Title:

CD19/CD22 targeting with co-transduced CAR T-cells to prevent antigen negative relapse after CAR T-cell therapy of B-ALL

Running Title:

CD19/CD22 CAR T-cells for therapy of B-ALL

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ABSTRACT

CD19-negative relapse is a leading cause of treatment failure after Chimeric antigen receptor (CAR) T-cell therapy for ALL. We investigated a CAR T-cell product targeting CD19 and CD22 generated by lentiviral co-transduction with vectors encoding our previously-described fast-off rate CD19CAR (AUTO1) combined with a novel CD22CAR capable of effective signalling at low antigen density. Twelve patients with advanced B-ALL were treated (CARPALL study, NCT02443831), a third of whom had failed prior licensed CAR therapy. Toxicity was similar to that of AUTO1 alone, with no cases of severe cytokine release syndrome. Ten of 12 patients (83%) achieved a Measurable Residual Disease (MRD) negative complete remission at 2 months post infusion. Of 10 responding patients, 5 had emergence of MRD (2) or relapse (3) with CD19 and CD22 expressing disease associated with loss of CAR T-cell persistence. With a median follow-up of 8.7 months there were no cases of relapse due to antigen-negative escape. Overall survival was 75% (95%CI: 41-91%) at 6 and 12 months. Six and 12-month event free survival (EFS) were 75% (95%CI: 41-91%) and 60% (95%CI: 23-84%). These data suggest dual targeting with co-transduction may prevent antigen negative relapse after CAR T-cell therapy.

INTRODUCTION

CD19CAR T-cell therapy has transformed relapsed/refractory (R/R) B-cell acute lymphoblastic leukemia (ALL) outcomes. However, event-free survival (EFS) is 40-50% [1–4] and antigen loss is a key cause of treatment failure (36-68% of cases) [1, 3, 4]. For example, AUTO1, a fast off-rate CD19 CAR T-cell therapy, previously demonstrated therapeutic efficacy, favorable safety, and excellent persistence[5] but 5/14 treated patients relapsed with CD19-negative leukemia. Dual antigen targeting of both CD19 and CD22 represents a logical approach to preventing this. A

variety of dual targeting approaches have been tested but to date none has improved on ELIANA outcomes or entirely eradicated antigen-negative relapse.

Relapses post CD22CAR infusion are associated with CD22 down-regulation[6, 7]. We developed a highly sensitive CD22CAR responding to low CD22 levels (250 molecules/cell[8]). We incorporated this into a novel CAR T-cell product generated by co-transduction of T-cells with separate lentiviral vectors encoding the CD19 and CD22 CARs, resulting in a product containing single and dual transduced populations. Unlike other dual CAR formats[9], our CD19/CD22CAR T-cells effectively targeted CD19-negative NALM6 leukemia demonstrating the efficacy of the CD22 component. We have now tested these CD19/22 co-transduced CAR T-cells in children with with R/R ALL in a phase 1/2 study.

METHODS

The CARPALL study (NCT02443831) was a UCL-sponsored academic multi-centre, single arm, open label phase I study. Details of CAR T-cell manufacture, study design, and analyses are in Supplementary Material. Patients (age $\leq$24 years) with high risk, relapsed $CD19^{+}$ and/or $CD22^{+}$ haematological malignancies were eligible. All enrolled had B-ALL. Patients received a single dose of 10^{6} CAR+ve T-cells/kg following lymphodepletion with fludarabine/cyclophosphamide. Primary endpoints were incidence of grade 3-5 toxicity causally associated with CAR T-cells and proportion of patients achieving a molecular MRD-negative bone marrow remission with complete response of disease at any relevant extramedullary sites, (assessed radiologically or by evaluation of the CSF). OS was the time from infusion to time of death. Patients were censored on day last seen alive. EFS was defined as in the ELIANA study: events included no response, morphological relapse after having complete remission with or without incomplete hematologic recovery (CR/CRi) or death,

whichever occurred first. Patients were censored if they received further therapy or at the date last seen alive. EFS was also more stringently defined where emergence of MRD and need for further therapy were included as events. Clinical data were analysed in STATA 17.0 with time-to-event outcomes per Kaplan-Meier analysis. Toxicity was reported using maximum grade experienced with cytokine release syndrome (CRS) and neurotoxicity due to Immune Effector Cell-related Cytotoxicity Syndrome (ICANS) graded as per American Society for Transplantation and Cellular Therapy (ASTCT).[10]

