product skewed towards naïve and memory T-cells (TN/MEM). They reported an EFS of 40% at 18 months. Activity of the tandem construct against the 2 antigens did not seem to be impaired with reports of high overall responses and CAR-T cell persistence over 6 months.

A group in Wisconsin[70,78] also designed a CD19-20 tandem construct and reported equivalent outcomes to single antigen targeting data with an EFS at 24 months of 44%. In CAR-naïve patients with DLBCL, EFS increased to 50%. For patients who showed an initial complete response and then relapsed (6/12), these occurred late (>180 days), which is not the usual pattern seen with tisa-cel[14] or axi-cel[8]. Early expansion seems to correlate with durable responses, as suggested by this study[78],

data from the CD19 NIH product with a CD28 co-stimulatory domain[15], and data from ZUMA-1 with axi-cel[84]. On the matter of patterns of resistance, Shah et al.[70] highlights a patient who relapsed with detectable circulating CAR-T cells and available relapse biopsy material. When co-cultured in vitro, frozen CAR-T cells were able to kill CD19+/CD20+ Raji cells, however, did not show any activity against bright CD19+/CD20+ biopsy material. This suggests other mechanisms of resistance in the tumour microenvironment in B-NHL beyond antigen loss or downregulation.

Finally, a group from Beijing[71,79] performed detailed in vitro screening of different tandem CAR construct candidates by measuring F-actin accumulation at the immunological synapse (IS) and polarisation of the microtubule organising centre (MTOC)[71]. TanCAR7 proved to have the most stable IS and delivered the most effective target cell lysis and was thus selected for further in vivo studies. In a Phase 1-2 study of TanCAR7 in 87 patients with B-NHL they reported an EFS of 61% at 12 months with a median EFS of 27.6 months. Median persistence was around 100 days, and no significant difference was seen between patients who relapsed or who maintained a response. Interestingly, from 12 patients with available post-relapse biopsy samples, 5 patients still had detectable CAR-T cell in the tissue, but only 1 showed CD19 and CD20 antigen loss.

5. Summary and future directions

In comparison to the experience with single antigen CD19-targeting CAR-T cells, dual-targeting strategies have shown equivalent initial expansion rates and have proven to be a safe approach with an equivalent toxicity profile. To date, the current generation of dual targeting CAR-T cell studies have not resulted in significantly improved outcomes compared to targeting CD19 alone. This may reflect both the heterogeneity in approaches used and the fact that dual targeting per se does not address other mechanisms of resistance beside antigen escape. Nonetheless, important lessons have been learned.

If a CD22 CAR is used, it needs to target low antigen density. Clinical studies with CD22 CARs alone[37] have shown high rates of relapse associated with CD22 down-regulation. A number of studies suggest[57,60] that optimising the CD22 CAR domain to recognise low-antigen density targets and enhancing its potency is an important next step in improving efficacy.

Co-transduction can lead to skewed in vivo expansion. Different transduction efficiencies can lead to heterogeneous products (of CD19, CD22 and CD19/22 CAR-T cells) that can further show skewed and unpredictable expansion of the different cellular components in vivo.

Designing a tandem CAR that functions optimally for both targets is challenging. With a variety of possible designs and conformations, it has proven difficult to optimise function against 2 different antigens, perhaps reflecting differences in the distance of the epitopes from the cell membrane. Studies exploring size and rigidity of the CAR construct[77], or the stability of the immunological synapse (IS)[71] have proven useful in selecting CARs with the most effective target cell lysis, but in vitro assays do not necessarily recapitulate functionality in vivo.

It is possible that expression of two CARs on the surface could trigger cell death. The clinical application of bicistronic vectors has led to products with a differentiated T-cell phenotype and a high proportion of early CAR-T cell loss. It is possible that expressing 2 CARs on a single cell could accelerate activation-induced cell death and/or exhaustion. Further studies are needed to

investigate this possibility: if this is the case then co-administration may be preferrable to bicistronic or co-transduction approaches. Indeed, on the basis of the available data at present, co-administration strategies have shown the most promising outcomes in B-cell ALL.

Evasion mechanisms by malignant cells and their microenvironment could be a major barrier for the success of dual-targeting CAR-T cells. Though poorly characterised, studies hint at other mechanism of disease resistance aside from loss of persistence and antigen loss/downregulation. For example, Zhang et al.[79] describe four patients with relapsed B-NHL and antigen positivity despite persisting CAR-T cells in the biopsied tissue. Possible causes for such cases could be the inhibition by Tregs and myeloid-derived suppressor cells (MDSCs) in the bone marrow microenvironment[20], upregulation of immune checkpoint molecules via mutations in the IL-6/JAK/STAT3 signalling pathway[85], abnormalities in the apoptotic pathway[86], downregulation of cGAS-STING signalling[87], or production of adenosine by tumour cells[88].

Poor CAR-T cell persistence remains a key challenge. Several mechanisms have been suggested, such as poor CAR-T cell fitness, exhaustion, and immune rejection of the product.

