CAR T-cell immunotherapy of B-cell malignancy: the story so far

Leena Halim and John Maher

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***Abstract*:** Chimeric antigen receptor (CAR) T-cell immunotherapy has achieved unprecedented efficacy in the treatment of chemotherapy-resistant or refractory B-cell malignancies. Promising results from pivotal anti-CD19 CAR T-cell phase II trials have led to landmark approvals of two CD19-specific CAR T-cell products by the United States Food and Drug Administration and European Medicines Agency. However, several issues associated with CAR T-cell treatment remain unresolved, such as the management of severe toxicities and the frequent occurrence of both antigen-positive and antigen-negative relapse. Nonetheless, pre-clinical research is advancing at an unprecedented pace to develop innovative solutions to address these issues. Herein, we summarise recent clinical developments and outcomes of CD19-targeted CAR T-cell immunotherapy and discuss emerging strategies that may further improve the success, safety and broadened applicability of this approach.

Keywords: B-cell, CD19, chimeric antigen receptor, clinical trial, leukaemia, lymphoma

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Introduction

Chimeric antigen receptor (CAR) T-cell immunotherapy has radically altered the treatment of refractory B-cell malignancies.[1] Therapeutic efficacy of CAR T-cell immunotherapy has proven unprecedented in this setting, with response rates of up to 90% in patients with relapsed/refractory B-cell acute lymphoblastic leukaemia (B-ALL) and up to 60% of patients with B-cell non-Hodgkin's lymphoma (NHL).[2] Following the attainment of these breakthrough clinical results in multicentre pivotal trials, the United States Food and Drug Administration (US FDA) and European Medicines Agency (EMA) have both approved two anti-CD19 CAR T-cell products for the treatment of patients with relapsed/refractory B-cell malignancies.

Design of chimeric antigen receptors

CARs are recombinant proteins composed of an antigen recognition domain, most commonly a single chain variable fragment (scFv), linked *via* a hinge or spacer and transmembrane domain to an intracellular signalling domain.[3] Several CAR iterations have been developed and tested in the laboratory and in the clinic (Figure 1). The prototype configuration, now known as a first generation CAR, provides a T-cell receptor (TCR)-like 'signal 1' alone, typically *via* CD3ζ or Fcεr1γ.[4] However, lack of T-cell persistence, expansion and limited anti-tumour efficacy in pre-clinical and clinical trials led to further modifications of CAR design.[4,5] Pioneered by Finney *et al.*, second generation CARs have proven to be transformative upon subsequent clinical development.[6] This modification entailed the introduction of a single intracellular costimulatory module placed above CD3ζ, thereby integrating both signals 1 and 2. When included in a linear CAR arrangement, costimulatory receptors such as CD28 and 4-1BB promote enhanced interleukin-2 (IL-2) secretion and T-cell proliferation. Third-generation CAR constructs combine two or more costimulatory domains such as CD28, 4-1BB, OX40 or ICOS. However, there is limited clinical data to support the superiority of the third-generation CAR platform and true enablement of this technology remains open to question.[7,8]

Correspondence to:
Leena Halim
King's College London, School of Cancer and Pharmaceutical Sciences, Guy's Hospital, London, UK
leena.halim@kcl.ac.uk

John Maher
King's College London, School of Cancer and Pharmaceutical Sciences, Guy's Hospital, London, UK

Department of Clinical Immunology and Allergy, King's College Hospital NHS Foundation Trust, London, UK

Department of Immunology, Eastbourne Hospital, East Sussex, UK

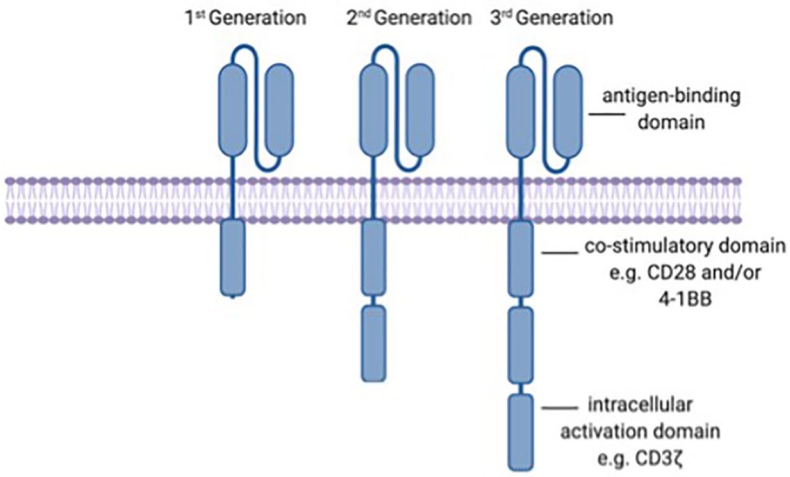

Figure1. Evolution of CAR design over generations. First generation CARs consist of an antigen binding domain, usually an scFv, fused to a CD3ζ activation domain. Second generation CARs contain an additional intracellular costimulatory domain, usually CD28 or 4-1BB (CD137). Third generation CARs combine two or more costimulatory domains.
CAR, chimeric antigen receptor.

Targeting CD19 with CAR T-cells

As indicated above, most clinical success achieved using this technology pertains to the use of second generation CAR T-cells targeted against CD19. CD19 is a transmembrane glycoprotein required for normal B-cell development in humans and is expressed on the cell surface from early pro-B development through to the onset of terminal plasma cell differentiation.[9,10] CD19 is an attractive therapeutic target since it is expressed in over 95% of B-cell malignancies, including B-ALL, chronic lymphocytic leukaemia (CLL) and B-cell NHL. Furthermore, expression of CD19 is restricted to the B-cell lineage. Whereas B-cells are required for antibody production, clinical experience of primary immunodeficiency disorders in which B-cells are absent (e.g. X-linked agammaglobulinaemia) indicates that B-cell aplasia can be managed effectively using intravenous or subcutaneous immunoglobulin replacement therapy.[9] Consequently, in patients with refractory malignancy, it may be viewed that the induction of B-cell aplasia as a necessary on-target off-tumour toxicity of CAR T-cell immunotherapy could be considered to constitute a justifiable toxicity.

