A novel low affinity CD19CAR results in enhanced expansion and prolonged CAR T cell persistence in pediatric ALL

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Abstract

We generated a novel CD19CAR (CAT) with a lower affinity than FMC63, the binder utilised in many clinical studies. CAT CAR T cells showed increased proliferation/cytotoxicity *in vitro* and enhanced proliferative capacity and anti-tumor activity than FMC63 CAR T cells in a xenograft model. In a clinical study (CARPALL, NCT02443831), 12/14 patients with relapsed/refractory pediatric B-ALL obtained molecular remission after CAT CAR T cell therapy. CAR T cell expansion compared favourably with published data on other CD19CARs and persistence was demonstrated in 11 of 14 patients at last follow-up. Toxicity was low with no severe cytokine release syndrome. At a median follow up of 14 months, 5/14 patients (37%) remain in molecular CR with circulating CAR T cells.

Introduction

T cells engineered to express chimeric antigen receptors (CARs) targeting CD19 have shown unparalleled responses in relapsed/refractory acute lymphoblastic leukemia (ALL), leading to FDA approval. Nonetheless, significant challenges remain. Toxicity is significant with a 25-45% incidence of severe (≥grade 3) Cytokine Release Syndrome (CRS))[1–5] and variable rates of severe neurotoxicity[1–5] and variable rates of severe neurotoxicity)[1–5] and variable rates of severe neurotoxicity[1–5] which limit broader application of CAR T cell therapy in earlier stage disease. Moreover, while 70-90% of patients with ALL respond, 40-60% ultimately relapse due to poor CAR T cell persistence or emergence of $CD19^-$ clones. The optimal CAR design has yet to be determined. A number of factors, including the length of both the spacer between heavy and light chains of the single chain Fv[6], the extracellular spacer[7] and choice of costimulatory domain[4,5,8] have a profound effect on CAR T cell function and persistence. However, little is known about the impact of the affinity of CAR binding.

To date, clinical studies of CD19CAR T cells have utilized CARs with high affinity binders such as FMC63. However, studies of both TCR and CAR T cells suggest that an affinity ceiling exists above which increased affinity does not augment and may adversely affect T cell responses[9–11]. We developed a CD19CAR with lower affinity binding to CD19 resulting in a shorter half-life of CAR-CD19 interaction and have studied this novel CAR *in vitro*, in a xenogeneic mouse model and in a clinical study.

Results

CAT19 CAR.

We derived a CD19 specific scFv (CAT) from the CAT131E10 hybridoma. In Surface Plasmon Resonance (SPR) analysis (Extended Data Figure 1), a CAT derived scFv-Fc showed

substantially (>40 fold) lower affinity than scFv-Fcs derived from FMC63[12,13]. The higher KD with CAT (14 nM) was the result of a much faster off-rate (CAT $3.1x10^{-3}$ s^{-1}, FMC 6.8×10^{-5} s^{-1}), whereas, the on-rate was equivalent (CAT 2.2×10^5, FMC 2.1×10^5).

To determine key CD19 residues required for CAT and FMC63 binding, the 3 loops identified in the crystal structure[14] were mutated and tested for loss of binding by flow cytometry (Extended Data Figure 2A and B). Both antibodies shared important residues within loops 1 (AA 97-107) and 2 (AA 155-166). Specifically, CAT and FMC63 shared key binding residues L96, C97, W159 and R163, while CAT binding was also affected by residues Y157, K161 and D162. Taken together, these data suggest that FMC63 and CAT bind to the same or overlapping epitopes on CD19.

Thermal stability of CAT19 and FMC63 scFv-Fcs, assessed by differential scanning fluorimetry melting temperature (Tm) analysis, was similar (55.1°C and 57.7°C, Extended Data Figure 2C). To compare cell surface stability, both scFvs with a V5tag were cloned into the 4-1BBz CAR format[12]and co-expressed with mCherry in a bicistronic lentiviral vector (Extended Data Figure 2D). CAR expression could then be detected independent of CAR-antigen affinity through detection of the V5 tag, and compared against the transduction efficiency as assessed by mCherry. The relative fluorescent intensity of V5 staining on mCherry+, transduced T cells was identical for both CARs (Extended Data Figure 2D). Thus the stability of FMC63 and CAT appear similar.

T cells transduced with a low affinity CD19CAR demonstrate enhanced cytotoxicity and proliferative responses

We then compared the function of T cells lentivirally transduced with FMC63 and CAT binders in an identical 2nd generation CAR format with a CD8 derived stalk/transmembrane

region, a 4-1BB co-stimulatory domain and CD3ζ. To control for transduction efficiency, mCherry fluorescent protein was co-expressed using a 2A-peptide (Supplementary Figure 1A, B and C).

The cytotoxicity of CAT CAR T cells against a CD19 expressing cell line (SupT1CD19) was significantly greater than FMC63 CAR T cells (Figure 1A). To determine the activity of CAT CAR T cells against target cells expressing CD19 at physiological or low levels, flow based killing assays were performed against NALM-6 cells and SUPT1 cells engineered to express low levels of CD19 (Supplemental Figure 2A-C). CAT CAR and FMC63 CAR T cells showed equivalent cytotoxicity against low density CD19 expressing targets.

We next compared proliferative responses of CAT and FMC63 CAR T cells following stimulation with $CD19^+$ targets. As demonstrated in Figure 1B, CAT CAR T cells showed significantly greater antigen-specific proliferation than T cells transduced with the FMC63 CAR (mean cpm ± SEM: Raji: CAT 63158±7159, FMC 27582±2776 *n=4*, *p<0.01*; NALM-6: CAT 49237±14006, FMC 13097±4047 *n=4*, *p<0.05*). Cytokine production by CAT and FMC CAR T cells in response to stimulation with $CD19^+$ targets was similar except that CAT CAR T cells secreted significantly more TNF-α than FMC63 CAR T cells (mean CAT 750.7 ± 103.3 pg/ml, FMC : 292.1 ± 36.51 pg/ml, *n=4, p<0.01*).

Improved in vivo efficacy of low affinity CD19-CAR^+ T cells against ALL in a xenograft model

Next, we tested FMC63 and CAT CARs in a NALM-6 tumor model in immunodeficient NOD/SCID/$\gamma^{-/-}$(NSG) mice (Figure 2 and Extended Data Figure 3A). Control mice receiving non-transduced T cells showed rapid, disseminated tumor infiltration. FMC63 CAR T cells slowed but did not prevent tumor growth. In contrast, equivalent numbers of CAT CAR T cells resulted in tumor regression. On day 12 post T cell injection substantial differences

were seen in tumor burden (CAT CAR: $1.1x10^8 \pm 9.3x10^7$, FMC63 CAR $3.2x10^9 \pm 7.7x10^8$, mean photons/sec/cm^2, n=18, p<0.001).