As to CAR-T cell fitness, clones derived from naïve populations (T naïve and T stem cell memory) are thought to play a critical role in long-term functional CAR-T cell persistence[24,89]. Biasco et al.[24] showed that stem cell memory T-cell subpopulations contributed the most to the clonal pool at late timepoints of patients with long-term persisting CAR-T cells. Some strategies to improve the functionality of the product include optimising CAR design by reducing the affinity of CAR-T binding to antigens[5], the use of CD3zeta domains with reduced number of ITAM domains[90], shortening duration of ex-vivo culture[76,91], using AKT inhibitors[92,93], or by modifying the culture medium by including IL-21[94], increasing the potassium concentration[95], or adding N-acetylcysteine[96].

Exhaustion has been suggested as a possible mechanism through methylation profiling of CD19 CAR-T cells post-infusion[27]. Addition of checkpoint inhibitors in the B-NHL population has yielded mixed results. Re-infusion of CAR-T cells followed by Nivolumab is currently being

investigated (NCT05310591), while there are pre-clinical studies on gene-edited CAR-T cells with down-regulation of DNMT3A[97] or PRDM1[98].

Finally, immunogenicity of the CAR product must be considered since most CAR-T cells utilise an antigen recognition domain derived from murine antibodies. Turtle et al.[25] observed no expansion or persistence after CD19-targeted CAR-T cell re-infusion in adult B-cell ALL patients despite the use of lymphodepleting chemotherapy in 4/5 patients. They were able to demonstrate CAR-specific cytotoxic T cell responses in an in vitro model and define possible antigenic epitopes within the CAR construct. Immune-mediated rejection may explain the relatively low rate of long-term responses to re-infusion of Tisagenlecleucel for early B-cell recovery[99]. Since dual-targeting products incorporate two scFvs and are frequently given after single-antigen targeted CAR-T cell therapies with mostly the same constructs, there is an increased potential for immune-mediated rejection and immunogenicity should be monitored. Humanisation of CARs[100], and optimising exposure to Fludarabine[101,102] are being explored as strategies to reduce CAR-T cell rejection.

Importantly, whilst in B-cell ALL there is strong evidence that persistence is key for durable remissions[19,103-105], in B-NHL however this is not as well established. Interestingly, most patients with LBCL still relapse with antigen-positive disease following CAR-T cell therapy, which warrants further investigation if products with longer persistence profiles could deliver more durable responses in LBCL.

Whilst dual targeting has not yet fully eradicated CD19-negative relapse or improved outcomes, the studies to date have given important insights into the challenges to overcome. Building on these lessons, the next generation of dual targeting CAR-T cell studies are well placed to fully achieve the potential of this approach. Subsequent studies should utilise CD22CARs which recognise low antigen density targets and incorporate strategies to enhance CAR T cell persistence. For example, in our next study in paediatric B-cell ALL, we plan to combine optimised lymphodepletion with fludarabine therapeutic drug monitoring with the use of CAR-T cells

transduced with CD19CAR and CD22CAR vectors separately generated with a rapid manufacturing protocol. Such approaches may increase the regulatory complexity and cost of CAR-T cells but if they achieve sufficiently improved long-term outcomes compared to existing licensed products this investment will be justified. Moreover, as we move forward, the lessons learned in dual targeting of B-lineage ALL and NHL may give us important insights in to how best to deliver dual targeting CAR-T cells for other malignancies.

Authorship

Contribution: G.C. prepared manuscript draft, tables and figures. C.R. and P.A. both reviewed and contributed to the final version of the manuscript. P.A. supervised the writing process.

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Tables and Figures:

Table 1: Main trials in single antigen targeted CAR-T cells for B-cell ALL

Reference	Trial, phase	CAR construct	N* (age range)	In vivo expansion	Rate of CR or CRi	Toxicity	Persistence	Relapse incidence and phenotype	EFS/OS
B-cell ALL – CD19									
Maude et al. 2018[4] Updated by Laetsch et al. 2021[23] ELIANA study	2	Tisagenlecleucel FMC63 scFv – 41BB – Cd3z	n = 79 (3 – 21 years)	AUC 0-28: 318,000 mean copies/µg Cmax 34,700 copies/µg in responders[64]	CR: 45/79 (60%) CRi: 16/79 (21%) 65/79 (82%) MRD-negative at 3 mo	CRS G3/4: 46% NTx G3/4: 13%	Median time to B-cell recovery in responders 35.3 months BCA 12 mo: 71% BCA 24 mo: 59%	51% (33/65) CD19+: 2/33 (6%) CD19-: 16/33 (48%) CD19+/-: 3/33 (9%) Unknown: 12/33 (36%)	Median EFS 23.7 mo EFS 44% at 3 y OS 63% at 3y
Gardner et al.[3] 2017	1-2	FMC63-4-1BB-CD3z *Defined 1:1 ratio of CD4+/CD8+ CAR-T cells*	n = 45 (1 – 27 years)	Peak 10 days. No correlation peak expansion with cell dose. Higher expansion with >15% CD19 disease in marrow.	40/45 (89%) MRD-negative CR by day 21.	CRS G3/4: 10/43 (23%) NTx G3/4: 9/43 (21%)	BCA ≈ 30% at 6 months	18/40 (45%) CD19+: 11/18 (61%) CD19- : 7/18 (39%)	Median EFS ~ 13 mo EFS 50.8% at 12mo OS 70% at 12mo
Ghorashian et al.[5] CARPALL study	1-2	CAT19 scFv – 41BB – CD3z	n = 14 (< 25 years)	AUC 0-28: 1,721,355 mean copies/µg Cmax 128,012 mean copies/µg	86% (12/14) CR MRD- at 3 mo	No G3/4 CRS NTx G3/4: 1/14 (7%)	B-cell aplasia 21% at 12 mo CAR detectable qPCR 79% (11/14) at last follow-up Median duration 215 d (14 – 728d)	50% (6/12) CD19+: 1/6 (16%) CD19- : 5/6 (83%)	Median EFS 9 mo EFS 46% at 12 mo OS 63% at 12 mo
Park et al.[106] 2018	1	FMC63 scFv - CD28 – CD3z	N = 53 (23 – 74 years)	Higher expansion in patients with pre-infusion MRD-negative complete remission	44/53 complete remission at day 21 32/48 MRD-	CRS G3/4: 26% (14/53) NTx G3/4/5: 22/53**	Short persisting CAR-Ts. Median duration of CAR-T cell detection: 14 days Majority CAR-T cells lost before day 40.	25/53 CD19+: 21/25 (84%) CD19-: 4/25 (16%)	Median EFS 6.1 mo EFS ~ 18% at 24 mo Median OS 12.9 mo
Shah et al.[22] 2021 ZUMA 3	2	Brexucabtagene autoleucel (KTE-X19) FMC63 scFv – CD28 – CD3z	N = 55 (28 – 52 years)	Median peak: 40.47 cells/µL (IQR 6.04 – 76.70)	39/55 (71%) at median of 1 month	CRS G3/4: 13/55 (24%) NTx G3/4/5: 14/55 (25%) †	B-cell recovery in 10/12 ongoing responders at month 12.	Relapse incidence: 12/55 (22%) CD19+: 6/9 (67%) CD19-: 3/9 (33%) (only 9 patients with available data)	Median EFS 11.6 mo OS 71% at 12 mo 9/55 proceeded to HSCT
B-cell ALL – CD22									
Fry 2018[37], Updated and expanded by Shah 2020[39]	1	*Anti-CD22 m971 scFv – 41bb – CD3z* → Shah et al incorporated CD4/CD8 selection to manufacturing	58 (4 - 30 years) 36/58 (62 %) previous aCD19 CAR-T 39/58 (67%) previous HSCT	Median peak: 480.5 CAR-T cells/µL (range 39.7 – 11346/µL)	40/57 (70%) at 1 month	CRS G3/4: 12/58 (24%) NTx G3/4/5: 1/58 (2%) → 19/58 (33%) developed HLH (HLH incidence increased after incorporating CD4/CD8 selection at target dose)	NR	30/58 (75%) Downregulation of cD22 expression in most patients.	Median EFS 6 mo Median OS 13.4 mo 14 patients proceeded to HSCT

BCA: B-cell aplasia; Cmax: peak serum concentration; scFv: single-chain fragment variable; mo: months; NR: not reported; NTx: neurotoxicity

*Showing final number of infused patients †used ASTCT consensus criteria for CRS grading and CTCAE grading for neurotoxicity

Table 2: Main trials in single antigen targeted CAR-T cells for B-NHL

Reference	Trial, phase	CAR construct	N* (age range) and diagnoses	In vivo expansion	Best ORR and CR	Toxicity	Persistence	Relapse incidence and phenotype	EFS/OS
B-NHL CD19									
Neelapu et al.[8] 2017 (ZUMA-1)	2	*Axicabtagene ciloleucel CD19 scFv – CD28 – CD3z*	101 (25-76 years) - DLBCL: 77 - PMBCL: 8 - tFL: 16	Peak at 14 days (peak 10 – 100 copies/µL)	ORR: 82/101 (82%) CR: 54/101 (54%)	CRS G3/4: 13/101 (13%) NTx G3/4/5: 28/101 (28%) †	Most patients with detectable CAR-T cells at 180 days.	58/101 (58%) 11 patients available CD19-status: 7/11 CD19+ disease 3/11 had CD19- disease	Median PFS 5.8 mo 41% PFS at 15 mo. OS 52% at 18 mo
Abramson et al.[13] 2020 (TRANSCEND)	2	*Lisocabtagene maraleucel CD19 scFv – 4-1BB – CD3z (sequential CD8+ then CD4+ components at equal doses)*	268 (18-86 years) - DLBCL NOS: 131 - HGBCL: 33 - tFL: 54 - t iNHL: 18 - PMBCL: 14	Peak at 12 days (Cmax 23928 copies/µL)	ORR: 186/256 (73%) CR: 136/256 (53%)	CRS G3/4: 6/268 (2%) NTx G3/4/5: 27/268 (10%) †	CAR-T cells detectable at 1 year in 35/67 patients (52%) B-cell aplasia at 1 year in 51/70 (73%)	NR	Median PFS 6.8 mo 44% PFS at 12 mo. Median OS 21.1 mo
Schuster et al.[9] 2019 (JULIET)	2	*Tisagenlecleucel CD19 scFv – 4-1BB – CD3z*	93 (22 – 76 years) - DLBCL NOS: 88 - tFL: 21 - Other: 2	Peak at 9 days (Cmax 5530 copies/µg)	ORR: 48/93 (52%) CR: 37/93 (40%)	CRS G3/4: 24/93 (22%) NTx G3/4/5: 13/93 (14%) †	Not quantified. Long-term persistence up to 2 years observed.	NR	PFS 65% at 12 mo
B-NHL – CD20									
Till et al.[42] 2012	1	*CD20 scFv – CD28-41BB-CD3z* *3rd generation CAR*	4 Indolent lymphomas	1 patient no expansion	2 patients no evaluable disease 1 partial response	No grade 3/4 toxicities.	9 – 12 months detectable CAR-T cells	1 progression after partial response	NR
Wang et al.[43] 2014	1	*CD20 scFv – 41BB – CD3z*	7 (37-85 y) Diffuse large B-cell lymphoma	-	1/7 complete remission 4/7 partial response	CRS G3/4: 1 No NTx Reported delayed-onset CRS and toxicities in tumour involvement sites.	NR	NR	NR
Zhang et al.[44] 2016	2	*CD20 scFv – 41BB – CD3z*	11	Peak levels at 4 weeks (range: 800 – 255,044 copies/µg DNA)	Objective response rate: 9/11 (82%) CR: 6/11 (55%) PR: 3/11 (27%)	No CRS or NTx. *Excluded patients with intrapulmonary involvement, GI involvement or refractory to debulking therapy.*	NR	Relapse incidence: 6/11 All with loss of persistence and recovery of CD20+ B-cells	Median PFS 6 mo
B-NHL – CD22									
Baird et al.[40] 2021	1	*CD22 scFv (m971) – 41BB – CD3z*	3	Peak levels at 14 days	Complete response 3/3 at 6 months	CRS G3/4: 0/3 NTx G3/4: 0/3	3/3 detectable at last assessment at 6 months	No relapses at 6 mo	NR