Clinical results of anti-CD19 CAR T-cell therapy for the treatment of B-cell NHL

NHL is a heterogenous disease comprising several subtypes and is the most frequent B-cell malignancy worldwide.[11] Lymphomas of B-cell origin represent 86% of all diagnosed NHLs. Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype and accounts for approximately 40% of all B-cell NHLs worldwide.[12] Whereas 5-year survival rates have improved significantly in recent years, up to 50% of patients with DLBCL are refractory to standard immunochemotherapy treatments or will relapse after achieving complete response (CR).[13] The prognosis for patients with relapsed/refractory DLBCL is poor and there is a clear need for new treatment approaches to address this gap. In this context, the development of CD19-targeted CAR T-cell immunotherapy represents a highly attractive approach given its superior efficacy to alternative more traditional treatment schedules.

The first case report of effective anti-CD19 CAR T-cell immunotherapy was published in 2010, and described a patient with advanced follicular lymphoma (FL) who was treated at the US National Cancer Institute (NCI).[14] Prior to the CAR T-cell infusion, the patient received a conditioning chemotherapy regimen of fludarabine and cyclophosphamide to ensure lymphocyte depletion. The autologous T-cells were transduced retrovirally to express a CAR containing an anti-CD19 scFv known as FMC63 coupled with a CD28 costimulatory domain and CD3ζ.[15,16] Following treatment, the patient achieved a partial response (PR)

lasting 32 weeks. Upon disease progression the patient was retreated with CAR T-cells and again achieved PR and remained progression free for 7 years.[17] Subsequently, the NCI conducted the first trial that demonstrated the broader efficacy of anti-CD19 CAR T-cell immunotherapy for DLBCL.[18] In this small study, 15 patients with various B-NHL subtypes received a single infusion of retrovirally transduced FMC63.28z CAR T-cells. Eight patients achieved CR, four patients achieved PR and one experienced stable lymphoma. Of the four CR, three patients still had ongoing CR at the last reported follow up.[18]

Based on these successful results, a pivotal multicentre phase II trial, known as ZUMA-1 [ClinicalTrials.gov identifier: NCT02348216], was conducted by Kite/Gilead in which 101 patients with DLBCL, primary mediastinal B-cell lymphoma and transformed follicular lymphoma (tFL) were treated with the same product, now designated Axicabtagene ciloleucel (axi-cel).[19,20] Prior to axi-cel infusion, patients received three doses of fludarabine and cyclophosphamide conditioning chemotherapy. As per the protocol, patients requiring bridging therapy were excluded from the trial. Recently published long-term follow-up data revealed an overall response rate (ORR) of 83% with 58% of patients achieving a CR after a median follow up of 27.1 months as assessed by trial investigators. Importantly, grade 3 or higher cytokine release syndrome (CRS) occurred in 11% of patients, whereas grade 3 plus neurotoxicity occurred in 32% of patients as per criteria of Lee and colleagues. Toxicities were linked to CAR T-cell expansion. Moreover, two treatment-related deaths due to CRS were reported. Nonetheless, it was concluded that the durable responses observed represented a major improvement in clinical outcomes for these patients who were refractory to several lines of treatment. Outcome for patients with refractory DLBCL is poor, with a median overall survival of approximately 6 months and a response rate of only 20% with conventional treatments.[21,22] Based upon these promising results, the US FDA and EMA in 2017 and 2018, respectively, approved axi-cel for the treatment of relapsed/refractory DLBCL after two preceding lines of therapy.

In addition to axi-cel, another anti-CD19 CAR T-cell therapy has been approved for the treatment of relapsed/refractory large B-cell lymphoma in the US and EU. Tisagenlecleucel (Novartis) was originally developed by investigators at the University of Pennsylvania (UPenn) and is also a second-generation CAR. However, it employs 4-1BB as a costimulatory domain as opposed to CD28 and is delivered by a lentivirus.[23,24] In 2017, results were published from an academic study in which 28 adult patients with DLBCL or FL were treated with tisagenlecleucel. The ORR was 64% with 57% of patients achieving CR. Furthermore, response was maintained in 86% of patients with DLBCL and 89% of FL patients at a median follow-up interval of 28.6 months. Notably, four patients who achieved PR at 3 months later converted to CR at 6 months. Severe CRS (grade 3 or higher) occurred in 18% of patients and severe encephalopathy occurred in 11%, of which one case proved fatal.

JULIET [ClinicalTrials.gov identifier: NCT02445248] is an international phase II trial of tisagenlecleucel for the treatment of relapsed/refractory DLBCL involving 27 centres in 10 countries. Adult patients who experienced disease progression after haematopoietic stem-cell transplantation (HSCT) or who were ineligible for HSCT were enrolled in the study, and a total of 111 patients received an infusion. Bridging therapy and lymphodepleting chemotherapy was given at the treating physicians discretion. Nevertheless, over 90% of patients enrolled received both. Of 93 evaluable patients, the best ORR was 52%, with 40% of patients attaining CR as determined by an independent review committee. At 12 months, overall survival was 49%. Median progression free survival (PFS) has not been reached for those patients who achieved CR. The estimated probability of survival among patients who achieved a CR was 90%. Importantly, no deaths were attributed to tisagenlecleucel, with grade 3 CRS only occurring in 22% of patients and grade 3 neurotoxicity in 12% as determined by Penn grading.[25]