RESULTS AND DISCUSSION

All 13 screened patients were enrolled and a CART-cell product generated (Supplementary Figure 1). One patient withdrew before lymphodepletion (uncontrolled adenoviraemia). Median transduction efficiency, based on expression of either or both CARs was 83.2% (range 60.8-92.6%). Products showed a predominance of central memory (Tcm median 91.5%, range 50.3-95.5%, Tn/scm median 0.5%, range 0.06-1.3%, Supplementary Figure 2a). The majority of CAR T-cells were CD19/22 dual transduced T-cells (median 54.4%, range 14.1- 70.0%) with lower, balanced populations of CD19 (median 13.1%) and CD22 (median 11.6%) single-positive CAR T-cells (Supplementary Figure 2b).

Median patient age was 12 years (range 3.7-20.5). This was a heavily pre-treated cohort with a median of 3 prior therapies (range 2-6). Half had relapsed post allogeneic SCT, 4 post tisagenlecleucel. Three patients had CD19-negative disease, one of whom had an additional 5% CD22-negative population (Table 1). All patients were ineligible for tisagenlecleucel.

Supplementary table 1 and 2 detail toxicities. Eleven of 12 patients developed cytokine release syndrome (CRS, grade 1 n=5, grade 2 n=6), with 5 receiving tocilizumab. No severe CRS (≥grade 3) or CRS-related ICU management occurred. Cytokine profiles are in

Supplementary Figure 3. Grade 1-2 ICANS occurred in 5 patients. One developed grade 4 neurotoxicity/ICANs 6 weeks post-infusion, resembling fludarabine-related leukoencephalopathy although ICANS could not be excluded. Prolonged cytopenia was noted in 10/12 patients, with one needing a CD34+ donor stem cell infusion but only 4 instances of grade 4 infection were seen. No toxicity-related deaths or haemophagocytic lymphohistiocytosis were noted, unlike other CD22 CAR studies[11]. This may relate to the generally mild CRS manifestations and limited cytokine disturbance found with this product. No increased toxicity from dual-targeting was evident.

Figure 1 summarizes outcomes. One month post CAR T-cell infusion, 10/12 (83%) patients achieved CR/CRi (including 3 in continuing CR/CRi). By the second month, all responders were MRD negative. Two of 3 patients with prior CD19-negative disease achieved MRD negative CR/CRi, validating CD22 CAR T-cell efficacy. Two patients failed to respond, one with CD19+/CD22+ disease and another with progression of double CD19-/CD22- disease present as a minor population pre-infusion. Both succumbed to disease.

Of 10 patients achieving MRD-negative CR/CRi, 3 relapsed with CD19+/CD22+ disease. In 2 cases, emerging MRD (CD19+CD22+) prompted further therapy (allo-SCT n=1, maintenance chemotherapy n=1), both achieving subsequent molecular CR. In all 5 patients with recurrent disease this was CD19+CD22+ and this was associated with loss of CAR T-cell persistence in 4/5 cases. Two further patients received additional therapy for early CAR T-cell persistence loss (allo-SCT n=1, maintenance chemotherapy n=1) whilst in molecular CR (Figure 1a). Crucially, with a median follow up of 8.7 months, there have been no cases of leukemic relapse in responding patients due to antigenic escape, although leukaemic relapse without antigen modification was seen. Whilst it is possible this may occur with longer follow up, it is noteworthy that in cohort 1 the longest interval to CD19 negative relapse was 7 months. This

suggests dual targeting may have prevented antigen-negative relapse as this contrasts with our prior experience with CD19 CAR T-cells alone, where 5/14 patients relapsed with CD19-negative disease within 7 months post-infusion, as well as with other dual CAR studies, either due to suboptimal CD22 CAR function[9, 12], or poor persistence[13, 14]

