LETTERS
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Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR

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Chimeric antigen receptor (CAR)-modified T cells targeting CD19 demonstrate unparalleled responses in relapsed/refractory acute lymphoblastic leukemia (ALL)[1–5], but toxicity, including cytokine-release syndrome (CRS) and neurotoxicity, limits broader application. Moreover, 40–60% of patients relapse owing to poor CAR T cell persistence or emergence of CD19$^-$ clones. Some factors, including the choice of single-chain spacer[6] and extracellular[7] and costimulatory domains[8], have a profound effect on CAR T cell function and persistence. However, little is known about the impact of CAR binding affinity. There is evidence of a ceiling above which increased immunoreceptor affinity may adversely affect T cell responses[9–11]. We generated a novel CD19 CAR (CAT) with a lower affinity than FMC63, the high-affinity binder used in many clinical studies[1–4]. CAT CAR T cells showed increased proliferation and cytotoxicity in vitro and had enhanced proliferative and in vivo antitumor activity compared with FMC63 CAR T cells. In a clinical study (CARPALL, NCT02443831), 12/14 patients with relapsed/refractory pediatric B cell acute lymphoblastic leukemia treated with CAT CAR T cells achieved molecular remission. Persistence was demonstrated in 11 of 14 patients at last follow-up, with enhanced CAR T cell expansion compared with published data. Toxicity was low, with no severe CRS. One-year overall and event-free survival were 63% and 46%, respectively.

We have developed a new CD19 single-chain variable fragment (scFV) called CAT, which has shown substantially (>40-fold) lower affinity to CD19 than existing scFvs derived from FMC63 (ref. [12,13]) (Extended Data Fig. 1). The higher equilibrium dissociation constant (K_D) of CAT (14 nM) was the result of a much faster off-rate, which measures how quickly an antibody dissociates from its antigen, for CAT (CAT, 3.1×10^{-3} s^{-1}; FMC, 6.8×10^{-5} s^{-1}). The on-rate, which characterizes how fast an antibody binds to its target, was equivalent between the two (CAT, 2.2×10^{5} M^{-1} s^{-1}; FMC, 2.1×10^{5} M^{-1} s^{-1}). Key CD19 residues required for CAT and FMC63 binding were identified by sequential mutation analysis. Both antibodies shared important residues within loops 1 (AA 97–107) and 2 (AA 155–166), suggesting that FMC63 and CAT bind to the same or overlapping epitopes on CD19 (Extended Data Fig. 2a,b). The thermal stability and cell-surface stability of CAT and FMC63 single-chain variable fragments linked to single-chain constant fragments (scFv-Fcs) were similar (Extended Data Fig. 2c,d).

We compared the function of T cells that were lentivirally transduced with either FMC63 or CAT in identical second-generation CAR formats. These cells had a CD8-derived stalk/transmembrane region, a 4-1BB co-stimulatory domain and a CD3ζ chain, and they co-expressed mCherry fluorescent protein to control for transduction efficiency (Supplementary Fig. 1a–c). The cytotoxicity of CAT CAR T cells against a CD19-expressing cell line (SupT1CD19) was significantly greater than that of FMC63 CAR T cells (Fig. 1a). CAT CAR and FMC63 CAR T cells showed equivalent cytotoxicity against low-density CD19-expressing targets (Supplementary Fig. 2a–c). CAT CAR T cells showed significantly greater antigen-specific proliferation than T cells transduced with the FMC63 CAR (Fig. 1b). 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 tumor necrosis factor-α (TNF-α) than FMC63 CAR T cells (Fig. 1c).

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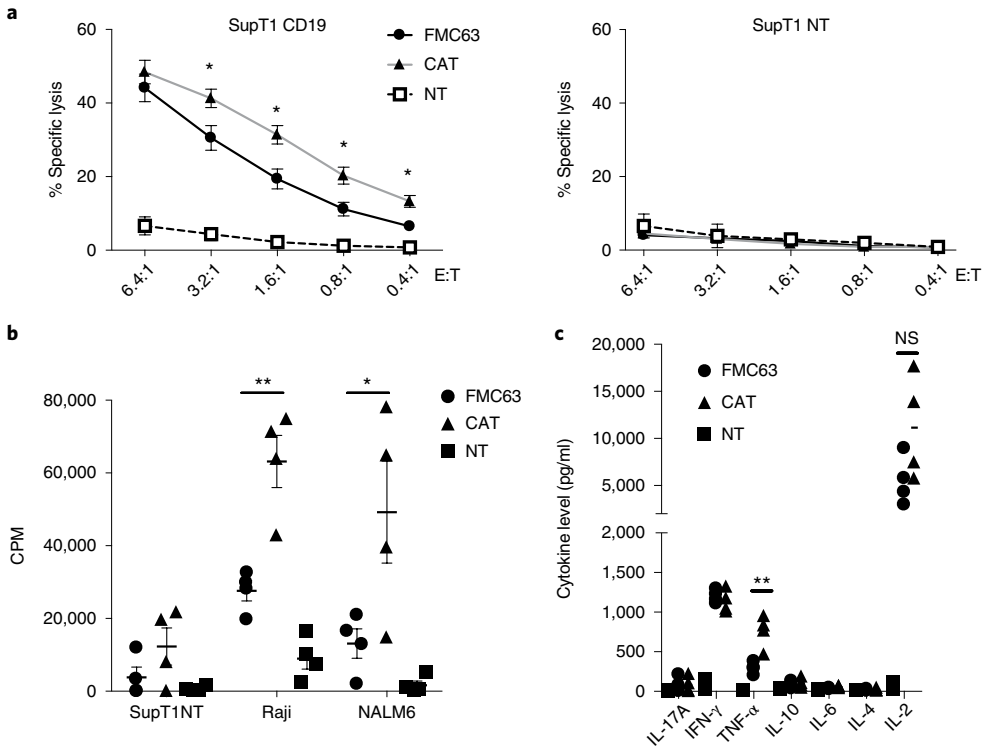