Researchers at the Fred Hutchinson Cancer Research Center (FHCRC) engineered an alternative 4-1BB-based second generation CAR that contains a distinct IgG4-based hinge and is being developed by Juno as JCAR017. Academic investigators conducted a phase I clinical trial in which anti-CD19 CAR T-cells were individually formulated from purified $CD4^+$ and $CD8^+$ T-cells, which were expanded and then infused at a defined

1:1 CD4:CD8 ratio.[26] In preclinical studies, results suggested that CAR T-cells manufactured from purified $CD4^+$ or $CD8^+$ central memory (T_{CM}) or naïve (T_N) T-cells had more potent anti-tumour activity compared with effector memory (T_{EM}) cells. Furthermore, enhanced CAR T-cell proliferation and tumour elimination was seen when a defined ratio of $CD4^+$ and $CD8^+$ CAR T-cells was administered, when compared with unselected T-cells or either subset alone.[27] In a phase I single institute academic study, 32 patients with relapsed/refractory B-cell NHL were treated with CAR T-cells following lymphodepletion with cyclophosphamide alone or with fludarabine.[26] Patients who received fludarabine-based chemotherapy had markedly increased response rates (50% CR, 72% ORR) compared with patients who did not receive fludarabine (8% CR, 50% ORR). The improved therapeutic efficacy in the lymphodepleted patients was associated with increased CAR T-cell expansion and persistence.[26]

The aforementioned academic study was followed by an international multicentre phase II trial sponsored by Juno (TRANSCEND [ClinicalTrials.gov identifier: NCT02631044]). The product was designated lisocabtagene maraleucel (JCAR017; axi-cel) and was evaluated in patients with aggressive relapsed/refractory NHL.[28] A total of 102 patients received axi-cel after lymphodepletion and an interim analysis of the trial reported ORR rates of 49% and 46% CR at 6 months.[29] Liso-cel was very well tolerated, with grade 3 or above CRS only occurring in 1% of patients and severe neurotoxicity in 15%. A recent update described results from 268 patients treated with liso-cel in which all primary and secondary efficacy endpoints were met with a CR rate of 53%.[30] Grade 3 or above CRS occurred in 2% of patients, and grade 3 or above neurological events in 10%.

Follicular lymphoma (FL) is the second most common NHL subtype diagnosed in the US and Europe.[31] Progression of disease within 2 years after first-line chemotherapy treatment or development of histological transformation (tFL) is associated with poor survival outcome.[32] A phase I/II trial reported durable CR rates in FL in patients with relapsed/refractory FL and tFL [ClinicalTrials.gov identifier: NCT01865617].[33] A total of 21 patients received lymphodepletion with fludarabine and cyclophosphamide followed by anti-CD19 CAR T-cells with a defined 1:1 CD4:CD8 ratio. A higher CR rate was reached in FL (88%) compared with 46% in tFL. All FL patients who attained CR remained in remission at a median follow up of 24 months, whereas the median PFS in tFL patients was 10.2 months. No severe adverse events wee occurred (grade $\geqslant 3$) in either FL or tFL patients.

In total, three anti-CD19 CAR T-cell therapies have been approved or are under investigation for the treatment of relapsed/refractory B-cell NHL. However, comparisons of efficacy, toxicity and durability across trials are difficult to analyse because of relatively small patient numbers, variations in patient populations, differences in prior treatment and in conditioning regimens used prior to CAR T-cell infusion. Cross-trial comparisons are further complicated due to differences in CAR design, cell manufacturing, toxicity grading and inclusion criteria.

A recent meta-analysis of all published studies in lymphoma has concluded that doses of less than 10^8 cells/m^2, absence of IL-2 administration and the inclusion of fludarabine in conditioning regimens all contributed to improved efficacy and safety.[34] The success of phase II studies has initiated progression towards phase III trials, such as TRANSFORM [ClinicalTrials.gov identifier: NCT03575351], to examine the efficacy and safety of anti-CD19 CAR T-cell therapy compared with standard salvage therapy with autologous stem cell transplantation.[35] A summary of phase II clinical trials of CD19 CAR T-cell immunotherapy of B-NHL is provided in Table 1.

Clinical results of anti-CD19 CAR T-cell therapy for the treatment of B-ALL

B-cell ALL is an aggressive disease that can occur at any age, but is most prevalent in children and young adults. In children under 15 years of age, ALL accounts for 25% of all malignancies and 19% of cancers in young adults aged 15–19 years.[36] Survival rates in paediatric and young-adult patients with ALL have improved greatly to more than 90%.[36,37] However, the prognosis of adult ALL has remained poor, with only 30–40% of adult patients achieving long-term remission.[38] Furthermore, despite an improvement in survival of young patients, 10–15% will relapse after chemotherapy treatment,

Table 1. Phase II clinical trials in B-NHL.

Trial	JULIET/Novartis	ZUMA-1/Kite	Transcend/Juno
CAR-T	Tisagenlecleucel *Kymriah*	Axicabtagene ciloleucel *Yescarta*	Liscoabtagene maraleucel
Co-stimulatory domain	4-1BB	CD28	4-1BB: defined 1:1 CD4:CD8 ratio
Patients	111	108	268
Disease	DLBCL tFL	DLBCL tFL, PMBL	Aggressive B-NHL
ORR	52%; 33% at 6-months	82%; 38% at 27.1-months	73%
CR	40%; 29% at 6-months	58%; 37% at 27.1-months	53%
CRS grade ⩾3	22%	11%	2%
Neurotoxicity grade ⩾3	12%	32%	10%
CRS grading system	Penn	Lee	Lee
Response assessed by	Independent review committee	Investigators	Independent review committee

B-NHL, B-cell non-Hodgkin's lymphoma; CAR-T, chimeric antigen receptor T-cell immunotherapy; CR, complete response; CRS, cytokine release syndrome; DLBLC, diffuse large B-cell lymphoma; ORR, overall response rate; tFL, transformed follicular lymphoma;

and approximately 3% will initially present with refractory disease.[37,39] As such, ALL patient outcomes remain unsatisfactory and emerging new strategies for treatment are necessary.