Two weeks after CAR T cell infusion, blood and bone marrow (BM) were analyzed for residual tumor and persisting CAR T cells. A higher number of NALM-6 tumor cells were observed in the BM (Mean NALM-6 cells/ml: $3x10^2$ in CAT, $2.8x10^5$ in FMC63 cohort, *n=9 p<0.001*; Figure 2C) and blood (Mean: CAT 13.2, FMC63 594.5, *n=9, p<0.001*; Extended Data Figure 3B) of recipients of FMC63 CAR T cells. Conversely, a significantly greater absolute number of CAT CAR T cells were seen in BM compared to FMC63 CAR T cells (mean CAR T cells/ml: $5.1x10^4$ CAT CAR; $2.0x10^4$ FMC63 CAR, *n=9*, *p<0.05*; Figure 2D) and blood (Mean: CAT 18743, FMC63 2843, *n=9*, *p<0.001*; Figure 2E).

Expression of exhaustion markers LAG3, PD-1 and TIM3 on CAR+ T cells was similar on CAT or FMC63 CAR T cells (Extended Data Figure 3C). Intracellular staining of Th1-like cytokines revealed greater expression of TNF-α in CAT transduced T cells, reflecting our *in vitro* findings (Figure 2F). CAR T cells from the BM and blood showed significantly higher levels of CD127 (IL7-Rα) and intracytoplasmic expression of the anti-apoptotic molecule Bcl-2 (Figures 2G and H; Extended Data Figure 3D and E) in CAT CAR treated mice. Together, these results indicate that, under conditions designed to give CAR T cells a numeric disadvantage, lower affinity CAR T cells mediate enhanced anti-tumor responses and expansion compared to high affinity CAR T cells.

Clinical Trial

Based on these data, we implemented a clinical study utilizing the CAT CAR in patients age <25 with high risk CD19$^+$ ALL (CARPALL). Eligibility criteria are outlined in Supplementary Table 1. Seventeen patients were enrolled and 14 received an infusion of CAR T cells

(Supplementary Figure 3). Patients were followed up to a data cut-off of 19/12/2018 with a median follow up of 14.4 months.

Patient characteristics

These are summarized in Extended Data Tables 1 and 2. The median age was 9 years and all patients had advanced ALL with a median of 4 lines of prior treatment. Ten of 14 patients had relapsed post allogeneic SCT. Eight patients were treated in 2nd relapse, 5 in > 2nd relapse and 3 had relapsed after prior Blinatumomab or Inotuzumab. Two patients had ongoing CNS disease at registration. Four patients were in morphological relapse (17-81% blasts) pre-lymphodepletion, 6 had MRD level disease and 4 were MRD negative in the BM. MRD negative patients had isolated CNS relapse post TBI conditioned SCT (*n=3*)/cranial irradiation (*n=1*) and were not salvageable with standard therapies.

CAR T cell product and lymphodepletion

We were able to generate a product in 14 of 17 patients (82%). Twelve of 14 patients received the target dose of 10^6/kg, 2 received 0.73-0.78 x10^6/kg (Extended Data Figure 4B). CAR T cells showed a predominantly central memory or naïve/stem cell memory phenotype with a low level of dual expression of PD-1 and TIM-3 (Extended Data Figure 4A). All patients received lymphodepletion with fludarabine 150mg/m^2 and cyclophosphamide 1.5g/m^2 except CPL-05 in whom a reduced dose fludarabine (90mg/m^2) was used because of prior leukoencephalopathy.

Toxicity

This is summarized in Table 1, Extended Data Tables 2-3 and Supplementary Table 2.

Cytokine release syndrome (CRS)

Thirteen of 14 patients (93%) developed CRS (graded by Lee *et al.*[15] criteria, Table 1, Extended Data Table 2) at a median of 7 days from CAR T cell infusion (range 1-11). CRS was generally mild (grade 1 *n=9* and grade 2 *n=4*) and lasted a median of 5 days. No grade 3 or 4 CRS occurred and no patient required Tocilizumab therapy or admission to Intensive Care. Using the UPenn scale, 3 patients had grade 3 by virtue of hypotension requiring fluid boluses/oxygen requirement < 40% (Extended Data Table 2). Commensurate with the absence of severe CRS and in contrast to reported data with FMC63 CAR T cells, we saw only modest increases of pro-inflammatory cytokines IFN-γ, IL-6 as well as IL-10, as measured by cytometric bead array in the blood of a minority of patients and most patients showed no elevation of these cytokines (Extended Data Figure 5A). IL-2, IL-4 and TNFα levels were not raised in any patient. CRP levels were generally low except in patients with concomitant infection. We retrospectively cross-validated these results using a more sensitive 30-plex cytokine panel[16]. Using this methodology, we demonstrated modest increases in IFN-γ, IL6, IL8, sIL2Rα in patients who had more significant CRS manifestations (eg CPL-01,-02 and -05) (Extended Data Figure 5, Supplementary Table 3).

Neurotoxicity

Neurological side effects occurred in 7 patients and were generally mild. Six patients experienced CTCAE grade 1-2 neurotoxicity (Table 1, Extended Data Table 2, Supplementary Table 2) including dysarthria, paresthesiae and somnolence. Grade 4 encephalopathy occurred in 1 patient (CPL-12),37 days post CAR T cells and the timing of leukoencephalopathy, the absence of significant prior CRS and the presence of white matter changes on MRI were more consistent with fludarabine than CAR-associated neurotoxicity.

Cytopenias

Cytopenias were common (Table 1, Supplementary Table 2), reflecting the heavy pre-treatment of this cohort and lymphodepletion. Cytopenias persisting beyond 28 days, or new cytopenias developing after initial count recovery in the absence of relapse were analyzed separately as these might relate to CAR T cell therapy. Ten patients had grade 3-4 cytopenia (particularly neutropenia) persisting beyond day 28 or recurring after this. Of these, 6 patients had grade 3 -4 neutropenia (42.9%, 95% CI 17.7% to 71.1%) and 3 patients (21.4%, 95% CI 4.7% to 50.8%) had grade 3 -4 thrombocytopenia persisting beyond day 28. One patient with prolonged neutropenia and multiple infections including refractory HSV as well as grade 4 encephalopathy ultimately died in remission from sepsis. Another patient with a fungal chest infection prior to CAR T cell therapy developed a grade 4 fungal chest infection during prolonged neutropenia. There were 2 other grade 3 infections associated with prolonged neutropenia.

B cell aplasia

A correlate of CD19CAR T cell persistence is B cell aplasia which occurred in 13/14 patients. The median duration of B cell aplasia was 7.6 months (0.9-23.9) months and 12/14 patients had B cell aplasia at last follow-up (Figure 4E, Extended Data Table 2). Hypogammaglobulinemia (IgG<3g/L) was noted in 11 patients and warranted immunoglobulin replacement in 6.

Efficacy

At 30 days post CAR T cell infusion, 10 out of 13 evaluable patients (77%) were in molecular complete remission (CR) or continuing CR assessed by PCR for leukemia-specific IgH gene rearrangements (Figure 3). Two patients had stable disease and subsequently progressed with $CD19^{+}$ disease and in 1 patient insufficient DNA was obtained for analysis. By month 3,

12/14 patients (86%) had achieved molecular CR. On an intent-to-treat basis including the 3 patients who did not receive CAR T cells, the overall molecular remission rate was 12/17 (71%).