*Showing final number of infused patients †used ASTCT consensus criteria for CRS grading and CTCAE grading for neurotoxicity

CR: complete remission, DLBCL NOS: diffuse large B-cell lymphoma not otherwise specified; HGBCL: high-grade B-cell lymphoma; HLH: haemophagocytic lymphohistiocytosis; HSCT: hematopoietic stem cell transplant; mo: months; ORR: objective response rate; PFS: progression-free survival; PMBCL: primary mediastinal B-cell lymphoma; tFL: transformed follicular lymphoma; t iNHL: DLBCL transformed from indolent non-Hodgkin lymphoma other than follicular lymphoma; NR: not reported; NTx: neurotoxicity

Figure 1: Strategies for delivery of dual targeting CAR-T cells. CD19 and CD22 are shown as an example of antigenic targets. a. Co-administration: two independent products are generated and infused into patients. B. Co-transduction: T-cells are transduced with two different vectors, generating one single product with a mixed population of single antigen targeted and bi-specific CAR-T cells. C. Bicistronic vector: one single vector with binding domains for two different antigens is used. The vector is then cleaved and generates CAR-T cells with one CAR for each antigen on their surface. D. Bivalent tandem CAR: one vector generates one single CAR on the surface on the cell. That CAR has binding domains for two different antigens.

Table 3: Summary of advantages and disadvantages of the different strategies

Variants	Advantages	Disadvantages
Co-administration	- Minimal optimisation - allows for combination of two single CAR constructs. - Co-administration: Dose can be adjusted for each single CAR product. - Only one vector (lower cost). - Homogeneous product. - Single activation signal.	- High manufacturing cost. - Coordination and regulation around 2 infusions of 2 different products.
Co-transduction		- High manufacturing cost. - Heterogeneity in product composition may result in uneven expansion in vivo.
Bicistronic vector		- Large vector size can result in lower transduction efficiency. - Impact of increased CAR density/signalling uncertain.
Bivalent CAR		- Optimisation of construct to ensure efficient targeting of both antigens challenging.

Adapted from: Cordoba et al[57] and Xie et al[47].

Table 4: Main trials using dual targeting CAR-T cells for CD19 and CD22 in B-cell ALL