In 2013, investigators at Memorial Sloan-Kettering Cancer Center (MSKCC) were the first group to report impressive clinical outcomes in adult patients with relapsed B-ALL treated with anti-CD19 CAR T-cells.[1] Five patients were treated with autologous second generation (CD28-containing) anti-CD19 CAR T-cells, administered after cyclophosphamide conditioning therapy. Impressively, all five patients responded with rapid tumour eradication and achieved minimal residual disease (MRD) negative CR. One patient relapsed with $CD19^{+}$ disease 90 days after infusion, likely due to a loss of circulating CAR T-cells. Elevated serum cytokine levels were correlated with higher tumour burden at the time of CAR T-cell infusion and some patients required corticosteroid treatment to ameliorate toxicities. Severity of CRS was also correlated with T-cell expansion. Four of five patients went on to receive HSCT, raising the possibility that CAR T-cell immunotherapy might emerge as a bridging therapeutic approach to successful HSCT.[1] Indeed this approach is now being pursued by other centres who have reported that both event-free and relapse-free survival may be prolonged when CD19 CAR T-cell immunotherapy is employed prior to allogeneic HSCT.[40] Intriguingly however, further updates from the MSKCC group have indicated that overall survival was similar in patients who underwent allo-HSCT and those who did not.[41,42] However, there was a surprisingly high rate of treatment-related toxicities in patients treated with HSCT.[43]

Shortly after the MSKCC group published their initial clinical experience with anti-CD19 CAR T-cell immunotherapy of B-ALL, researchers at UPenn reported the successful treatment of two paediatric relapsed/refractory ALL patients with CTL019 (now marketed as tisagenlecleucel) CAR T-cells.[2] Complete remission was achieved

in both cases, although accompanied by severe CRS that was amenable to treatment with the anti-IL-6 receptor antibody, tocilizumab and anti-tumour necrosis factor-α agent, etanercept. Patient 1 remains in long-term remission 7 years after treatment. Patient 2 relapsed with CD19-negative disease 2 months after therapy, most likely due to the selective pressure exerted by the anti-CD19 CAR T-cells. A single centre phase I/IIA study was subsequently conducted at UPenn that included 30 patients with relapsed/refractory ALL. In contrast to the MSKCC experience, 25 of these patients were aged ⩽ 22 years and 5 were 26–60 years of age. At the first assessment undertaken 1 month after CAR T-cell infusion, 90% of patients were in CR and overall survival at 6 months was 78%. Long-term remission of up to 2 years was associated with CAR T-cell persistence, as inferred by sustained B-cell aplasia, which was consistent with continued T-cell effector function. However, all patients suffered CRS and eight individuals required treatment in intensive care because of severe toxicities. In the global Novartis-sponsored 25-centre phase II study ELIANA [ClinicalTrials.gov identifier: NCT02435849], 75 paediatric and young-adult patients with relapsed/refractory B-cell ALL were treated with tisagenlecleucel.[44] At enrolment, patients were aged between 3 and 23 years with at least 5% bone marrow lymphoblasts. Patients infused with tisagenlecleucel had a median of three previous therapies and 61% were post allo-HSCT. At 3 months, the overall remission rate was 81%, and all patients in remission were MRD negative. At 12 months, the rate of event-free survival was 50% and overall survival was 76%. As reported previously, all patients with a response to treatment had B-cell aplasia that was treated with immunoglobulin replacement therapy. CRS occurred in the majority of patients (77%) and neurotoxicity occurred in 40% as determined by the Penn grading system. For management of CRS, 48% of patients required tocilizumab treatment. However, 22 patients succumbed to relapse, most of which involved CD19-negative disease (15/22). Moreover, tisagenlecleucel CAR T-cells were detectable for as long as 20 months after initial infusion, indicating long-term perisistence.[44]

In parallel with these developments, MSKCC investigators published their updated experience involving 53 adult patients with relapsed/refractory B-cell ALL, reporting on longer-term outcome and safety [ClinicalTrials.gov identifier: NCT01044069]. Fifty-two of the patients were evaluable of whom 67% achieved MRD negative CR. Disease remission was significantly associated with higher peak CAR T-cell expansion.[43] Median follow-up was 29 months and median overall survival was 12.9 months. Four patients relapsed following the attainment of MRD negative CR with a loss of CD19 expression by leukaemic blasts. Interestingly, patients with low disease burden prior to treatment had distinctly enhanced survival outcomes compared with patients with more than 5% bone marrow blasts. Furthermore, patients with a high disease burden had more toxicity such as CRS and neurotoxicity and one patient died from severe CRS and multi-organ failure 5 days after treatment.

Recently, results of an MSKCC-sponsored multicentre CD19 CAR T-cell trial in paediatric/young adult patients were published [ClinicalTrials.gov identifier: NCT01860937].[45] Twenty five patients received conditioning chemotherapy with either high- or low-dose cyclophosphamide. Patients who received high-dose cyclophosphamide achieved higher response rates (94%) compared with the low-dose cohort (38%), once again associated with superior peak CAR T-cell expansion. Importantly, the more intense conditioning treatment did not lead to an increase in CAR associated toxicity. These results suggest a more powerful lymphodepletion regimen translates into improved response perhaps due to greater CAR T-cell expansion and reduced immune suppression of the infused cells.