Among those achieving CR, 6 subsequently relapsed, 5 with $CD19^-$ disease, 1 with $CD19^+$ disease. Next generation sequencing of BM DNA from patients with $CD19^-$ relapse showed mutations in the CD19 gene predicted to result in loss of surface expression in 4/5 patients (Extended Data Table 4). These mutations were not detectable prior to CAR T cell therapy.

With a median follow-up of 14 months, 5/14 patients (36%) are alive and disease-free. Overall survival was 84% at 6 months and 63% at 12 months and event free survival assessed by the criteria used in the ELIANA study (defined as the time from CAR T cells to the following events: no response or, morphological relapse after having CR/CRi) was 67% and 46% (Figure 3). Using more stringent criteria where an event is defined as molecular relapse, molecular EFS was 55% and 31%. The median duration of morphological remission in responding patients was not reached and of molecular remission was 7.4 months.

CAR T cell expansion and persistence

CAR T cell expansion/persistence were assessed in the peripheral blood (PB) on days 0, 2, 7, 14, 28, monthly up to 6 months, 6 weekly to 1 year then 3 monthly up to 2 years post infusion by transgene-specific qPCR and flow cytometry using an anti-idiotype antibody (Figure 4). Bone marrow (BM) was assessed monthly for the first 6 months and then at the same intervals as for blood (Extended Figure 6). Robust PB CAR T cell expansion (Cmax >50,000 copies/µg DNA) was seen in 12/14 (86%) patients with a median time to maximal expansion of 14 days (Table 2). The mean peak concentration of CAR T cells in PB (Cmax) was 128,911 copies/µg DNA and the mean area under the concentration-time curve in the

peripheral blood within the first 28 days post infusion (AUC 0 to 28) in all 14 treated patients was 1,721,355 copies/µg DNA (Table 2). Similar levels of CAR T cells were noted in the BM (Extended Figure 6A and B). This marked expansion of CAR T cells was also documented by flow cytometry. At the point of maximal expansion, a median of 41% of circulating T cells were CAR^+ (Figure 4A and B, *n=10* evaluable). Following this, the proportion of CAR T cells contracted. Nevertheless, CAR T cells continued to be detectable by qPCR in 11 of 14 (79%) patients at last follow up (up to 24 months post infusion in 2 patients) and by flow cytometry in 8 of 14 (57%). The median duration of persistence of CAR T cells at data cut-off was 215 days (range 14-728) and the median half-life of CAR T cells was 34 days (range 3-102) in 14 evaluable patients (Table 2).

Poor expansion was seen in 2 patients. In CPL-01 age (15 months) and prior intensive therapy for infant ALL may have contributed. In CPL-15, early expansion was seen in the first week but CAR T cells were absent by flow cytometry and qPCR in the blood by 2 weeks. A further patient (CPL-10) showed excellent initial expansion of CAR T cells up to 28 days post infusion but abruptly lost CAR T cells after this. PBMC from CPL-10 and 15 showed cytotoxicity against CAR-expressing autologous targets (Extended Figure 6E), suggesting lack of persistence in these cases was due to cell-mediated rejection of CAR T cells as previously described[2]. No human anti-mouse antibodies were detected.

Discussion

We report a novel, low affinity CD19CAR incorporating a CD19 specific scFv with a faster off-rate than the FMC63 CD19 binder used in many clinical studies[1,3,4,17]. T cells expressing our low affinity CAT CAR showed greater cytotoxic and proliferative responses *in vitro*. These *in vitro* data were supported by enhanced CAR T cell proliferation and anti-leukemic activity

with our low affinity CAT CAR in a xenogeneic model of ALL. Low affinity CARs directed against ErbB2 have previously been reported[18,18] to give equivalent anti-tumor responses to high affinity CARs in *in vivo* models of ovarian cancer. Similarly, in a mouse model of human thyroid carcinoma with CARs derived from LFA-1 the ligand of ICAM-1, CARs with lower affinity had superior anti-tumor efficacy compared to high affinity CARs[19]. The enhanced anti-leukemic effect seen in our model may reflect both the increased cytotoxicity and increased numbers of CAR T cells. The mechanism for increased antigen-induced expansion of CAT CAR T cells is unclear and we are currently studying this further. It is possible that serial triggering due to shorter receptor-ligand interaction may result in enhanced signalling through proliferative pathways. In addition to increased proliferation, improved survival through decreased apoptosis and IL-7 signalling may also play a role. This was evidenced by the greater expression of BCL-2 and CD127 on CAR T cells from mice receiving CAT CAR, though increased CD127 expression may also reflect enhanced tumor clearance and hence loss of ongoing antigenic stimulation of the FMC63 CAR T cells. We did not observe differences in expression of LAG-3, PD-1 and TIM3 between CAT and FMC63 CAR T cells in our model suggesting exhaustion was not contributory.

We then tested our low affinity CAR in pediatric patients with advanced relapsed/ refractory ALL. In this heavily pre-treated cohort, a single dose of CAR T cells resulted in molecular CR in 10/13 (77%, 95% CI 46-95%) at D28 and 12/14 patients (86%) overall. The lower limit of these 95% CI is higher than the rate of <20% molecular CR seen in historical controls treated with chemotherapy. These response rates are comparable to published studies with other 2nd generation CD19CARs in pediatric[1,3,4,17] and adult[2,5,20] ALL. With a median follow-up of 14 months, 5/14 patients (36%) remain in CR/CCR with ongoing persistence of CAR T cells and associated B cell aplasia. Sustained responses were seen in patients with multiple CNS

relapses after both cranial irradiation and SCT, indicating that CART are effective in CNS relapse. Overall/event free survival were 84%/67% at 6 months and 63%/46% at 12 months. These results are comparable to published data[3,21]. In the pivotal ELIANA study of Tisagenlecleucel[4], 81% of patients achieved CR with a 1 year OS of 76% and EFS of 50%. In all these studies a variable proportion of patients were consolidated with SCT whereas in our trial CAR T cells were designed as a stand-alone therapy and none of the patients were transplanted.

Strikingly, in concordance with our *in vitro* and murine data, CAT CAR T cells showed excellent expansion in patients. CAR T cells comprised up to 84% of circulating T cells at maximal expansion. The mean maximal concentration of CAR T cells in the blood (128,912 copies/µg DNA) was 3 x higher and the cumulative exposure to CAR T cells in the first 28 days as assessed by mean AUC (1,721,355 copies/µg DNA) 5 x higher than that reported for Tisagenlecleucel[17,22], despite the fact that these results were calculated on the complete cohort (including non-responding patients in whom expansion was poor). These results are particularly remarkable given that the majority of our patients had a lower tumor burden which has previously been associated with decreased CAR T expansion[2] and because in most cases expansion was seen without significant elevations of pro-inflammatory cytokines, suggesting that expansion of low affinity CAR T cells may be less cytokine dependent. The median half-life of CAT CAR T cells (34 days) was > 2 x that reported for Tisagenlecleucel (14.2 days). CAR T cells were detectable by flow and qPCR in 11/14 patients (79%) at last follow up. Supporting our persistence data, recovery of B cells was seen in only 2 patients. The median persistence of CAR T cells was 215 days which is similar to that reported in the ELIANA study (168 days). Our CAR incorporated a 4-1BB costimulatory domain which may confer prolonged persistence compared to CD28 by preventing T cell exhaustion through