Fig. 1 | CAT CAR T cells show enhanced CD19-specific cytotoxicity at low effector:target (E:T) ratios and exhibit enhanced functional avidity compared with FMC63 CAR T cells, as determined by proliferative response and cytokine production following stimulation with CD19⁺ target cells. CAT CAR T cells were generated by lentiviral transduction of human T cells with a transfer vector encoding the CAT-41BBZ-mCherry CAR cassette, as shown in Supplementary Fig. 1a. **a**, Antigen-specific killing of CD19⁺ tumor cells by CD19 CAR⁺ T cells (FMC63 or CAT) or non-transduced (NT) T cells, as measured by standard 4-h ^{51}Chromium-release assay. CAR⁺ T cell cytotoxic activity against SupT1 cells that are engineered to express CD19 (SupT1CD19, left) as well as target-antigen-negative non-transduced SupT1NT (right). Data are shown as mean ± s.e.m., $n = 5$ donors; $^{*}P = 0.03$, two-way ANOVA. **b**, Proliferation as measured by the incorporation of [^{3}H]thymidine following a 72-h 1:1 coculture with irradiated CD19-positive (Raji and NALM-6) and CD19-negative (SupT1) cell lines. Raji mean, CAT 63,158 ± 7,159, FMC63 27,582 ± 2,776. $^{*}P = 0.005$. NALM-6 mean, CAT 49,237 ± 14,006 counts per minute (CPM), FMC63 13,097 ± 4,047. $^{*}P = 0.05$, $^{**}P = 0.004$. Data are shown as mean ± s.e.m., $n = 4$ donors, two-tailed paired Student's t-test. **c**, Production of cytokines in response to 1:1 coculture of CAR cells with irradiated Raji cells, measured by a cytokine bead array of culture supernatants taken at 48 h. No significant differences were seen in cytokine production between groups, except for that of TNF-α (CAT mean, 750.7 ± 103.3 pg ml^{-1}; FMC63 mean, 292.1 ± 36.51 pg ml^{-1}. $n = 4$, $P < 0.01$). Data shown as mean ± s.e.m; $n = 4$ donors. $^{**}P = 0.01$; NS, non-significant, two-tailed paired Student's t-test. Data are representative of at least two independent experiments.

Next, we tested FMC63 and CAT CARs in a NALM-6 tumor model in immunodeficient NOD/SCID/$\gamma^{-/-}$ (NSG) mice (Fig. 2 and Extended Data Fig. 3a). FMC63 CAR T cells slowed but did not prevent tumor growth, but an equivalent number of CAT CAR T cells resulted in tumor regression. On day 12 post T cell injection, substantial differences were seen in tumor burden between the two groups of mice. Higher numbers of NALM-6 tumor cells were observed in the bone marrow (BM) (Fig. 2c) and blood (Extended Data Fig. 3b) of recipients of FMC63 CAR T cells at 2 weeks after CAR T cell infusion. Conversely, significantly greater absolute numbers of CAT CAR T cells than of FMC63 CAR T cells were seen in BM (Fig. 2d) and blood (Fig. 2e). Levels of expression of the exhaustion markers lymphocyte-activation gene-3 (LAG-3), programmed death-1 (PD-1) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) on CAR⁺ T cells were similar between CAT and FMC63 CAR T cells (Extended Data Fig. 3c). There was greater expression of TNF-α in CAT-transduced T cells, consistent with our in vitro findings (Fig. 2f). CAR T cells from the BM and blood showed significantly higher levels of CD127 (IL7-Rα) and expression of B cell lymphoma-2 (Bcl-2) (Fig. 2g,h and Extended Data Fig. 3d,e) in mice treated with CAT CAR T cells. Together, these results indicate that, under conditions designed to give CAR T cells a numeric disadvantage, low-affinity CAR T cells mediate enhanced antitumor responses and expansion relative to high-affinity CAR T cells.