As indicated previously, the group at FHCRC have pursued the approach of infusing 4-1BB-containing second generation CAR T-cells at a defined 1:1 $CD4^+$:$CD8^+$ T-cell ratio. Applying this approach to relapsed/refractory ALL, they treated 30 adults with in their initial study[46] [ClinicalTrials.gov identifier: NCT01865617]. Independent manufacture of purified $CD4^+$ and $CD8^+$ CAR T-cells was successful in 27 patients despite poor lymphocyte numbers normally associated with disease and intensive pre-treatment. After infusion, 93% of patients achieved CR as determined by flow cytometry. Fludarabine was incorporated into the conditioning regimen for the last 17 patients entered into the study which increased CAR T-cell expansion and persistence thus leading to an improved disease-free survival rate. Similar to the MSKCC experience, severe

Table 2. Phase II trials in B-ALL.

Author/ trial	Centre	CAR	Patient	MRD-neg CR	CRS grade ⩾3	Neurotoxicity grade ⩾3	CRS grading system
Maude/ ELIANA	Novartis/ Multicentre	4-1BB	75 paediatric and young adult	81%	46%	13%	Penn
Park	MSKCC	CD28	53 adult	67%	26%	42%	MSKCC
Lee	NCI	CD28	21 paediatric and young adult	67%	16%	0%	NCI
Turtle	FHCRC	4-1BB	30 adult	86%	28%	50%	Lee
Gardner	Seattle Children's	4-1BB	45 paediatric and young adult	93%	23%	23%	Lee

B-ALL, B-cell acute lymphoblastic leukaemia; CR, complete response; CRS, cytokine release syndrome; FHCRC, Fred Hutchinson Cancer Research Center; MRD, minimal residual disease; MSKCC, Memorial Sloan-Kettering Cancer Center; NCI, National Cancer Institute; neg, negative.

CRS and neurotoxicity were both correlated with higher tumour burden. Furthermore, one fatality at the highest dose level led to risk-adapted CAR T-cell dosing for the remainder of the study. Dosing was based on disease burden, which resulted in reduced toxicity in almost all patients with high-tumour burden. Relapse with $CD19^+$ disease occurred in a subset of patients. This was linked to a lack of persistence of CAR T-cells in the blood, who were then retreated with a second CAR T-cell infusion. However, retreatment did not result in anti-tumour activity or persistent CAR T-cells because of a $CD8^+$ T-cell mediated immune response against the CAR transgene product.

In a trial at the Seattle Children's Research Institute, a defined 1:1 CD4:CD8 was also used to treat 45 children and young adults with relapsed/ refractory ALL.[47] A major objective of this phase I trial was to determine the feasibility of manufacturing highly purified CAR T-cells within distinct T-cell subsets. To accomplish this objective, the CAR encoding vector co-expressed EGFRt thus allowing for cetuximab-mediated purification of transduced cells using the CliniMACS platform.[40] The rate of MRD negative CR achieved was 89% based on intention-to-treat analysis and 93% among patients who received anti-CD19 CAR T-cells. A total of 14 patients received fludarabine and cyclophosphamide lymphodepletion prior to treatment and all achieved MRD negative CR. There were no deaths attributed to toxicity. Severe CRS and/or neurotoxicity occurred in 23% of patients with a trend towards greater toxicity in those patients with a higher disease burden. Nonetheless, 18 patients who obtained CR relapsed within the follow-up time, 7 of whom had CD19-negative disease.[47] A summary of phase II trials of CAR T-cell immunotherapy is provided in Table 2.

CAR T-cell therapy-associated toxicities

The most common toxicities to occur following CAR T-cell therapy are CRS, neurotoxicity and B-cell aplasia. Incidence of severe CRS and neurotoxicity across different trials is varied but symptoms usually occur soon after the infusion and, despite the use of cytokine blocking agents such as tocilizumab, toxicity can be fatal.[2]

Cytokine release syndrome

Factors that are associated with higher risk of CRS include high disease burden and increased CAR T-cell dose. Unsurprisingly, higher peak blood CAR T-cell expansion is correlated with severity of CRS, which is further promoted by intensity of prior lymphodepletion. Patients suffering with CRS typically present with fever, hypotension, cardiac dysfunction, renal failure and other

toxicities. CRS is associated with elevated serum levels of cytokines such as interferon gamma (IFNγ), interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF) and interleukin-10 (IL-10), leading to uncontrolled systemic inflammation.[48,49] Treatment of CRS is dependent on its severity; patients with grade 3 or 4 CRS require immediate intensive treatment with vasopressors and immunosuppressive agents such as tocilizumab and corticosteroids. Circulating IL-6 levels are often correlated with CRS severity, and administration of the IL-6 receptor antagonist tocilizumab has become the standard approach for the management of CRS. The use of prophylactic tocilizumab and corticosteroids has been evaluated as an approach to reduce the incidence of severe CRS. A safety management study was incorporated into the ZUMA-1 trial, and patients received prophylactic treatment of levetiracetam and tocilizumab on day 0 and 2 of axi-cel infusion.[50] One patient experienced grade ⩾3 CRS; however, grade ⩾3 neurological events occurred in 38% of patients and one patient died of cerebral oedema. Accordingly, the authors concluded that prophylactic use of tocilizumab may reduce the occurrence of severe CRS but not severe neurological events.

Neurotoxicity

Neurological toxicities are another common complication of CAR T-cell immunotherapy, and symptoms include confusion, tremors, seizures and expressive aphasia.[51] Recently, immune effector cell-associated neurotoxicity syndrome (ICANS) grading has been used to grade this toxicity and link this to treatment recommendations.[51] The exact cause of ICANS remains unclear, but it has been suggested that endothelial activation and leakage of elevated cytokine levels across the blood-brain barrier, thereby promoting pericyte stress, may be contributory factors.[52] Using a murine model of CRS established by van der Stegen *et al*., recent studies have shown that IL-1 and IL-6 produced by activated macrophages were key contributors to CRS and neurotoxicity.[53–55] These findings suggest that intervention to block both IL-1 and IL-6 could protect patients from lethal toxicity.