tonic signalling[8,23]. However, the presence of 4-1BB is not sufficient for prolonged CAR T cell persistence[3]. While our pre-clinical data, which utilised the human phosphoglycerate kinase 1 (hPGK1) promoter to drive expression of both FMC63 and CAT CAR T cells suggest an intrinsic difference in antigen-induced proliferation, in our clinical study we used a PGK promoter as this gave better CAR expression across both CD4/CD8 subsets whereas the ELIANA study utilised the elongation factor EF1α promoter. It is therefore possible that differences in CAR expression due to use of the different promoters may have contributed to the observed differences between the studies. Further, our production methodology, which did not utilise cytokines in 13/14 patients, resulted in CAR T cells with a balanced CD4:8 ratio and predominantly naïve/central memory phenotype and this may have contributed to favourable expansion and persistence of CAR T cells on the study. However, it is likely that the higher expression of IL-7R and Bcl-2 in CAT CAR T cells, promoting homeostatic proliferation and preventing apoptosis, may also enhance persistence of CAR T cells. Importantly, CAT CAR T cells and Tisagenlecleucel are the only CD19CAR T cell products which reliably persist long-term, whereas with other CD19CAR T cells persistence has been limited to around 2 months[1,3,20]. This is of central importance in determining whether CAR T cells are used as a stand-alone or bridging therapy to SCT, which can be achieved in a significant proportion of patients with simpler agents such as Blinatumomab/ Inotuzumab.

The safety profile of CAT CAR T cells appears excellent. In our study, no patient developed grade 3-5 CRS using the Lee criteria[15] and no patient required Tocilizumab therapy or admission to intensive care for CRS. These results however need to be interpreted with caution as 10/14 patients in our study had a low leukemic burden which has been associated with a decreased incidence of severe CRS[2,24] and larger studies with patients with

higher leukemic burden will be needed to confirm this finding. Nonetheless the cytokine profile we observed appears different from that reported with high affinity CARs, suggesting there may be intrinsic differences in cytokine secretion with a low affinity CAR which become more apparent in the bone marrow microenvironment than *in vitro*, perhaps reflecting differential activation of downstream signaling pathways or other immune effector cells. Neurotoxicity was seen in 7 patients but was generally mild and self-resolving, as previously described in children. Cytopenias were comparable to Tisagenlecleucel: 6 patients (42.9%) had grade 3/4 neutropenia persisting beyond day 28compared to 53% on the ELIANA study. These mostly self-resolved by 2 months and were only associated with significant infections in 2 patients. The mechanism of late neutropenia is unclear although it is likely to be CAR related.

Six responding patients relapsed. In one of these (CPL-10) as well as 1 non-responding patient (CPL-15), relapse was $CD19^{+}$ and associated with abrupt loss of CAR T cells after robust initial expansion. Specific cytotoxic responses directed against the CAR were detected in these patients suggesting T cell mediated rejection, as previously reported[2]. The major cause of treatment failure was $CD19^{-}$ relapse(5/14 patients) which appeared more frequent in patients with a higher tumor burden. As previously described[25], this was due to outgrowth of clones with a variety of CD19 mutations predicted to lead to a truncated protein not expressed at the cell surface under selective pressure from CAR T cells. Potentially, $CD19^{-}$ relapse could be prevented by targeting 2 antigens simultaneously and we are now evaluating dual targeting of CD19 and CD22 in a further clinical study.

Our work demonstrates that a lower affinity CD19CAR with similar epitope, structure and stability to the FMC63 CAR shows enhanced proliferative capacity/anti-leukemic responses in preclinical studies and greater expansion in patients than reported with Tisagenlecleucel

as well as excellent persistence. Our preclinical data suggest that this may in part be mediated through enhanced IL-7R and Bcl-2 expression rather than prevention of CAR T cell exhaustion though further work is needed to fully determine the mechanisms underlying these differences. More broadly, it illustrates the potential for modulating CAR functionality by changing the affinity of binding to the cognate antigen.

Materials and methods

Study Design

Single chain variable fragments (scFvs) from the FMC63 and CAT13.1E10 hybridomas were generated and their binding to CD19 were assessed. Chimeric antigen receptors in a 4-1BB containing second generation format were generated from the FMC63 and CAT scFvs. A pre-clinical assessment of the in vitro responses of CAT CAR versus FMC63 CAR T cells (cytotoxicity, proliferation and cytokine production) was carried out as well as a comparison of their anti-tumour efficacy within a xenogeneic model of ALL, involving transfer of NALM6 tumour cells to immunodeficient mice. Next, a clinical study of CAT CAR T cells in the treatment of high risk B lineage ALL was implemented in children and young adults.

Cell lines

Raji, K562 and 293T cell lines were obtained from ATCC. SupT1 cells were purchased from ECACC and transduced with an SFG vector to express human CD19 (SupT1-CD19) and single cell selected by flow cytometry to generate a cell line, NALM6 expressing GFP and firefly luciferase were provided by Dr. Hilde Almasbak[26].

CAR, scFv and transfer vector engineering

The DNA sequence for CD19 scFvs was obtained from previous publications[27] or derived through 5' Rapid amplification of cDNA ends (RACE) using primers for the constant regions of the heavy and light chains of hybridoma cells. An scFv was then generated by linking the variable regions of the heavy and light chains together via a $(SGGGGS)_3$ linker. For affinity measurements, competitive binding assays and differential scanning fluorimetry analyses, anti-CD19 scFvs were fused to mouse IgG2a-Fc and generated in a secreted format using an SFG-eBFP **γ**-retroviral vector to transfect Human Embryonic Kidney (HEK) 293T cells. Supernatant containing the secreted scFvs was then purified on a protein A column.

For comparisons of CD19CARs in human T cells, scFvs were cloned into the 4-1BBz CAR format[12] and co-expressed in a bicistronic lentiviral vector with the mCherry fluorescent protein via an in-frame 2A ribosomal skipping sequence.[28] Transgene expression was driven by the hPGK1 promoter in all cases. To compare stability of CAR expression, CD19-41BBZ CARs were tagged with a V5 tag (Supplementary figure 2D). However, for all other pre-clinical comparisons, an untagged version of the CD19-41BBZ CAR was used (vector schematic shown in Supplementary Figure 3A).

Affinity/scFv binding assessment

To determine the binding kinetics of anti-CD19 scFvs, surface plasmon resonance (SPR) was performed on a Biacore T200 instrument. Anti CD19 scFv-Fc (mIgG2a) constructs were coupled to a CM5 sensor chip at a target density of 100 response units (RU) and various concentrations of CD19 protein injected over the flow cell at a flow rate of 30 µl/min. BIAevaluation software Version 2.0 (GE Healthcare) was used for data processing. Kinetic rate constants were obtained by curve fitting according to a 1:1 Langmuir binding model.