On the basis of these data, we designed a clinical study using the CAT CAR in patients younger than 25 years of age with high-risk CD19⁺ ALL (CARPALL). Eligibility criteria are outlined in Supplementary Table 1 and in the study protocol (Supplementary Appendix). Seventeen patients were enrolled, and 14 received an infusion of CAR T cells (Extended Data Fig. 4). Patients were followed up to a data cut-off of 19 December 2018, with a median follow-up of 14.4 months. The median age was 9 years, and all patients had advanced ALL with a median of 4 lines of prior treatment (Supplementary Table 2). Ten of 14 patients had relapsed following allogeneic stem-cell transplant (SCT). All patients had advanced-stage disease (Supplementary Tables 2 and 3). Before lymphodepletion, 4 patients were in morphological relapse (17–81% blasts), 6 had minimal residual disease (MRD)-level disease and 4 were MRD-negative in their BM. MRD-negative patients had isolated central nervous system (CNS) relapse post total body irradiation (TBI)-conditioned SCT ($n = 3$) or cranial irradiation ($n = 1$), and could not be successfully treated with standard therapies.

We were able to generate a product in 14/17 patients (82%). Twelve of 14 patients received the target dose of 10^6 cells per kg body weight, and 2 received 0.73–0.78 × 10^6 cells per kg body weight