At 8.7-month median follow-up (95%CI: 3.9 to 12.2), 5/10 responders are alive and disease-free. Six- and 12-month OS was 75% (95%CI: 41-91%) (Figure 1c); EFS was 75% (95%CI: 41-91%) and 60% (95%CI: 17-84%), respectively (Figure 1d). Despite a high-risk cohort (including patients failing prior CD19 CAR, having CD19-negative disease, non-CNS extramedullary disease, and prior blinatumomab recipients, all factors associated with poor CAR T-cell outcomes) [15], our study's 12-month OS and EFS were comparable to the ELIANA study. Six- and 12-month stringent EFS (including further therapy for MRD emergence or further therapy for early CAR T-cell loss) were 75% (95%CI: 41-91%) and 38% (95%CI: 9-67%) (Figure 1e). Median remission duration in responders was 9.9 months.

Rapid CAR T-cell expansion was noted, peaking 14 days post-infusion. Median time to loss of single CD19 and double CD19/22 CAR T-cells by flow cytometry was 5 months and for CD22 CART-cells, 7 months (Supplementary Figures 4 and 5). We observed balanced expansion of CD19 single positive, CD22 single positive, and double positive CAR T-cell populations, contrasting studies where one CAR T-cell population dominated post-infusion[16]. Pharmacokinetics using qPCR (Supplementary Figure 6, Supplementary Table 3) confirmed excellent cumulative CAR T-cell exposure in the first 28 days (AUC0-28 CD19CAR: 9,492,498 copies/ug DNA; CD22CAR: 2,586,767 copies/ug DNA), higher than that noted with AUTO1 CD19CAR T-cells alone[5]. These data are encouraging since studies using a tandem CAR with binding sites for both CD19 and CD22 have been limited by suboptimal signalling in response to CD22[9, 12]. Another approach to overcome suboptimal T-cell signalling due to complex CAR

design is the delivery of CAR T-cell cocktails or sequential CD19 and CD22CAR T-cells, though here there are regulatory challenges in delivering multiple CAR T-cell products. In a recent multicentre study, 192/225 paediatric patients achieved an MRD negative CR. 17/43 relapsing patients had antigen-negative relapse and again persistence of CD22CAR T-cells was suboptimal. 78 had consolidative stem cell transplant (SCT), potentially confounding impact of dual targeting[17].

CAR T-cell persistence is a pre-requisite to assess dual-targeting and this has been a major limitation of studies to date. In our previous study with a bicistronic vector, short CAR T-cell persistence led to a high rate of CD19/22+ve relapses[18]. Within the cohort presented here receiving AUTO1/22, CD19 CAR T-cells were detectable by qPCR at last follow-up in 7/12 and CD22CAR T-cells in 5/12 patients. Seven of 12 patients experienced ongoing B cell aplasia; median duration of B cell aplasia was not reached. The median duration of CAR T-cell persistence by qPCR in the blood (CD19CAR T-cells 135 days, CD22CAR T-cells 105 days) was similar to tisagenlecleucel (102 days) in ELIANA and ENSIGN studies[19]. This is the first study we are aware of in which antigen-negative relapse was not observed and sufficient expansion and persistence of CAR T-cell populations occurred to allow full assessment of a dual-targeting approach.

We acknowledge a risk factor for CD19 negative relapse in patients treated with tisagenlecleucel[4] includes high disease burden and that the cohort presented here generally had a low bone marrow disease burden (Table 1). However, the lack of CD19 negative relapses in this report contrasts sharply with our prior experience of our single CD19CAR T-cell product alone (AUTO1) in patients with a similarly low disease burden in which 5/6 relapses were with CD19 negative disease.

Our data suggests co-transduced CD19/22 targeting CAR T-cells are well tolerated and highly effective in advanced ALL including in those failing prior tisagenlecleucel. Whilst acknowledging that the small size of this study may lead to sampling bias, to date we have observed no cases of relapse in a responding patient due to antigen modulation, suggesting that our dual CAR product may represent a promising approach to prevent this form of leukemic relapse. Ultimately, we noted shorter persistence overall with our dual CAR product compared to that noted with our CD19 CAR T product (AUTO1) alone and 5/10 cases of relapse or MRD emergence without antigen modification. The median VCN in the dual CAR products was greater than that seen with AUTO1 (median vector copy number 5.5 (range: 3.39 – 8.00) *vs* 4 (range;1.2-8.0), thus it is possible that higher per cell CAR expression, particularly of the dual CAR population may have contributed to activation induced cell death (AICD) or exhaustion. We are currently investigating manufacturing methods to support longer persistence to fully realise the potential of dual targeting CAR T-cell therapy.