ScFv competitive binding assays

For competitive scFv binding assays, FMC63 scFv was first covalently immobilised on a CM5 chip then recombinant CD19 with a C-terminal polyhis tag was injected at a flow rate of 30µl/min, followed by recombinant CAT scFv or an anti-His monoclonal antibody (GE Life sciences). Regeneration was carried out with 2.5 M glycine pH 3.0 between each cycle.

Differential scanning Fluorimetry (DSF)

DSF was used to monitor the unfolding of scFv-Fc constructs during exposure to a temperature gradient. 22.5 µl of scFv-Fc in PBS was mixed with 2.5 µL of 10x SYPRO Orange solution (Life Technologies), diluted from 5000x stock in ddH2O. A BioRad CFX Connect Real-Time System (Bio-Rad, Watford, UK) was used to record fluorescence changes during DSF measurement in FRET scanning mode. Samples were incubated at 15 °C for 4 min before exposure to a temperature gradient of 20 to 95 °C in 0.5 °C increments, with an equilibration time of 30 s at each temperature followed by a fluorescence read. Protein unfolding was reported as the midpoint transition temperature at which hydrophobic regions become exposed (T_m). Mean Tm values were determined using first-order derivative curves of triplicate experiments with reference to a blank buffer as background.

T cell transduction for pre-clinical experiments

Lentiviral supernatants were generated by co-transfection of 293T packaging cells with second generation lentiviral packaging plasmids pMD2.G and pCMV-dR8.74 as well as the pCCL-PGK-CD19 CAR transfer vectors using GeneJuice transfection reagent (Calbiochem).

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation of healthy donor blood, obtained under an ethically-approved study protocol after obtaining

informed consent. Human T cells were transduced following overnight activation with CD3/CD28 Dynabeads (Dynabeads CTS, Thermo Scientific) at a 3:1 bead:cell ratio, either in plates or G-Rex Gas Permeable Cell Culture Device (Wilson-Wolf) at $1x10^6$ cells/ml at multiplicities of infection (MOIs) ranging between 1-10.

Flow Cytometry

Flow cytometry acquisition was performed with a BD LSR II, Aria or Canto II (BD Biosciences). Data analyis was performed using FlowJo vX (Tree Star, Inc., Ashland OR), or FACs DIVA 8.0.1. Expression of CAR was detected by binding to a recombinant CD19-rabbit IgG1 protein (Origene) and via a fluorochrome-conjugated anti-Rabbit-Fc secondary antibody or mCherry expression.

Reagents for phenotyping CAR T cells

The following reagents were used for phenotypic analysis of CAR T cells: CD2 APC (Miltenyi), CD3 PerCPCy5.5 (Biolegend), CD4 PE-Vio770 (Miltenyi), CD8 PE or FITC (Biolegend), CD19 BV605 (Biolegend), CD19 PE (Biolegend), CD45RA BV605 (Biolegend), CCR7 APC (Biolegend), CD107a FITC (BD), CD223 APC-eFluor 780 (LAG-3, eBioscience), CD279 BV421 (PD1 Biolegend), CD366 (TIM3 BV711), IFN-gamma APC, TNF-alpha BV421, IL-2 BV605 (Biolegend), Anti-Rabbit Goat F(ab')2 FITC (Jackson Immunoresearch), Anti-Rabbit IgG BV421 (Biolegend), Anti-Mouse IgG PE (Biolegend), Fixable viability dye Life technologies Aqua. Fluorescence minus one (FMO) controls were used to determine expression thresholds where required.

***In vitro* functional assays**

Standard 4-h chromium-release cytotoxicity assays were performed as described previously[29]. NK cell depletion was performed before assays using CD56 magnetic bead depletion (Miltenyi) according to manufacturer's instructions. Specific lysis was calculated as:

% Lysis = (experimental lysis - spontaneous lysis) / (maximum lysis - spontaneous lysis) x 100

Longer term flow cytometric cytotoxicity assays were also performed in which effector and target cells were co-cultured at varying ratios for 24 hours. Countbright beads (Thermo Fisher) were added, cells stained for expression of CD2 and a live dead marker as an assessment of viability, to allow an assessment of the number of total remaining viable target cells.

Proliferation of CAR T cells was assessed by co-culturing effector and irradiated target cells at a 1:1 ratio in triplicates in 96 well plates. After 48 hours, the cells were pulsed with 1 µCi/well tritiated thymidine and processed as previously described Ricciardelli et al., 2014, #40175]. Specific proliferation was calculated as: CPM (effectors+targets) - CPM effectors only - CPM targets only

Cytokine production was analysed by obtaining supernatants after 48-hours of a 1:1 co-culture of effector and target cells in triplicate wells using a CBA Human Th1/Th2/Th17 Cytokine Kit (BD), according to manufacturer's protocol. Data were analysed using FCAP Array (Softflow, Inc.).

Xenograft model and Bioluminescence imaging

All animal studies were approved by the University College London Biological Services Ethical Review Committee and licensed under the Animals (Scientific Procedures) Act 1986 (ASPA). NOD^{-}SCID$^{-}\gamma^{-}$(NSG, female, aged 6-10 weeks) were obtained from Charles River Laboratory (Wilmington, MA) and raised under pathogen-free conditions. Mice were sublethally irradiated at 2.8 Gy 1 day prior to intravenous injection with 1x10^{6} F-Luc^{+} GFP^{+} NALM6 (CD19+ acute lymphoblastic leukemia). Disease engraftment was assessed at day -1 by bioluminescent imaging (BLI) and photon emission from FLuc^{+} NALM-6 cells was quantified using Living Image software. Cohorts were randomized and recipients with similar tumour burdens were distributed evenly across the groups prior to CAR T cell injection or non-transduced T cells as negative control. Photon emission from NALM6 cells expressed in photon per second per cm2 per steradian (p/s/cm2 /sr) was quantified using Living Image software (Xenogen) as previously described[30]. The experimental schema is shown in Figure 2A. Mice were closely monitored using a clinical scoring system every 1-3 days for signs of xenogeneic graft-versus-host disease and other toxicities. Mice were sacrificed according to the Protection of Animals Act, after which bone marrow and spleen were investigated for presence of disease and CAR^{+}T cells.

Clinical study

The CARPALL study (NCT02443831) was a multi-centre, non-randomised, open label Phase I A'Hern single stage clinical study which recruited eligible UK patients via a national referral pathway and was conducted in 3 hospitals. Eligible patients were children and young adults (age $\leq$24 years) with high risk, relapsed CD19^{+} haematological malignancies, though in practice, all patients screened for the study had B lineage ALL. Inclusion and exclusion

criteria for the study are given in Supplementary Table 1. The study was approved by the UK Medicines and Healthcare Products Regulatory Agency (Clinical Trial Authorisation number 20363/0361/001), London - West London & GTAC Research Ethics Committee (REC ref: 16/LO/0283) and by the Research and Development department of each participating NHS Trust. Written informed consent was obtained from patients or their carers prior to study entry.

Clinical lentiviral manufacture

A third generation self-inactivating (SIN) lentiviral vector[31], encoding the αCD19CAT-41BBz cassette under control of a human PGK promoter and incorporating, HIV CPPT, RRE, and mutated WPREwas manufactured in accordance with EMEA–Guidelines on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03) at Rayne Cell Therapy Suite (RCTS) at King's College London. The human PGK promoter was selected for optimal CAT CAR expression.