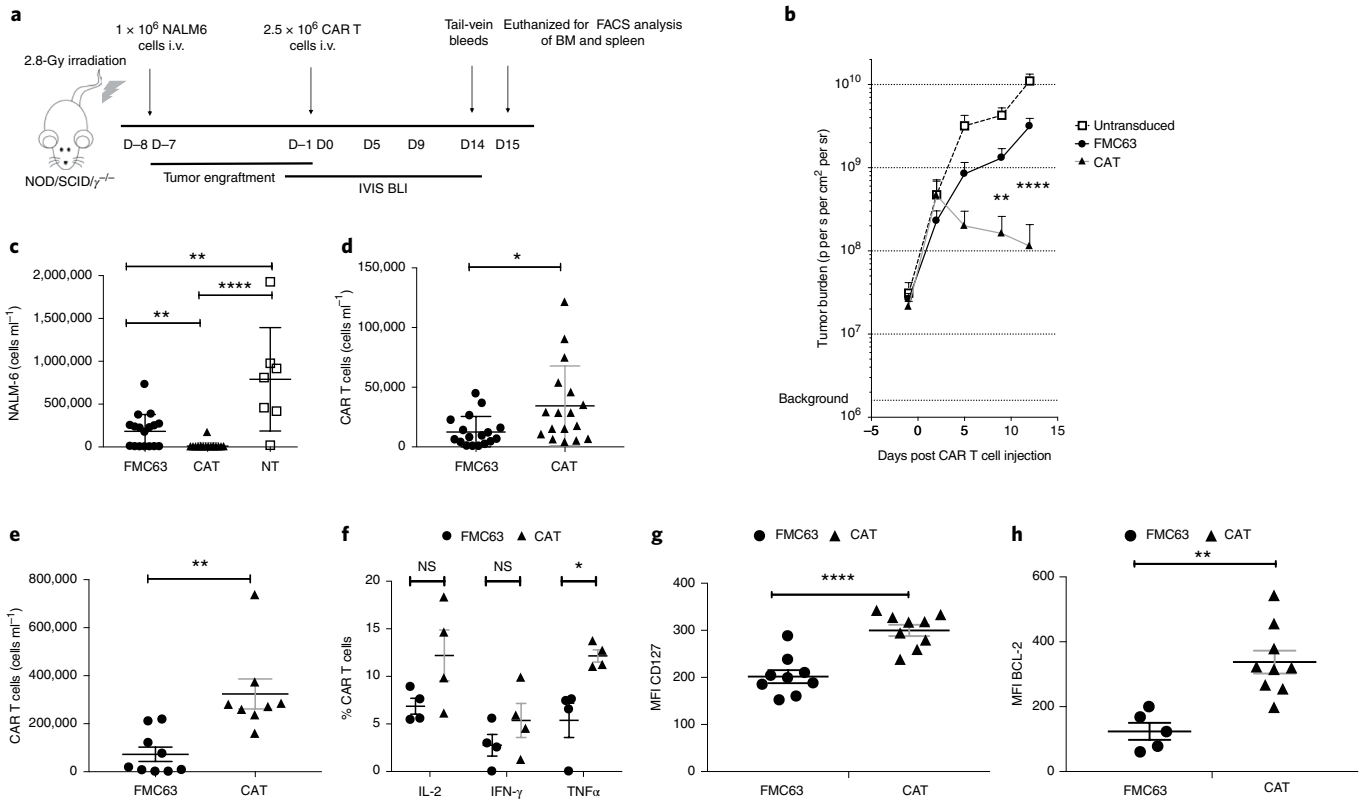

Fig. 2 | CAT CAR T cells show better disease control, accumulate in greater number in vivo and show enhanced cytokine elaboration after transfer to tumor-bearing hosts. CAT CAR T cells were generated by lentiviral transduction of human T cells with a transfer encoding the CAT-41BBZ-mCherry CAR cassette, shown in Supplementary Fig. 1a. **a**, To assess the ability of CD19 CAR T cells to kill NALM-6 tumor cells in an established model, NALM-6 cells were transduced with firefly luciferase imaging (BLI). The CAR T cell dose and engraftment interval were such that FMC63 CAR T cells resulted in partial, but not complete, tumor regression. Mice were injected with 1×10^6 GFP$^+$F-Luc$^+$ NALM-6 cells 24 h after sublethal irradiation and 7 d prior to CAR T cell injection (FMC63 or CAT CAR T cells, or nontransduced T cell control). Post termination, the spleen and BM were analyzed by flow cytometry. **b**, Burden of F-Luc$^+$ tumor cells was quantified and measured as maximum photon per s per cm^2 per steradian (p per s per cm^2 per sr). Lines represent cumulative results of light emission values ± s.e.m. Bioluminescence was determined in two separate experiments. $n=18$ mice. At day 12, mice that received CAT CAR T cells had a significantly lower tumor burden. CAT CAR, $1.1\times10^8\pm9.3\times107$; FMC63 CAR, $3.2\times10^9\pm7.7\times108$, mean photons/s/cm^2. $n=18$ mice, two-sided Student's *t*-test, $^{**}P=0.01$, $^{****}P=1\times10^{-11}$. **c**, After euthanization at 16 d following infusion of CAR T cells, absolute numbers of NALM-6 cells were assessed in BM by flow cytometry. $n=18$ mice. Mean NALM-6 cells ml^{-1}, 3×10^2 in CAT, 2.8×10^5 in FMC63 cohort. $n=9$ mice. Data are depicted as mean ± s.d. Statistical analysis was done using a two-sided Student's *t*-test. $^{**}P=0.001$, $^{****}P=2\times10^{-5}$. **d**, CAR T-cells in BM at 16 days post infusion were quantified by flow cytometry. CAT mean, $3.4\times10^4\pm8.1\times10^3$; FMC63 mean, $1.3\times10^4\pm3.1\times10^3$. $n=18$ mice, $P=0.02$. Data are depicted as mean ± s.e.m. Statistical comparisons were made using a two-sided Student's *t*-test. **e**, There were greater numbers of CAT CAR cells than of FMC63 CAR cells in peripheral blood (CAT, $1.9\times10^4\pm3.1\times10^3$; FMC63, $2.8\times10^3\pm8.2\times10^2$, $n=9$ mice; data depicted as mean ± s.d.). Statistical comparisons were made using a two-sided Student's *t*-test; $^{***}P=0.0003$. **f**, Percentage of cytokine-producing CAR T cells in BM was determined by flow cytometry after gating on CAR$^+$ T cells. Mean percent producing ± s.d., $n=4$; two-sided Student's *t*-test; $^*P=0.04$. **g**,**h**, Mean fluorescence intensity (MFI) of CD127 ($n=9$) (**g**) and Bcl-2-positive (**h**) cells in BM ($n=9$ CAT CAR, $n=5$ FMC63 CAR), as determined by flow cytometry after gating on CAR$^+$ T cells. Data are shown as mean ± s.d. $n=5$–9 mice for FMC63 and $n=9$ mice for CAT; $^{**}P=0.001$, $^{****}P=6\times10^{-5}$, two-sided Student's *t*-test. Data are representative of at least two independent experiments.

(Extended Data Fig. 5b). CAR T cells showed a predominantly central memory or naive stem cell memory phenotype, with a low level of dual expression of PD-1 and TIM-3 (Extended Data Fig. 5a). All patients received lymphodepletion with fludarabine and/or cyclophosphamide lymphodepletion.

Toxicity is summarized in Extended Data Fig. 6 and Supplementary Tables 3–6. Thirteen of 14 patients (93%) developed CRS (assessed with criteria in Lee et al.[14]; Extended Data Fig. 6) at a median of 7 d from CAR T cell infusion (range, 1–11 d). CRS was generally mild (grade 1, $n=9$; grade 2, $n=4$) and lasted a median of 5 d. No grade 3 or 4 CRS occurred, and no patient required tocilizumab therapy or admission to intensive care. Using the University of Pennsylvania scale, we determined that 3 patients had grade 3 CRS by virtue of hypotension requiring fluid boluses/oxygen requirement <40% (Supplementary Table 3). Commensurate with the absence of severe CRS and in contrast to reported data on FMC63 CAR T cells, we saw only modest increases of proinflammatory cytokines interferon- γ (IFN-γ) and interleukin-6 (IL-6), as well as IL-10, in the blood of a minority of patients, and most patients had no elevation of these cytokines (Extended Data Fig. 7a). IL-2, IL-4 and TNF-α levels were not elevated in any patient. C-reactive protein (CRP) levels were generally low, except in patients with concomitant infection. Using a more sensitive 30-plex cytokine panel[15] for retrospective crossvalidation, we demonstrated that there were modest increases in the levels of IFN-γ, IL-6, IL-8 and soluble IL-2 receptor in patients who had more significant manifestations of CRS (for example, patients CPL-01,-02 and -05) (Extended Data Fig. 7 and Supplementary Table 5).