Reference	Trial, phase	CAR construct	N*	In vivo expansion	Rate of complete remission	Relapse phenotype				Persistence	EFS/OS
						CD19 + CD22 +	**CD19 +** *CD22 –*	*CD19 –* **CD22 +**	*CD19 – CD22 –*		
Wang et al.[52]† 2020 Wuhan, China	1	**Co-administration** 3rd generation CAR Sequential, day 0 - 4	51 (ages 9 – 62y)	-	48/51 (96%) on day 30	23/24	0	0	1/24 ($CD19^{-}$ /$CD22^{dim}$)	Short persistence (4 months median time to recovery of bone marrow B-cell haematogones)	53% 12m RFS
Pan et al.[54] 2020 Beijing, China	1	**Co-administration** Sequential, separated by 1.65 months, once CAR19 undetectable	20 (ages 1 – 16y)	-	20/20 (100%) MRD-negative on day 28	1/3 (downregulation)	0	2/3	0	Good persistence (17/20 patients showed >1 year CAR-T cell persistence)	80% 18m RFS
Liu et al.[55] 2021 Beijing, China	1	**Co-administration** Sequential, separated by at least 1 month	27 infusion 1 21 infusion 2 (ages 1.6 – 55y)	Similar expansion after CD19 product and CD22	23/27 CR after infusion 1 20/21 CR after infusion 2	4/21	0	2/21	0	B-cell aplasia (median): 10 months 75% lost CD22 CAR-T cells on day 60 50% had CD19 CARs on day 60	65% 18m EFS 84% 18m OS
Wang et al.[49] 2022 Shanghai, China	2	**Co-administration** 2nd generation CAR Pooled 1:1 7-day manufacture	225 (<20y)	Earlier and more robust expansion for CD19-CAR T cells	192/194 (99%) MRD-negative on day 28	24/43	0	16/43	1/43	B-cell recovery: - median 74 days - 60% by 6m	74% 12m EFS 88% 12m OS
Zhang et al.[53] 2022 Tianjin, China	1	**Co-administration** Sequential, days 1 and 2. *HIB22 CD22 CAR*	4 (ages 18 – 40)	Peak 14 – 21 days	4/4 (100%) MRD-negative on day 28	2/4	0	0	1/4 (CD19-/CD22dim)	9 months CAR-T cell presence in peripheral blood of two patients alive and without HSCT. Both relapsed with CD19 and CD22 expression.	25% 18m EFS 50% 18m OS
Pan et al[56] 2023 Beijing, China	2	**Co-administration** Sequential, separated by 39 days CD19 murine CD22 humanised	81 (79 received both infusions) (ages 1 -18y)	CD19: Peak at 9 days CD22: peak at 12 days Peak not related to dose or bone marrow burden	79/81 (98%) MRD-negative or CRi at 3 months	11/79	0	2/79	1/79	20% B-cell recovery at 12 months 40% CAR-T cell loss at 12 months (as undetectable CAR transgene)	79% 18m EFS 96% 18m OS
Gardner et al.[65] *(PLAT-05, SCRI-CAR19x22v1)* 2018	1	**Co-transduction** aCD19(FMC63)-41BBz aCD22(m971)41BBz	7	Selective expansion of CD19 components - CD19 9.1% - CD22 1.2% - CD19/CD22 2.4%	4/7 (57%) MRD negative on day 21	1/4	0	2/4	1/4	-	*No follow-up time reported*
Annesley et al.[66] *(PLAT-05, SCRI-CAR19x22v2)* 2021	1	**Co-transduction**	12	Product skewed towards CD22. In vivo expansion mostly CD22	11/12 (91%) MRD negative	-	-	-	-	-	*No follow-up available yet.*
Ghorashian et	1	**Co-transduction**	12	Balanced expansion	10/12 (83%)	5/10	0	0	0	qPCR in blood (median):	60% 12m EFS

al.[58] *(CARPALL study)* 2024 London, UK		aCD22-9A8-41BBz aCD19-CAT-41BBz	(<24y)	of all three components	MRD-negative at 2m (molecular MRD)					- CD19 CAR-T: 135 days - CD22 CAR-T: 105 days Less persistence than equal CD19 CAR product	75% 12m OS
Cordoba et al.[57] 2021 London, UK (AMELIA study)	1	**Bicistronic vector** *Humanised CAR (AUTO 3)*	15 (ages 4 – 16y)	Kinetics of expansion like Tisagenlecleucel	13/15 (86%) MRD-negative at 2 months	6/13	0	2/13	1/13	119 days median time to last detection in blood (lower than Tisagenlecleucel)	32% 12m EFS
Dai et al.[107] 2020 Beijing, China	1	**Tandem CAR**	6 (ages 17 – 44 y)	Peak at 2 weeks	6/6 (100%) MRD-negative at 1 month	2/6			1/6 (CD19-/CD22^{dim})	5/6 patients less than 6 months persistence	
Spiegel et al.[61]† 2021 Stanford, USA	1	**Tandem CAR**	17 (ages 25 – 78y)	Peak at 10 – 14 days Higher expansion of CD8 compared to CD4	15/17 (88%) MRD-negative at 6m (10^{-4} sensitivity)	4/15 (1 no CD22 status reported)	0	4/15	0	All CAR-T present at day 60. No measurements undertaken thereafter.	33% 6m EFS
Hu et al.[108] 2021 Hangzhou, China	1	**Tandem CAR** *Universal CRISPR/Cas9-engineered*	6 (ages 26 – 56 y)	Peak at 10 – 14 days	5/6 (83%) MRD-negative on day 28	0	1/6 (CD19+/CD 22dim)	0	0	Patients with ongoing remission (2 patients) persistent CAR-T cells >90 days Relapsed patient lost CAR-T cells <60 days.	-
Cui et al.[68] 2023 Suzhou, China	1/2	**Tandem CAR** CD22 VL – CD19 VH, VL – CD22 VH – 41BB	47 (ages 6 – 56 y)	-	40/47 (85%) MRD-negative on day 28	10/47	0	2/47	0	35 patients (75%) underwent consolidative HSCT at median of 2 months from CAR-T cell infusion	69% 24m RFS 74% 24m OS
Niu et al.[109] 2023 Shanghai, China	1	**Tandem CAR** CD19 VL – CD22 VH – VL – CD19 VH – 41BB	15 (ages 23 - 70) First-line MRD-positive patients And relapsed MRD-positive patients	Peak at 10 days. Higher in patients with sustained remission than in those who relapsed.	14/15 (94%) MRD-negative on day 28	4/15	0	1/15	0	3 patients with CAR-T cell persistence > 90 days	77% 12m RFS 86% 12m OS
Shalabi et al.[60] 2022 Bethesda, USA	1	**Tandem CAR**	20 (ages 5 – 34y)	Lower expansion than CD22 CAR alone	16/20 (80%) MRD-negative at 1m (but 4 patients residual or progressive EMD)	3/12 (CD19+, no CD22 status reported)	0	0	1/12 (CD19-, no CD22 status reported)	Less persistence compared to patients receiving CD22 CAR alone (median 28 days vs 88 days)	58% 12m RFS in responders