Manufacture of Advanced Therapeutic Investigational Medicinal Product

Products were generated from autologous PBMCs after leucapheresis of the patient. PBMCs were washed and activated with Dynabeads CD3/CD28 CTS at a 3:1 bead: lymphocyte ratio. Lentiviral transduction was performed in Retronectin-coated cell culture bags and on day 4 transduced lymphocytes were washed and expanded for up to 3 days in a WAVE bioreactor (GE healthcare). Dynabeads were magnetically removed on day 7 of manufacture and the cell product either rested overnight or cryopreserved the next day. Throughout manufacture, cells were cultured in X-VIVO15 media (Lonza) supplemented with 5% Human AB serum (Seralab). Exogenous cytokines were not routinely supplemented (13/14 products

infused) except in 1 case where the peripheral lymphocyte was <$0.5x10^9$/L or where poor expansion was noted post-transduction. Release assays performed prior to infusion included assessments of sterility (Gram stain, bacterial culture, Mycoplasma PCR), endotoxin levels (LAL), residual bead count, viability and transduction efficiency by flow cytometry. Cellular material was separately tested for viral copy number.

Lymphodepletion and CAR T cell infusion-

Patients received lymphodepletion with fludarabine (30mg/m^2 days -7 to -3, total 150mg/m^2) and cyclophosphamide (0.5g/m^2 days -4 to -2, total 1.5g/m^2). One patient (CPL-05) received 90mg/m^2 of fludarabine because of prior leukoencephalopathy. CAR T cells were infused at a single time point, the target cell dose was $1x10^6$ CAR T cells/kg patient weight with an additional 20% cryopreserved to allow for thawing losses. Where this dose could not be achieved, it was possible to infuse a dose from $0.5x10^6$/kg up to the target dose. Doses cryopreserved for each patient are given in Supplementary figure 7.

End points

Primary endpoints included the incidence of severe toxicity causally-related to CAR T cell infusion (grades 3-5 toxicity according to CTCAE v4.03, except for CRS, which was graded according to the criteria developed by Lee *et. al*[15]) as well as biological efficacy in terms of the proportion of patients achieving an MRD negative bone marrow remission. This was determined by a nationally-accredited qPCR assay for leukaemia-specific IgH gene rearrangements or by flow cytometry. Where the CSF was previously involved, CNS remission status was also determined.

Secondary endpoints included the proportion of patients in an MRD negative remission without the need for further therapy at 2 years, kinetics of CAR T cell persistence, incidence and duration of hypogammaglobulinemia and disease-free as well as overall survival at one and two years post infusion.

Overall survival was measured as the time from infusion of CAR T cells to time of death. Patients were otherwise censored at the date last seen alive. Event free survival was defined as reported in the ELIANA study where events of interest included no response or morphological relapse before response was maintained for at least 28 days *(n=2)*, or morphological relapse after having complete remission with or without incomplete hematologic recovery, whichever occurred first. Patients *(n=3*) were censored if they received further therapy or at the date last seen alive. Event free survival was secondarily defined by more stringent criteria in which events of interest included failure to achieve remission, morphological or molecular relapse after remission, or death, whichever occurred first.

Clinical laboratory evaluations

Serum cytokine measurements were assessed on days 0, 2, 5, 7, 9, 12, 14 post-CAR T cell infusion by an ISO-accredited method using cytometric bead array analysis of IL-2, IL-4, IL-6, IL-10 TNF, IFNγ (BD Biosciences). The validated lower limit of this assay is 50pg/ml. Cryopreserved serum samples from each time point were also analysed for a panel of 30 cytokines using the more sensitive MAGPIX Reader and Human Cytokine Magnetic 30-Plex Panel Kit (IL1RA, FGF-Basic, MCP1, G-CSF, IFNγ, IL12, IL13, IL7, GM-CSF, TNFα, IL1β, IL2, IL4, IL5, IL6, IFNα, IL15, IL10, MIP1α, IL17, IL8, EGF, HGF, VEGF, MIG, RANTES, Eotaxin, MIP1β,

IP10, IL2R) from Invitrogen according to the manufacturer's instructions. A Biotek 405 TS Plate Washer was used for automated plate washing with R&D Systems Wash Buffer. Measurements were performed using standard product protocol with sample dilution at 1:4. Data was analysed using Milliplex Analyst software.

Assessments of CAR T cell persistence were carried out on peripheral blood and bone marrow at monthly intervals until 6 months post CART cell infusion, 6 weekly from 6-12 months post infusion and 3 monthly from 12-24 months post CAR T cells. CAT CAR T cells were detected using a validated qPCR assay detecting a transgene-specific sequence. Genomic DNA was isolated and sequencing reactions carried out with transgene-specific primers and Taqman probes (Applied Biosystems), using a minimum of 0.25 µg genomic DNA where possible. A control qPCR assay using primers and probes for albumin was carried out in parallel to allow calculation of actual DNA present per sample. Results were reported as copies of the transgene per µg genomic DNA, with a detection limit of 100 copies/µg DNA. Circulating CAR T cells in blood and bone marrow were also analyzed by flow cytometry using an anti-CAT CAR anti-idiotype antibody . Absolute T cell numbers were obtained using a Trucount method (BD Biosciences) and staining for viable, CD45+CD3+ cells. Reagents used were 7-AAD, Fc gamma block CD45 FITC (BD Biosciences), CD3 APC-Cy7 (Biolegend). The percentage of CAR+ T cells was separately assessed using an anti-CAT CAR anti-idiotype (Evitria) and secondary anti-rat IgG antibody (Biolegend), with co-staining to allow detection of viable CAR+ CD45+ CD3+ cells (gating strategy shown in Supplementary Figure 9D). From this, the absolute CAR T cell count was established. Normal donor PBMC were used as negative controls. The threshold for detection was 0.1% CAR T cells.

Analysis of mechanisms of CD19- relapse

For patients relapsing with CD19- negative disease, paired bone marrow DNA samples were available from prior to CAR T cell therapy and at the time of CD19- relapse. Following centrifugation on Ficoll-Hypaque, DNA was extracted from bone marrow mononuclear cells according to standardised protocols using QIAamp DNA Mini Kit (Qiagen). DNA concentration was estimated by spectrophotometry (Nanodrop, Thermo Fisher Scientific), then accurately quantitated using a fluorometer (Invitrogen Qubit, Thermo Fisher Scientific), and integrity checked using TapeStation Genomic DNA tape (Agilent). One hundred nanograms of DNA from each time point was used to produce sequencing library by 10-plex capture using Cell3 exome kit (Nonacus). Libraries were sequenced on 1 lane of a HiSeq3000 (Illumina) using 75bp paired end sequencing to a depth of at least 120x. Fastq files were trimmed using Trimommatic to remove adaptor and low quality sequencing (trimmed when a sliding window of 4 bases fell below a median Q-score of Q20). Bowtie-2 was used to align to the human genome (Genome Reference Consortium Human Build 38) and aligned data was then deduplicated using Picard tools before variant calling with Strelka2[32]. Predicted functional effects of variants were annotated using SnpEff software[33].