Neurological side effects were generally mild. Six patients experienced Common Terminology Criteria for Adverse Events (CTCAE)

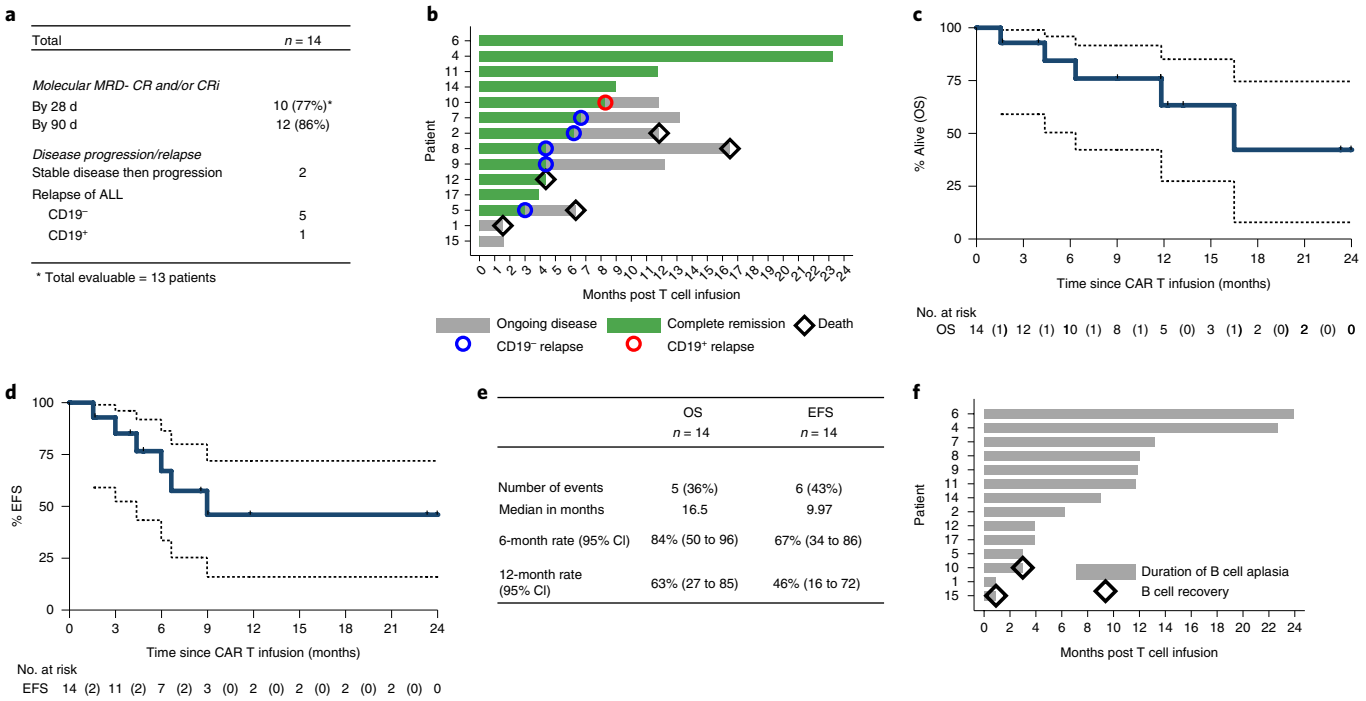

Fig. 3 | Anti-leukemic efficacy of CAT CAR T cells and response duration. a, Summary table of MRD-negative CR rate as assessed by qPCR for leukemia-specific IgH gene rearrangement, as well as the number of patients with progressive disease, relapse and the nature of relapses. **b**, Swimmer plot showing responses of individual patients infused with CAT CAR T cells, duration of response and nature of relapse and death. **c**, Kaplan–Meier plot of OS in 14 patients infused with CAT CAR T cells. **d**, Kaplan–Meier plot of EFS in the 14 patients who received infusions. Events of interest were defined as no response or relapse before response was maintained for at least 28 d ($n=2$), or morphological relapse after having CR 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 parentheses are those censored at each time point. The blue line is the estimated survival curve, and dashed lines are the 95% confidence bands. **e**, Tabulated results of 6- and 12-month survival rates. **f**, CAR T cell persistence is correlated with duration of B cell aplasia, shown as a swimmer plot.

grade 1–2 neurotoxicity (Extended Data Fig. 6 and Supplementary Tables 3 and 6), including dysarthria, paresthesia and somnolence. Grade 4 encephalopathy occurred in 1 patient (CPL-12) on day 37 post transfusion of CAR T cells. The timing of leukoencephalopathy, the absence of significant prior CRS and the presence of white-matter changes on magnetic resonance images were more consistent with fludarabine than CAR-associated neurotoxicity.