An alternative potential solution to mitigate the toxicity associated with CD19 CAR T-cells entails the further engineering of the CAR to reduce cytokine production. A variant anti-CD19 CAR has been described recently in which an altered CD8α hinge/transmembrane domain was introduced. As a result of this modification, significantly lower levels of inflammatory cytokines were produced whilst cytotoxicity against $CD19^+$ tumour cells was maintained.[56] A phase I study of this CD19-BBz(86) was conducted whereby 25 patients with refractory NHL were treated with fludarabine and cyclophosphamide before receiving CAR T-cells. At the highest dose, 6/11 patients achieved complete remission that was durable with a median duration of response of >181 days. Importantly, neurotoxicity was not observed in any of patients, and the only adverse event reported was grade 1 CRS. Furthermore, serum cytokine levels of IL-6, IFNγ and TNFα remained at basal level at different time points following CAR T-cell infusion. Another approach to limit toxicity is to reduce CAR binding affinity. The 'CAT' CAR was developed with a substantially lower affinity for CD19 compared with FMC63 which translated into enhanced anti-tumour activity *in vitro*.[57] In a clinical study, 14 paediatric patients with relapsed/refractory B-ALL were treated with the CAT CAR after lymphodepletion with fludarabine and cyclophosphamide (CARPALL [ClinicalTrials.gov identifier: NCT02443831]). At 3 months post-treatment, 12/14 patients had achieved a CR and OS was 63% at 12 months, which is comparable with the tisagenlecleucel trial and other published data.[44,47] Strikingly, CAT CAR T-cells had heightened expansion and increased persistence; detectable at up to 24 months post infusion. Toxicity was also reduced as no patient developed severe CRS or neurotoxicity in contrast to FMC63 CAR T-cells.[43,44,46] These studies demonstrate the potential for modulating CAR toxicity and functionality by altering CAR structure and binding affinity.

Universal CD19 CAR T-cell therapies

Thus far, only autologous CAR T-cell therapies have been approved for clinical use. However, manufacture of autologous CAR T-cell products is not always feasible, for example, in heavily pretreated or lymphopenic patients.[58] Furthermore, autologous manufacturing processes remain time-consuming, expensive and require access to complex facilities. Given these considerations, there has been considerable interest in the development of universal 'off the shelf' CAR T-cells from

healthy nonmatched donors. Using the genome-editing technology TALEN (transcription activator-like effector nuclease), investigators have disrupted the constant region of the T-cell receptor α (TRAC) in donor T-cells in order to minimise graft-*versus*-host disease (GVHD).[59] A second TALEN pair was used to target the CD52 gene, the target antigen of the depleting monoclonal antibody, alemtuzumab. By this means, alemtuzumab could be used to achieve selective depletion of recipient T-cells, thus promoting survival of donor CAR T-cells. In 2015, two infants with relapsed/refractory B-ALL were treated successfully with universal anti-CD19 CAR T-cells, UCART19, that had been engineered in this manner. Both patients achieved molecular remission within 28 days and went on to receive allo-HSCT. Phase I clinical trials for UCART19 are now underway in paediatric (PALL [ClinicalTrials.gov identifier: NCT02808442]) and adult (CALM [ClinicalTrials.gov identifier: NCT02746952]) relapsed/refractory B-ALL patients. A recent update in 2018 stated that 21 patients had received UCART19 and that overall CR rate was 67%.[60] In 17 patients in whom the lymphodepletion regimen included alemtuzumab, the CR rate was 82% and 71% achieved MRD. Among the four patients who did not receive alemtuzumab, minimal CAR T-cell expansion was observed. None of these patients achieved clinical response, suggesting that $CD52^+$ cell depletion is important for allogenic CAR T-cell survival. One treatment-related death 15 days post infusion was reported in the CALM study, attributed to severe grade 4 CRS and neutropenic sepsis. Importantly, only two cases of mild GvHD were observed, which was acute and easily controlled. Furthermore, all of the other patients had moderate and clinically manageable CRS. These findings provide encouragement for the future development of allogeneic CAR T-cells. Other gene editing approaches such as zinc-finger nucleases and CRISPR/Cas-9 directed genome editing hold great promise for advancing CAR T-cell therapy.[61,62] These innovative gene-editing developments suggest that off-the-shelf allogenic CAR T-cell therapy will be more widely accessible in the future.

Overcoming the challenge of relapse following CD19 CAR T-cell immunotherapy

Anti-CD19 CAR T-cell immunotherapy has achieved remarkable response rates in patients with relapsed/refractory B-cell malignancies, but many patients relapse with CD19-negative disease. One tactic that is under investigation in an attempt to prevent tumour escape due to antigen loss is to target other or multiple B-lineage antigens, such as CD22.

Multi-targeted CAR T-cell therapies

Fry *et al.* described the use of CD22-targeted CAR T-cells to treat B-ALL, including patients who had failed prior therapy with CD19 CAR T-cell immunotherapy.[63] Lymphodepletion with fludarabine and cyclophosphamide was implemented and, of 52 treated patients, the CR was 72.5%.[63,64] The study included 30 subjects who previously received anti-CD19 CAR T-cell therapy and 28 patients who had CD19-negative disease at enrolment. Patients with no prior CD22 targeted therapy had a superior response compared with those treated with an anti-CD22 monoclonal antibody. Moreover, patients with diminished CD19 expression responded to anti-CD22 CAR T-cells and reached CR, indicating that prior immunotherapy did not negatively impact response. The median time to relapse was 2 months compared with 6 months if patients had no prior CD22-targeted therapy. Relapse was largely due to down-modulation of CD22 expression without detectable mutation. The majority of patients experienced CRS (88.4%) and unique toxicities occurred in a minority of participants, including capillary leak syndrome and hemophagocytic lymphohistiocytosis. This trial demonstrates proof of concept for the efficacy of CD22 targeting in ALL patients. However, similar to CD19 CAR T-cell immunotherapy, relapse due to diminished antigen expression suggests targeting of multiple B-lineage antigens may be more effective.