Assessment of anti-CAR immune responses

Anti-CAR antibody responses were assessed by detection of human anti-mouse antibodies (Biolegend) in accordance with manufacturers' instructions. For detection of cytotoxic responses against CAR T cells, cryopreserved or fresh PBMCs post-CAR T cell infusion were stimulated twice with irradiated autologous CAR-transduced T cells at 1:1 ratio for 7 days in G-Rex 24-well plates. On day 14, untransduced and CAR-transduced lymphocytes were labelled with ^{51}Cr and co-cultured at different target: effector ratios (in triplicate) for 6

hours. Supernatant was harvested and incubated overnight with scintillant (Optiphase, Perkin Elmer) before being read. Specific lysis was calculated as described above (In vitro Functional Assays)

Analysis of cellular kinetics

Analysis of CAR T cell kinetics was performed on from the CAR transgene Area under the curve analysis of CAR T cell levels up to 28 days (AUC 0-28) was estimated by a trapezoidal algorithm and represented early CAR T cell expansion. Cmax was the peak concentration of CAR T cells documented, Tmax was the time in days from infusion to maximal CAR T cell concentration, Tlast was the time from infusion to the last documented detection of CAR T cells. T1/2 was the half-life of CAR T cell persistence over the contraction phase, as measured in patients with a minimum of 3 data points documented after Tmax.

Data analysis

Unless otherwise stated, pre-clinical data are expressed as mean ± SE, analyses were performed in GraphPad Prism, version 7. No custom computer code was generated in the analysis of data. Statistical analyses of in vitro assays were undertaken by 2-way ANOVA with donor matching and Tukey post-test for multiple comparisons or 2-tailed Student t-test, as indicated in figure legends. Significance of findings are defined as follows: ns=not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

Stata 15.1 was used for clinical data analysis. Categorical variables are reported in terms of frequencies and percentages and continuous variables in terms of medians and ranges. The molecular response rate (Molecular MRD- CR/CRi) at 28 days and at 3 months post infusion is reported along with exact binomial 95%CI. Time to event outcomes were summarised

using the Kaplan Meier method. Swimmer plots represent duration of B cell aplasia and remission experience. Toxicity events are reported at the maximum grade experienced. No large data sets were generated or analysed during the current study. Data supporting findings of this study are available on request from the corresponding author.

ACKNOWLEDGEMENTS

This work was supported by Children with Cancer UK, Great Ormond St Children's Charity, the JP Moulton Foundation and supported by the National Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London. P.J.A. is a recipient of an NIHR Research Professorship which also supported SG. MP is supported by the UK National Institute of Health Research University College London Hospital Biomedical Research Centre. F.F.'s group at King's is supported by CRUK, the Experimental Cancer Medicine Centre, and the NIHR Biomedical Research Centres (BRC) based at King's Health Partners. F.C. was supported by the Stylian Petrov Foundation. We thank Professor Malcolm Brenner, Drs John Moppett and Wendy Qian for providing oversight of the study as the IDMC, Professor Waseem Qasim for technical support in GMP CAR T cell manufacture.

AUTHOR CONTRIBUTIONS

S.G. performed pre-clinical experiments, participated in writing study documentation, developed the manufacturing protocol and clinical assays, analyzed data and wrote the manuscript. A.M.K. designed, performed and analysed pre-clinical experimental work. S.O. designed and performed surface plasmon resonance analysis, epitope mapping, thermal stability experiments, designed key reagents. GW carried out CAR T cell persistence analysis

by qPCR and disease endpoint assessment by molecular PCR, J.B. analysed exome sequencing data, R.R. and J.C.C carried out clinical study assays, manufactured products, analysed data. S.A performed manufacturing scale ups, carried out clinical study assays, manufactured products. B.P., F.C., K.C., K.V. wrote study documentation and provided trial management. J.Y. and W.V. carried out clinical study assays. P.A.W generated a key reagent. G.W.K.C. and L.M. performed pre-clinical experimental work. A.G. participated in manufacturing products. D.P, J.C., coordinated patient care and were responsible for data collection. G.L., J.S. O.C., A.L. R.C., K.R. provided medical care and contributed to data collection for study patients. S.I. provided flow cytometry assessment of disease status. K.G provided manufacturing and clinical assay expertise, G.A. was responsible for ATIMP storage and issue. M.F., S.M. performed epitope mapping and biacore experiments. T.B. performed next generation exome sequencing. A.L. provided statistical analyses and wrote the manuscript. A.H provided statistical analyses and contributed to study design. F.F. manufactured lentiviral vector and contributed to study documentation. D.B., S.S., N.G., A.V., contributed to study design, identified study patients and provided expertise in medical care for study patients. R.H., R.W. were Principal investigators for the study and provided medical care for study patients. M.A.P. conceived the idea, generated the CAR construct and participated in the design of experimental work.. P.J.A. designed the experimental work, wrote study documentation, analysed data, wrote the manuscript and was Chief Investigator for the study.

COMPETING INTERESTS STATEMENT

S.G., A.M.K., M.A.P and P.J.A. 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 knowhow from the CARPALL study. P.J.A. has research funding from Bluebird Bio Inc.. O.C. and P.V. have received funding from Servier. P.V. has research funding from Bellicum Pharmaceuticals. F.F. has founder shares in Autolus PLC and work in his laboratory is supported by Autolus funding. S.C.O., and M.A.P. are shareholders in and employees of Autolus PLC, which has licensed CAT CAR.

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FIGURE LEGENDS

Figure 1. CAT-CAR transduced T cells show enhanced CD19-specific cytotoxicity at low E:T ratios and exhibit enhanced functional avidity compared to FMC63 transduced T cells determined by proliferative response and cytokine production following stimulation with CD19+ target cells.

CAT CAR T cells were generated by lentiviral transduction of human T cells with a transfer encoding the CAT-41BBZ-mCherry CAR cassette shown in Supplementary Figure 3A. (A) Antigen-specific killing of CD19+ tumour cells by CD19-CAR+ T cells as measured by standard 4-hour ^{51}Chromium release assay. CAR+ T cell cytotoxic activity against SupT1 cells that are engineered to express CD19 (SupT1CD19) as well as target antigen negative supT1NT. Data, mean ± SEM, *n= 5*; *, *p<0.05*, 2-way ANOVA.; (B) Proliferation, as measured by the incorporation of ^{3}H-thymidine following a 72 hour 1:1 co-culture with irradiated CD19 positive (Raji & NALM-6) and CD19 negative (SupT1) cell lines. Data, mean SEM, *n=5*; *, *p<0.05*, ** p<0.01, statistical comparisons were made with a two-tailed paired Student's t-test; (C) Production of cytokines in response to 1:1 co-culture with irradiated Raji cells measured by Cytokine Bead Array of culture supernatants taken at 48 hours. Data, mean SEM, *n=4*; **, *p<0.01*; NS, non= significant, statistical comparisons were made with a two-tailed paired Student's t-test.

Figure 2. CAT-CAR transduced T cells show better disease control, accumulate in greater number in vivo and show enhanced cytokine elaboration after transfer to tumor bearing hosts.