Cytopenias were common (Extended Data Fig. 6 and Supplementary Table 6) owing to the heavy pretreatment of this cohort and lymphodepletion. Cytopenias persisting beyond 28 d and new cytopenias developing after initial count recovery in the absence of relapse were analyzed separately because these might be related to CAR T cell therapy. Ten patients had grade 3–4 cytopenia (particularly neutropenia) persisting beyond day 28 or recurring after this time point. Of these, 6 patients had grade 3–4 neutropenia (42.9%; 95% confidence interval (CI), 17.7–71.1%) and 3 patients (21.4%; 95% CI, 4.7–50.8%) had grade 3–4 thrombocytopenia persisting beyond day 28. One patient with prolonged neutropenia and multiple infections, as well as grade 4 encephalopathy, died in remission from sepsis. There were 3 other grade 3–4 infections associated with prolonged neutropenia. A correlate of CD19 CAR T cell persistence is B cell aplasia, which occurred in 13/14 patients. The median duration of B cell aplasia was 7.6 months, and 12/14 patients had B cell aplasia at the last follow-up (Fig. 3f and Extended Data Fig. 6). Hypogammaglobulinemia (IgG < 3 g L^{-1}) was noted in 11 patients and warranted immunoglobulin replacement in 6 of these patients.

At 30 d post infusion, 10/13 evaluable patients (77%) were in molecular complete remission (CR) or continuing CR, as assessed by PCR for leukemia-specific IgH gene rearrangements (Fig. 3). Two patients had stable disease and subsequently progressed to CD19⁺ disease, and in one patient, an insufficient amount of DNA was obtained for analysis. By month 3, 12/14 patients (86%) had achieved molecular CR. On an intention-to-treat basis that included the three patients who did not receive CAR T cells, the overall molecular remission rate was 12/17 (71%).

Among those who achieved CR, six subsequently relapsed: five with CD19⁻ disease and one with CD19⁺ disease. Next-generation sequencing of BM DNA from patients with CD19⁻ relapse revealed that 4/5 patients had mutations in the CD19 gene that were predicted to result in loss of surface expression (Supplementary Table 7). 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 (OS) was 84% at 6 months and 63% at 12 months, and event-free survival (EFS), assessed by the criteria used in the ELIANA[4] study (defined as the time from CAR T cells to the following events: no response or morphological relapse after having CR or CR with incomplete hematologic recovery (CRi)), was 63% and 46% at 6 and 12 months, respectively (Fig. 3). Using more stringent criteria, under which an event is defined as molecular relapse, molecular EFS was 55% and 31% at 6 and 12 months. The median duration of morphological remission in responding patients was not reached and was 7.4 months for molecular remission.

CAR T cell expansion and persistence were assessed in the blood and BM up to 2 years post infusion by transgene-specific quantitative PCR (qPCR) and flow cytometry using an anti-idiotype antibody (Fig. 4 and Extended Data Fig. 8a,b). Robust peripheral CAR T cell expansion (maximum concentration (C_{max}) > 50,000 copies per µg DNA) was seen in 12/14 (86%) patients (Fig. 4), with a median time to maximal expansion of 14 d and a mean area under the curve (AUC) from day 0 to day 28 of 1,721,355 copies per µg DNA (Extended Data Fig. 9). At the point of maximal expansion, a median of 41% of circulating T cells were CAR⁺ by flow cytometry