ALL: acute lymphoblastic leukaemia; CR: complete remission; EFS: event-free survival; EMD: extra-medullary disease; HSCT: hematopoietic stem cell transplant; iCR: complete remission with incomplete haematological recovery; LBCL = Large B-cell lymphoma; m: month; MRD: minimal residual disease; OS: overall survival; RFS: relapse-free survival; y: years

*Showing number of final infused patients †showing results for B-cell ALL cohort only

Table 5: Toxicity profile of main dual-targeting CAR products for B-cell ALL

Reference	Grading system used	n	CRS					Neurotoxicity (ICANS)				
			Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Wang et al.[52]	CTCAE[110]	89 (B-cell ALL + B-NHL)	66 (74%)	-	15 (17%)	3 (3%)	1 (1%)	11 (12%)	0	0	1 (1%)	0
Pan et al.[54]		20 Cycle 1 20 Cycle 2	17 (85%) 15 (75%)		1 (5%) 0		0	3 (15%) 3 (15%)	0	1 (5%) 0	0	0
Liu et al.[55]		27 1st 21 2nd	3 (11%) 8 (38%)	13 (48%) 3 (11%)	5 (19%) 0	1 (4%) 0	1 (4%) 0	1 (4%) -	1 (4%) -	1 (4%) -	-	-
Wang et al.[49]		225	133 (59%)		64 (28%)		1 (0.4%)	36 (16%)		9 (4%)	2 (0.8%)	
Zhang et al.[53]	ASTCT[111]	4	2 (50%)	0	1 (25%)	0	-	1 (25%)	0	0	0	-
Pan et al[56]		81 cycle 1 79 cycle 2	60 (74%) 54 (68%)		12 (15%) 2 (3%)	1 (1%) 2 (3%)	-	19 (23%) 13 (16%)	3 (4%) 1 (1%)	1 (1%) 0		-
Gardner et al.[65]		7	5 (71%)	-	-	-	-	2 (29%)	0	0	0	-
Annesley et al.[66]		12	5 (42%)	-	-	-	-	4 (33%)	0	1 (8%)	0	-
Ghorashian et al.[58]		12	5 (42%)	6 (50%)	-	-	-	4 (33%)	1 (8%)	0	1 (8%)	-
Cordoba et al.[57]		15	11 (73%)	1 (7%)	-	-	-	4 (27%)	0	0	0	-
Dai et al.[107]		6	4 (67%)	2 (33%)	0	0	-	0	0	0	0	-
Spiegel et al.[61]		17	5 (29%)	7 (41%)	-	1 (6%)	-	1 (6%)	1 (6%)	2 (12%)	1 (6%)	-
Hu et al.[108]		6	3 (50%)	2 (33%)	1 (17%)	0	-	0	0	0	0	-
Cui et al.[68]		47	33 (70%)		8 (17%)		-	1 (2%)	0	0	0	-
Niu et al.[109]		15	2 (13%)	2 (13%)	0	0	-	1 (7%)	0	0	0	-
Shalabi et al.[60]		20	7 (35%)	-	3 (15%)	-	-	0	0	1 (5%)	0	-

ASTCT: American Society for Transplantation and Cellular Therapy; CRS: cytokine release syndrome; CTCAE: Common Terminology Criteria for Adverse Events

Table 6: Main trials using **CD19/CD20** dual targeting CAR-T cells for B-cell lymphomas