CAT CAR T cells were generated by lentiviral transduction of human T cells with a transfer encoding the CAT-41BBZ-mCherry CAR cassette shown in Supplementary Figure 3A. (A) To assess the ability of CD19 CAR T cells to kill NALM-6 tumour in an established tumour model, NALM-6 cells were transduced with firefly luciferase imaging (BLI). THE CAR T cell dose and engraftment interval were designed such that FMC63 CAR T cells resulted in partial but not complete tumor regression. Anti-tumor responses of FMC63 and CAT CAR T cells from the same donors were compared. Mice were injected with 1x10^{6} GFP^{+} Fluc^{+} NALM-6 cells 24 hours after sublethal irradiation and 7 days prior to CAR T cell injection or non-transduced T cells as negative control. Post termination of the experiment the animals' spleen and bone marrow were analyzed by flow cytometry; (B) Photon emissions from FLuc^{+} tumor cells were quantified and measured as maximum photon/sec/cm2 /steradian (p/s/cm2/sr). Lines represent cumulative results of light emission values ± SEM. Bioluminescence was determined in 2 separate experiments, *n=18*, Student's t-test, **$p<0.001$, *** $p<0.001$; (C) After termination of the experiment at 16 days following infusion of CAR T-cells, absolute numbers of NALM-6 cells were assessed in bone marrow by flow cytometry, n=18, statistical analysis was done using a two-sided Student's t test; **, *p<0.01*, ***, *p<0.001*; (D) Bone

marrow CAR T-cells: Mean number ± SEM CAT $3.4x10^4$ ± $8.1x10^3$, FMC63 $1.3x10^4$ ± $3.1x10^3$ *n=18*, *p<0.05*. Statistical comparisons were made using a two-sided Student's t-test. (E) There were greater numbers of CAT CAR transduced cells compared to FMC63 in peripheral blood (CAT $1.9x10^4$ ± $3.1x10^3$, FMC63 $2.8x10^3$ ± $8.2x10^2$, *n=9*. (F) Percentage of cytokine-producing CAR T cells in bone marrow was determined by flow cytometry after gating on CAR+ T cells Mean percent producing ± SD, *n=4*; two-sided Student's t test; *$p < 0.05$ are shown. (G) Mean fluorescence of CD127 & (H) Bcl-2 positive cells in bone marrow as determined by flow cytometry after gating on CAR^+ T cells. Data, mean SD, *n=5* in FMC63 and *n=9* in CAT; *, $p < 0.05$, ***, $p < 0.0001$, two-sided Student's t test

Figure 3. Anti-leukemic efficacy of CAT CAR T cells and response duration

(A) Summary table of MRD negative complete remission rate as assessed by qPCR for leukaemia-specific IgH gene rearrangement, as well as number of patients with progressive disease, relapse and nature of relapses. (B) Swimmer plot showing responses of individual patients infused with CAT CAR T cells, duration of response, nature of relapse and death. (C) Kaplan Meier plot of overall survival among 14 patients infused with CAT CAR T cells. (D) Kaplan Meier plot of event-free survival in the 14 patients infused. Events of interest were defined as no response or relapse before response was maintained for at least 28 days (n=2), or morphological relapse after having complete remission with or without incomplete hematologic recovery, whichever occurred first. Numbers of patients contributing to the survival analysis are given under the plots and numbers in brackets are those censored at each time point. The blue line is the estimated survival curve, dashed lines are the 95% confidence bands. (E) Tabulated results of 6 and 12 month survival rates.

Figure 4. CAR T cell expansion and persistence in peripheral blood

Expansion of CAR T cells was assessed by flow cytometry of peripheral blood, as well as qPCR for a transgene-specific sequence at time points post infusion. (A) Shows flow cytometry plots (pregated on viable, CD45+ CD3+ lymphocytes) of CAR T cells in the peripheral blood of a representative patient (CPL-14). CAR expression (y axis) was detected by staining with an anti-idiotype antibody to CAT CAR, numbers in right upper quadrants give percentage of CAR-expressing T cells. (B) depicts percentage of CAR T cells within the T cell compartment of the peripheral blood in 13/14 infused patients at all evaluable time points. (C) Absolute numbers of CAR T cells detected by flow cytometry of the peripheral blood in 13/14 evaluable patients. CAR T cell expansion and persistence in peripheral blood as assessed by transgene specific qPCR is depicted in (D). CAR T cell persistence is correlated with duration of B cell aplasia, shown as a swimmer plot (E).

TABLES

	N(%) N=14
Maximum grade CRS (Lee criteria)	
Grade 1	9 (64%)
Grade 2	4 (29%)
Grade 3	0
Grade 4	0
Grade 5	0
Maximum grade neurotoxicity	
Grade 1	3 (21%)
Grade 2	3 (21%)
Grade 3	0
Grade 4	1 (7%)
Grade 5	0
Infection	
Grades 1-3	3 (21%)
Grade 4	1 (7%)
Grade 5	2 (14%)
B cell aplasia*	
At day 30	13 (93%)
At last follow-up (median 14 months)	12 (86%)
Hypogammaglobulinemia**	11 (79%)
Maximum grade neutropenia	
Grade 1-2	0
Grade 3	1 (7%)
Grade 4	12 (86%)
Maximum grade thrombocytopenia	
Grade 1-2	3 (21%)
Grade 3	1 (7%)
Grade 4	4 (29%)
Cytopenia not resolving by day 28 or recurring after day 28	
Grade 1-3	3 (21%)
Grade 4	8 (57%)

Table 1. Adverse events

Frequency of adverse events noted post CAR T cell infusion, by grade and type of toxicity. Cytopenias were defined as reduced neutrophil or platelet count since (B) lymphocyte depletion was an expected consequence of CAR T cell therapy. B cell aplasia was defined as B cells <5/µL post CAR T cell infusion. Hypogammaglobulinemia was defined as IgG<3g/L

PK analysis	Peripheral blood (N=14)
Time of last measurement (days)	
Median	267
Range	28 to 728
Cmax concentration (copies/ug DNA)	
Geometric mean	128,911.60
CV%	330.22
Time to Cmax (days)	
Median	14
Range	7 to 14
AUC (0 to 28), (copies/ug DNA)	
Geometric mean	1,721,355
CV%	506.16
AUC (0 to t), (copies/ug DNA)	
Geometric mean	2,668,150
CV%	737.45
Half life (days)	
Median	34.42
Range	3.37 to 102.46
Time CAR T persistence (days)	
Median	214.5
Range	14 to 728
CAR T concentration at last follow-up (copies/ug DNA)	
Geometric mean	249.92
CV%	4259.78
N patients with CAR T≥100 copies/ug at last follow-up	11
Median follow-up in months	14.42

Table 2. Summary of CAR T cell kinetic parameters as measured in peripheral blood by qPCR

Cmax, maximum concentration; AUC, area under the curve; AUC (0 to 28) AUC from time zero to day 28; AUC (0 to t) AUC from time zero until last measurement; Time to Cmax is the time to reach peak CAR T cell concentration. CAR T cell persistence was defined as the median interval in days from infusion to first value <100 copies/µg DNA, or the last follow up if this threshold level was not reached.