A single institution phase I study is underway to assess the manufacturing feasibility and safety of a bicistronic CAR, co-targeting CD19 and CD22, each with 4-1BB and CD3ζ intracellular signalling domains.[65] Six adult patients with B-ALL or DLBCL were treated at the lowest dose level following lymphodepletion with fludarabine and cyclophosphamide. This intervention led to the induction of CR in two patients (one each with ALL and DLBCL), whereas the same approach achieved CR in four of four paediatric patients with low burden B-ALL.[65,66] All patients

tolerated the treatment well and only mild CRS was reported in adults and infants. Dose escalation is ongoing in both studies. Amrolia *et al.* also developed a bi-cistronic vector encoding dual CARs against CD19 and CD22 with OX40 and 4-1BB costimulatory domains respectively.[67] To enhance sensitivity, a pentavalent hinge was used in the CD22 CAR and the product, AUTO3, was trialled in a phase I/II study. Ten heavily pretreated ALL patients received AUTO3 CAR T-cells and 9/10 achieved MRD-negative CR. All six patients who received higher doses ($\geqslant 3 \times 10^6$ cells/kg) had MRD-negative CR and the latest update reported no relapse due to antigen loss.[67] However, a recent press release indicates that development of this product for B-ALL has been discontinued owing to inferior efficacy compared with their anti-CD19 CAR.[68]

Enhancing durability of disease response

Another important mechanism of disease resistance relates to lack of CAR T-cell persistence, an issue that is unlikely to be solved by targeting of multiple antigens. Anti-transgene immune responses against CAR T-cells have been associated with their poor expansion and persistence. CARs with humanized scFv regions have been developed to decrease immunogenicity and thereby improve efficacy.[69,70] HuCAR-19 is a fully human CAR administered to nine patients with advanced NHL and reported an ORR of 86%.[70]

The intrinsic fitness of CAR T-cells has been implicated as the most important factor shaping the clinical response in patients with advanced CLL, a disease setting in which response to CD19 CAR T-cells varies between 26% and 71%.[71–73] Patients responding to anti-CD19 CAR T-cells showed enhanced transcription of genes related to early memory differentiation and had more robust expansion potential both *ex vivo* and *in vivo*. Additionally, the IL-6/signal transducer and activator of transcription 3 (STAT3) pathway was upregulated in CAR T-cells from responding patients and STAT3 signalling blockade diminished T-cell proliferation. In contrast, CAR T-cells from non-responding patients upregulated genes associated with effector T-cell differentiation, exhaustion and glycolysis. This study suggests CAR T-cell fitness may be used as a biomarker to determine likelihood of successful therapeutic activity.[72]

Research is continuing to determine factors associated with durable remissions after CAR T-cell therapy. Multivariable analysis of clinical and treatment characteristics of NHL patients showed that a favourable cytokine profile after lymphodepletion, consisting of higher levels of monocyte chemoattractant protein-1 (MCP-1) and IL-7, were associated with superior PFS.[74] Furthermore, this favourable cytokine profile was linked with high intensity fludarabine and cyclophosphamide lymphodepletion. In addition, the polyfunctionality of CAR T-cells pre-infusion has been correlated with toxicity and improved clinical outcome in NHL patients.[75] In adult patients with ALL, multivariable modelling showed better event-free survival (EFS) was associated with lower lactate dehydrogenase levels and higher platelet count pre-lymphodepletion in MRD-negative CR patients.[76] Moreover, inclusion of fludarabine in the lymphodepletion regimen and allo-HSCT after CAR T-cell therapy were also linked with improved EFS.

Other strategies to increase CAR T-cell activity include administering immune checkpoint inhibitors in combination with CAR T-cells. A case report in 2017 described a DLBCL patient who achieved a clinically significant response to programmed death 1 (PD-1) blockade (pembrolizumab) after relapse following tisagenlecleucel.[77] A phase I/II clinical trial is now investigating the safety and efficacy of pembrolizumab in NHL patients who fail to respond or relapse after tisagenlecleucel [ClinicalTrials.gov identifier: NCT02650999]. The best ORR among 11 patients was 27%, with 2 CR.[78] Furthermore, re-expansion peaks of tisagenlecleucel CAR T-cells were detected in responding patients.

The ZUMA-6 study is investigating the combination of an anti-PD-L1 antibody (atezolizumab) with axi-cel in DLBCL patients. So far, data from 10 patients who have received one or more doses of atezolizumab with axi-cel show an ORR of 90%. However, 50% of patients enrolled experienced grade 3 or higher neurotoxicity.[79] The ultimate goal for anti-CD19 therapy is the attainment of durable remissions in all treated patients. Currently, antigen-negative disease relapse is the most common form of disease recurrence and is challenging to treat. As such, multi-antigen targeting might be the way forward to optimize CAR T-cell therapy. Furthermore, future directions to

improve CAR T-cell technologies and enhanced understanding of the determinants of antigen-positive relapse will be crucial to create more effective treatments.

Other B-cell malignancies targeted with CAR T-cells

In addition to the aforementioned clinical experience in B-cell ALL and NHL, a number of clinical trials are investigating the use of CAR T-cells in additional B-cell malignancies.