CD 19 / CD 20										
Reference	Trial, phase	CAR construct	N*	Diseases	Patient characteristics	Response	In vivo expansion	Persistence	Progression / Relapses (and relapse phenotype if available)	EFS/OS
Sang et al.[50] 2020 Xuzhou, China	2	Co-administration, same day - aCD19 scFv – 41BB - aCD20 scFv – 41BB	21 (ages 23 - 72y)	DLBCL: 21	Refractory: 15 Previous autologous HSC: 1 Previous CAR-T: none Bridging: none	ORR: 17/21 (81%) CR: 11 /21 (52%) At day 90	Higher expansion in patients with response. No difference between CD19 and CD20 peak.	Not reported for the full cohort. Persistence around 6 months.	9/21 (43%) patients No CAR-T cells detected in relapsed patients. 5/9 patients had B-cell recovery.	25% 12m PFS 30% 12m OS
Larson et al.[69] 2023 UCLA, USA	1	Tandem CAR CD20 VL CD20 VH CD19 VH CD19 VL – 41BB	10 (ages 29 – 70y)	MCL: 1 FL: 3 DLBCL: 1 tFL: 3 PM LBCL: 1 DH HGBCL: 1	Refractory: 4 Previous autologous HSC: 1 Previous CAR-T: none Bridging: 9/10 (90%)	ORR: 9/10 (90%) CR: 7/10 (70%) At day 60	Peak at 14 days	All responders remained in B-cell aplasia at time of data cut-off. 6 patients >12 months B-cell aplasia	PD: 2/10 Relapse: 1/10	40% 18m PFS 70% 18m OS
Shah et al.[70] 2020, updated by Zurko et al.[78] in 2022 Wisconsin, USA	1	Tandem CAR CD20 – CD19 – 41BB *Fifteen patients received fresh non-cryopreserved products*	22 (ages 38 – 72 y)	DLBCL: 11 MCL: 7 CLL: 3 FL: 1	Previous autologous HSC: 8 Previous allogeneic HSCT: 3 Previous anti-CD19 CAR-T: 1 Bridging: 7/22 (32%)	ORR: 18/22 (82%) CR: 14/22 (64%) At day 28	Higher expansion in patients with response. Peak at 7-12 days	For patients with early CR, B-cell recovery was 42% at 6 months and 56% at 9 months.	PD: 8/22 Relapse: 5/22 All had biopsies and there was no CD19 or CD20 antigen loss.	Updated data for 16 patients that received target dose: 44% 24m PFS 69% 24m OS
Tong et al.[71] 2020, Extended by Zhang et al.[79], 2022 Beijing, China	1-2	Tandem CAR (TanCAR7) CD20 VH CD20 VL CD19 VL CD19 VH – 41BB *Fresh non-cryopreserved product in all infusions.*	87 (ages 16 – 70 y)	DLBCL: 58 FL: 13 tFL: 6 PMBCL: 5 CLL: 2 Small lymphocytic lymphoma: 2 MCL: 2 MALT: 1	Previous autologous HSC: 12 Previous anti-CD19 CAR: 9 Bridging: none	ORR: 68/87 (78%) CR: 61/87 (70%) At month 3.	Peak 7 – 14 days. Higher levels in patients who achieved response.	Median around 100 days. Up to 400 days in 30 patients with ongoing complete remission. No difference in CAR-T cell levels between patients with ongoing response and relapse at days 21-40 and 41-60.	Relapse: 16/87 PD: 18/87 Biopsy available in 12 relapsed patients: - 1 patient had CD19 and CD20 loss. - 7 patients did not have detectable CAR-T cells in tumour tissue or peripheral blood	Median PFS 27.6 months 61% 12m PFS 79% 12m OS

Table 7: Main trials using **CD19/CD22** dual targeting CAR-T cells for B-cell lymphomas

CD19 / CD22										
Reference	Trial, phase	CAR construct	N*	Diseases	Patient characteristics	Response	In vivo expansion	Persistence	Progression / Relapses (and relapse phenotype if available)	EFS/OS
Wang et al.[52] † 2020 *Wuhan, China*	1	Co-administration (3rd generation Sequential, day 0 - 4	38 (ages 9 to71 y)	DLBCL NOS: 23; DH HGBL: 4; HGBL NOS: 3; FL: 3; Burkitt Lymphoma: 2; PMBCL: 1; Others: 2	Refractory: 15 1st relapse: 11 2nd relapse: 4 ≥ 3rd relapse: 8 Bridging: allowed, but no data	OR: 26/36 (72%) CR: 18/36 (50%) at month 3	NR	NR	18/38 (7 were biopsied, showed CD19+/CD22+ disease)	50% 12m PFS 55.3% 12m OS
Cao et al. [51] 2021 *Wuhan, China*	1	High-dose chemotherapy with aHSCi, followed by aCD22 then aCD19 co-administration (days 2 and 3),	42 (ages 24 to 61y)	DLBCL NOS: 30 tFL: 7 DH HGBL: 2 Others: 3	PR: 10/42 PD: 23/42 SD: 9/42 Bridging: high-dose chemotherapy with aHSCi	OR: 38/42 (91%) CR: 34/42 (81%) at month 3.	Peak at 1 week	Median time to B-cell recovery 8.2 months	7/42 (5 were biopsied, showed CD19+/CD22+ disease)	83% 24m PFS 83% 24m OS
Wu et al. [72] 2021 *Wuhan, China*	1	High-dose chemotherapy with aHSCi followed by sequential CD19 and CD22 CART infusion for CNS	13 (ages 23 – 65y)	DLBCL with CNS involvement: 8 Primary CNS DLBCL: 4 ILBCL: 1	Refractory: 1 PR: 2 PD: 3 CNS relapse: 7 Bridging: permitted, no data available	OR: 9/11 (82%) CR: 6/11 (55%) at month 3.	Peak at 1 week	Median persistence <3mo	3/11	75% 12m PFS 83% 12m OS
Roddie et al[73] 2023 London, UK (ALEXANDER study)	1	Auto 3 **Bicistronic vector** *Humanised CAR + Pembrolizumab*	52 (ages 27 – 83 y)	DLBCL: 36; tFL: 10; PM LBCL: 1; t nodal MZL:1; HG BCL: 3	Previous autologous HSC: 16 Bridging: 37/51 (73%)	ORR: 31/47 (66%) CR: 23/47 (49%) at month 1.	Median peak at 12 days.	Median of 4.2 mo persistence	33/52 13 had biopsy: - Majority CD19+ - 7/13 CD22 lo/- - 2 cases of clear CD19 – (H-score heat mapping)	26% 12m EFS 54% 12m OS
Spiegel et al.[61]† 2021 Stanford, USA	1	Tandem CAR (CD19VH – CD22 VL – CD22 VH – CD19 VL – 41BB)	21 (ages 25-78 y)	DLBCL: 14 tFL: 4 PMBCL: 2 Richter: 2	Previous autologous HSC: 4 Previous CAR: none Bridging: permitted, no data available	ORR: 13/21 (62%) CR: 6/21 (29%) at month 3.	Peak at 10 – 14 days CD8 > CD4 expansion	NR	Relapse: 1/21 PD: 15/21 14 biopsied at progression: 4 patients $CD19^{-/lo}$	25% 12m PFS 65% 12m OS
Wei et al.[74] 2021 Hangzhou, China	1	Tandem (VL-VH-VL-VH)	16 (ages 23-68 y)	DLBCL: 13 B-LLy: 2 Burkitt Lymphoma: 1	Previous autologous HSCT: 1 Bridging: none	ORR: 14/16 (87.5%) CR: 10/16 (62.5%) at month 1.	Peak at 5-10 days	8/16 ongoing B-cell aplasia at 10 months 13/16 ongoing B-cell aplasia at 6 months	Relapse: 3/16 PD: 7/16 (2 were biopsied, showed CD19+/CD22+ disease)	40.2% 12m PFS 77.3% 12m OS
Zhang et al.[75] 2021 Suzhou, China	1	Tandem (CD22VL – CD19 VL – CD19 VH – CD22 VH – 41BB)	32 (no age range given) <60 y: 24 >=60 y: 8	DLBCL: 27 tFL: 2 PMBCL: 1 HGBL: 2	Primary refractory: 5 Previous autologous HSC: 4 Bridging: no data available	ORR: 22/29 (76%) CR: 10/29 (34%)	Peak 10-14 days Responders had higher expansion	Median 92 days persistence in peripheral blood (min 13, max 763)	10/29 PD No biopsy performed at time of progression.	40% 12m PFS 63% 12m OS
Zhang et al.[82] 2023 Suzhou, China	2	Tandem + Tislelizumab	16 (ages 19 to 70)	DLBCL: 13 Richter: 2 Burkitt Lymphoma: 1	Previous autologous HSC: 4	ORR: 14/16 (88%) CR: 11/16 (69%)	Peak at median of 12 days.	CAR-T cells present in 50% of patients at 6-month follow-up.	Relapse: 2/16 PD: 3/16	69% 12m PFS 81% 12m OS