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AUTHOR CONTRIBUTIONS

SG, GL contributed to study design, analyzed data, provided medical care, contributed to data collection for study patients and wrote the manuscript. SA and EG carried out CAR T-cell persistence analysis by qPCR and disease endpoint assessment by molecular PCR. RR, KN, CT, AG carried out clinical study assays, performed manufacturing scale ups, manufactured products, analysed data. BP, AL, SK, YN wrote study documentation and provided trial management. JY, EK designed and performed and analysed pre-clinical experimental work. DP, JC, KK, CW, KW coordinated patient care and were responsible for data collection. SG, GL, MBC, KM, AL, JS, VP, JB, AR, KR, provided medical care and contributed to data collection for study patients. SI, RT, CC provided flow cytometry assessment of disease status. KG provided manufacturing and clinical assay expertise. AL and AH contributed to study design, provided statistical analyses and wrote the manuscript. XX manufactured lentiviral vector and contributed to study documentation. VP, JB, AR AV, DB contributed to study design, identified study patients and provided expertise in medical care for study patients. RH, RW were Principal investigators for the study and provided medical care for study patients. MAP led pre-clinical experimental work on development of the CD22CAR. PJA led experimental work, provided medical care, analysed data, wrote the manuscript and was Chief Investigator for the study.

Declaration of interests

SG, MAP and PJA have patent rights for CAT CAR in targeting CD19 (patent application, World Intellectual Property Organization, WO 2016/139487 Al) and may receive royalties from Autolus PLC who have licensed the IP and know-how from the CARPALL study. EK owns shares

in Autolus. PJA has received research funding from Bluebird Bio Inc. MAP is a shareholder in and employee of Autolus PLC, which has licensed CAT CAR.

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TABLES

#1	2nd relapse	No	No	Yes	Yes/yes	6	0.12% 1.3 x 10e-3	+/+
#2	2nd relapse	CNS	Yes	No	Yes/no	5	0.39% 1 x 10e-2	+/+
#3	3rd relapse	No	Yes	Yes	No/no	6	ND/ 6x10e-2	+/+
#4	2nd relapse	CNS	Yes	No	No/no	3	0.069% 7x 10e-4	+/+
#5	2nd relapse	CNS	Yes	No	No/no	3	Negative/Negative	+/+
#6	2nd relapse	No	Yes	Yes	Yes/no	4	85%	+/+
#7	2nd relapse	Adenopathy/ pelvic mass	No	No	Yes/yes	3	12% 2.8x10e-1	10% Blasts CD19 neg/+
#8	1st relapse	No	No	No	No/no	2	2.3% /ND	-/+
#9	2nd relapse	No	No	Yes	No/no	4	18.6%	100% bBasts CD19 neg/5% blasts CD22 neg
#10	2nd relapse	Chest wall	Yes	No	Yes/no	3	Negative/Negative	+/+
#11	1st relapse	CNS and spine	No	No	Yes/no	3	7.4%	+/+
#12	1st relapse	CNS	No	No	No/no	2	Negative/Negative	+/+

Table 1. Patients' characteristics. CNS indicates central nervous system; EM, extramedullary; Mol, molecular; MRD, minimal residual disease; ND, not determined; SCT, stem cell transplantation

FIGURE LEGENDS

Figure 1. Outcomes
1a Swimmer plot representing post infusion course for each of the enrolled pts. **b** summary of response and relapses. Kaplan Meyer curves for **c** 12 months overall survival (OS) with 12 pts at risk and 3 events, **d** 12 months event free survival (EFS) with event being non-response, morphological relapse or death, 12 pts at risk and 4 events and **e** 12 months "stringent event free survival" with events being non-response, morphological relapse or emergence of MRD level disease, death and need for further therapy, with 12 pts at risk and 7 events.