An early study evaluated the use of tisagenlecleucel CAR T-cells for the treatment of relapsed/refractory CLL [ClinicalTrials.gov identifier: NCT01029366].[71] A total of 14 patients received a variety of lymphodepleting chemotherapy regimens before CAR T-cell infusion. In this cohort, the ORR was 57% with four patients attaining CR and remaining disease-free 40 months post-treatment. At MSKCC, eight patients with relapsed/refractory CLL were treated with or without cyclophosphamide lymphodepletion prior to CAR T-cells.[80] Patients who did not receive lymphodepletion had no clinical response, whereas those who did achieved PR or stable disease for several months. In a similar trial at the NCI, eight patients were treated with CAR T-cells, resulting in an ORR of 87% with four patients having a durable CR for up to 23 months.[18,81]

A follow up phase I study at MSKCC further investigated the efficacy and safety of CD19 CAR T-cells in CLL patients [ClinicalTrials.gov identifier: NCT00466531].[82] Almost all patients (13/16) received prior lymphodepletion with cyclophosphamide and 5 received ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor, at the time of T-cell collection or infusion. Among evaluable patients who received lymphodepletion 3/12 attained CR. Two of these successfully treated patients received ibrutinib at the time of leukapheresis and infusion and remained disease free at a median follow up of 53 months. There was a low incidence of toxicity, with 10% of patients experiencing severe CRS or neurotoxicity. Whereas response rates across different studies in patients CLL appear to be lower when compared with ALL and DLBCL,[73,80,83] those CRs that occur are commonly durable. Consequently, efforts to increase CR rates are of clear importance. In an effort to achieve this, ibrutinib has recently been combined with CAR T-cell therapy leading to improved efficacy.[84–86] These studies and others have shown the potential for CAR T-cell therapy to produce complete responses in CLL. However, further optimizing CAR T-cells or combination therapies are required to improve efficacy in other B-cell malignancies.

Targeting other B-cell markers

Targeting of B-cell maturation antigen (BCMA) using CAR T-cells for the treatment of multiple myeloma (MM) has emerged as promising new therapy since initial assessments.[87,88] The Bluebird Bio/Celegene product, bb2121, is a second-generation CAR that employs a 4-1BB costimulatory domain. Results from a phase I trial were recently reported [ClinicalTrials.gov identifier: NCT02658929].[89] In the cohort, 33 patients received three doses of fludarabine and cyclophosphamide and then a single infusion of CAR T-cells. In this study the ORR was 85% with 45% of patients achieving CR; however, 40% of patients who had a CR did relapse. The median PFS was 11.8 months, therefore a longer follow up of patients is needed to determine the durability of response. CAR-associated toxicities occurred in the majority of patients, with 76% of patients experiencing CRS and 46% exhibiting neurotoxicity.

Similar to bb2121, another BCMA-targeting CAR has been evaluated for safety and efficacy in China [ClinicalTrials.gov identifier: NCT03090659].[90] However, the LCAR-B38M is a dual epitope-binding CAR targeted against two distinct BCMA epitopes. In a multi-centre phase I study, cyclophosphamide alone was used as the lymphodepleting therapy followed by three split doses of CAR T-cells. In total, 57 patients were treated, and the ORR was 88% and CR was 68% at a median follow up of 8 months. In comparison with bb2121, almost all patients experienced CRS (90%); however, neurotoxicity was observed in only one patient.

BCMA expression has been identified on lymphoma cell lines and primary lymphomas.[91,92] Pre-clinical data has shown bb2121 CAR T-cells successfully eliminated tumours in mice bearing B-NHL lymphoma xenografts despite low BCMA expression.[92,93] This data supports initiation of

clinical trials of BCMA targeted CARs for the treatment of B-cell malignancies, particularly in patients relapsing with CD19-negative disease. Such trials have already begun in China [ClinicalTrials.gov identifier: NCT02954445] in patients with lymphoma, leukaemia and MM.

A CAR targeting the κ light chain was developed and a phase I clinical trial conducted with patients with relapsed/refractory NHL, CLL or MM.[94] By taking advantage of B-cell light Ig chain restriction, normal B-cells expressing the reciprocal λ light chain will be spared, thus avoiding hypogammaglobulinemia. No CAR-associated toxicities were observed, and two patients with NHL and CLL entered CR. Four patients with MM had stable disease. These trials targeting other B-cell antigens show promising efficacy and the potential for CAR T-cell therapy to successfully treat other malignancies. Lessons learned from anti-CD19 clinical trials will help to progress CAR T-cell therapy beyond B-cell malignancies.

Conclusion and future outlook

CAR T-cell therapies have transformed the treatment of patients with B-cell malignancies and are likely to become the new standard of care for refractory/relapsed patients. FDA and EMA approvals for tisagenlecleucel and axi-cel were achieved as a result of rapid pre-clinical and clinical development of these products, and it is anticipated that other B-cell specific CAR T-cell products will soon be approved. Nevertheless, many issues remain to be resolved, including high cost, complexity of manufacture and clinical delivery, toxicity and relapse with both $CD19^+$ and $CD19^-$ disease. Given the remarkable pace at which CAR T-cell immunotherapy of B-cell malignancy has developed in the past decade, rapid development and implementation of novel solutions are to be expected.

Authors' note

The views expressed are those of the authors and not necessarily those of the National Health Service (NHS), the National Institute for Health Research (NIHR) or the Department of Health.

Conflict of interest statement

JM is chief scientific officer of Leucid Bio. The other authors have no conflict of interest to declare.

Ethical statement

Our study did not require an ethical board approval because it is a review article with no human or animal work.

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ORCID iD

Leena Halim https://orcid.org/0000-0002-4565-2673

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