aHSCi: autologous hematopoietic stem cell infusion; B-LLy: B-cell lymphoblastic lymphoma; CLL: chronic lymphocytic leukaemia; DH HGBL: double-hit high-grade lymphoma; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; GCB: germinal-centre B-cell like; ILBCL: intravascular large B-cell lymphoma; MALT: mucosa-associated lymphoid tissue lymphoma; MZL: marginal zone lymphoma; NOS: not otherwise specified; NR: not reported; PD: progressive disease; PFS: progression-free survival; PM LBCL: primary mediastinal large B-cell lymphoma; PR: partial remission; SD: stable disease; tFL: transformed follicular lymphoma;

*Showing number of final infused patients †Showing results for B-cell lymphoma cohort only

Table 8: Toxicity profile of main dual-targeting CAR products for B-cell lymphoma

Reference	Grading system used	Total n	CRS					Neurotoxicity (ICANS)				
			Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Sang et al.[50]	ASTCT	21	15 (71%)		6 (29%)		-	3 (14%)		2 (10%)		-
Larson et al.[69]	ASTCT	10	6 (60%)	0	0	0	-	0	0	0	0	-
Shah et al.[70]	ASTCT CTCAE*	22	13 (59%)		1 (4%)		-	4 (18%)		3 (14%)		0
Tong et al.[71] Zhang et al.[79]	ASTCT	87	39 (45%)	13 (15%)	8 (9%)	1 (1%)	-	11 (13%)	2 (2%)	2 (2%)	0	-
Cao et al. [51]	ASTCT CTCAE*	42	26 (62%)	12 (29%)	2 (5%)	0	-	5 (12%)	2 (5%)	2 (5%)	0	0
Wu et al. [72]	ASTCT	13	9 (69%)	2 (15%)	0	0	-	2 (15%)	0	1 (8%)	0	-
Roddie et al[73]	ASTCT	52	11 (21%)	7 (13%)	1 (2%)	0	-	2 (4%)		2 (4%)		0
Spiegel et al.[61]	ASTCT CTCAE*	21	12 (57%)	3 (14%)	1 (5%)	0	-	5 (24%)	3 (14%)	1 (5%)	0	0
Wei et al.[74]	ASTCT	16	4 (25%)	11 (69%)	0	1 (6%)	-	0	0	0	0	-
Zhang et al.[75]	CTCAE	32	14 (44%)	6 (19%)	5 (16%)	3 (9%)	1 (3%)	1 (3%)	0	4 (13%)	0	0
Zhang et al.[82]	CTCAE	16	7 (44%)	0	1 (6%)	0	0	0	0	0	0	0

*Neurotoxicity graded by CTCAE in these studies

ASTCT: American Society for Transplantation and Cellular Therapy; CRS: cytokine release syndrome; CTCAE: Common Terminology Criteria for Adverse Events

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Figure 1