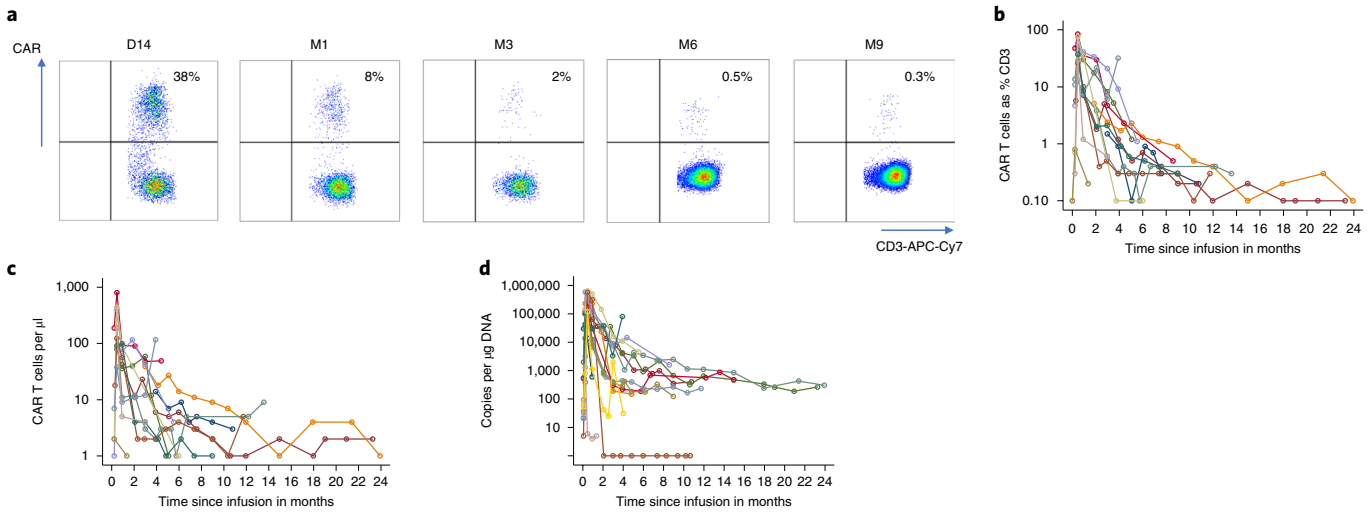

Fig. 4 | CAR T cell expansion and persistence in peripheral blood. Expansion of CAR T cells was assessed by flow cytometry of peripheral blood, as well as qPCR for a transgene-specific sequence on days 0, 2, 7, 14 and 28, monthly up to 6 months, every 6 weeks to 1 year then every 3 months up to 2 years post infusion. **a**, Flow cytometry plots (pre-gated 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 the right upper quadrants give percentage of CAR-expressing T cells. Data are representative of independent samples taken at multiple time points from $n = 13$ patients. **b**, The 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. **d**, CAR T cell expansion and persistence in peripheral blood, as assessed by transgene-specific qPCR.

(Fig. 4a,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 patients (57%). The median duration of persistence of CAR T cells at data cut-off was 215 d (range, 14–728 d), and the median half-life of CAR T cells was 34 d (range, 3–102 d) in 14 evaluable patients (Extended Data Fig. 9).

Poor expansion was seen in two 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 in the blood, as determined by flow cytometry and qPCR, by 2 weeks. Another patient (CPL-10) showed excellent expansion of CAR T cells at one month post infusion, but abruptly lost CAR T cells after. Peripheral blood mononuclear cells (PBMCs) from CPL-10 and -15 showed cytotoxicity against CAR-expressing autologous targets (Extended Data Fig. 8e), suggesting that cell-mediated rejection was occurring[2]. No human antimouse antibodies were detected.

In conclusion, we report on a new, low-affinity CD19 CAR incorporating a CD19-specific scFv with a faster off-rate than the FMC63 CD19 binder used in many clinical studies[1,3,4,16]. T cells expressing our low-affinity CAT CAR showed greater cytotoxic and proliferative responses in vitro. These data were supported by enhanced CAR T cell proliferation and antileukemic activity with our low-affinity CAT CAR in a xenogeneic model of ALL. Data on the impact of affinity on CAR T cell function are conflicting and may be target-specific[7,11]. Low-affinity CARs directed against ErbB2 (ref. [17]) and ICAM-1 (ref. [18]) have previously been reported to give equivalent or superior antitumor responses to high-affinity CARs in mouse models. The enhanced antileukemic effect seen in our model may reflect both 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. It is possible that serial triggering due to shorter-duration receptor–ligand interaction may result in enhanced signaling through proliferative pathways. In addition to increased proliferation, improved survival through decreased apoptosis and IL-7 signaling may also have a role, as evidenced by the greater expression of BCL-2 and CD127 on CAR T cells from mice that received the CAT CAR. We did not observe differences in expression of exhaustion markers between CAT and FMC63 CAR T cells in our model, suggesting this was not contributory to the enhanced expansion seen with CAT CAR T cells.

In a heavily pretreated cohort of pediatric patients with advanced relapsed/refractory ALL, CAT CAR T cells resulted in molecular CR in 10/13 patients (95% CI, 46–95%) at day 28 and 12/14 patients 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 and survival rates are comparable to those in published studies with other second-generation CD19CARs in pediatric[1,3,4,16] and adult[2,5,19] ALL. At a median of 14 months, 5/14 patients remain in CR/CCR with ongoing persistence of CAR T cells. Sustained responses were seen in patients with multiple CNS relapses after cranial irradiation/SCT, indicating that CAR T cells are effective in CNS relapse. One-year OS and EFS (63% and 46%) were comparable with published data[3,20] including the pivotal ELIANA study of tisagenlecleucel[4],an FMC63-based CAR T cell product now licensed for relapsed/refractory pediatric ALL (81% CR; 1-year OS and EFS, 76% and 50%). In 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 received SCT.

Strikingly, in concordance with our in vitro and murine data, CAT CAR T cells showed excellent expansion in patients. The mean maximal concentration of CAR T cells in the blood (128,912 copies per µg DNA) was 3× higher, and the cumulative exposure to CAR T cells in the first 28 d, as assessed by mean AUC (1,721,355 copies per µg DNA), was 5× higher than that reported for tisagenlecleucel[16,21], 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 is associated with decreased CAR T expansion[2], and because in most cases, expansion was seen without significant elevations

of proinflammatory 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 d) was >2× that reported for tisagenlecleucel (14.2 d). CAR T cells were detectable by flow/qPCR in 11/14 patients at last follow-up, and the median persistence of CAR T cells was 215 d (compared to 168 d on ELIANA study). Our CAR incorporated a 4-1BB costimulatory domain, which may confer prolonged persistence compared with CD28 by preventing exhaustion through tonic signaling[8,22], but the presence of 4-1BB is not sufficient for this[3]. While our pre-clinical data suggest an intrinsic difference in antigen-induced proliferation, in our clinical study, we used a phosphoglycerate kinase (PGK) promoter as this gave better CAR expression across both CD4/CD8 subsets, whereas the ELIANA study utilized the human elongation factor 1-α (EF1α) promoter, and it is possible that this may have contributed to the observed differences. Further, our production methodology, which did not utilize cytokines in 13/14 patients, resulted in CAR T cells with a predominantly naive and/or central memory phenotype, which may have contributed to favorable expansion and persistence of CAR T cells. Nonetheless, our preclinical data suggest that the enhanced expansion and excellent persistence of CAT CAR T cells may in part be mediated through higher expression of IL-7R and Bcl-2 in CAT CAR T cells, promoting homeostatic proliferation and preventing apoptosis. Importantly, CAT CAR T cells and tisagenlecleucel are the only reported CD19 CAR T cell products that reliably persist long-term beyond 2 months[1,3,19]. This is of central importance in determining whether CAR T cells should be used as a stand-alone therapy, or as a bridging therapy to SCT.

The safety profile of CAT CAR T cells is promising, with no severe CRS, but this needs to be interpreted with caution because 10/14 patients in our study had a low leukemic burden, which is associated with decreased likelihood of severe CRS[2,23]. Larger studies with patients with higher leukemic burdens will be needed to confirm this finding. Nonetheless, the cytokine profile we observed appears to be different from that reported for high-affinity CARs, suggesting that there may be intrinsic differences in cytokine secretion with a low-affinity CAR that become more apparent in the BM microenvironment than in the in vitro setting. Neurotoxicity was generally mild and self-resolving, as has been previously described in children. Cytopenias were comparable to those associated with tisagenlecleucel: 6 patients (42.9%) had grade 3/4 neutropenia persisting beyond day 28, compared with 53% in the ELIANA study. These mostly self-resolved by 2 months and were only associated with major infections in 2 patients. The mechanism underlying late neutropenia is unclear.

Six responding patients relapsed. In one of these patients, as well as in one non-responding patient, relapse was $CD19^{+}$ and was associated with loss of CAR T cells after initial expansion. CAR-specific cytotoxic responses 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), particularly in patients with a higher tumor burden. As previously described[24], this was due to outgrowth of clones with CD19 mutations that were predicted to lead to a truncated protein not expressed at the cell surface, which were under selective pressure from CAR T cells.

Our work demonstrates that a lower-affinity CD19 CAR with an epitope and structure and stability that are similar to the FMC63 CAR shows enhanced proliferative capacity and antileukemic responses in preclinical studies and greater expansion in patients than reported with tisagenlecleucel, as well as excellent persistence. More broadly, it demonstrates the potential for modulating CAR functionality by changing the binding affinity to the cognate antigen.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41591-019-0549-5.

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Author contributions

S.G. performed preclinical 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 analyzed pre-clinical experimental work. S.O. designed and performed surface plasmon resonance analysis, epitope mapping, thermal stability experiments and designed key reagents. G.W. carried out CAR T cell persistence analysis by qPCR and disease endpoint assessment by molecular PCR. J.B. analyzed exome sequencing data, and R.R. and J.C.-C. carried out clinical study assays, manufactured products and analyzed data. S.J.A. performed manufacturing scale ups, carried out clinical study assays and manufactured products. B.P., F.C., K.C. and 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 preclinical experimental work. A.G. participated in manufacturing products. D.P and J.C.-C., coordinated patient care and were responsible for data collection. G.L., J.S., O.C., A.L., R.C., K.R. and P.V. provided medical care and contributed to data collection for study patients. S.I. provided flow cytometry assessment of disease status. K.C.G. provided manufacturing and clinical assay expertise. G.A. was responsible for ATIMP storage and issue. M.F. and 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. and P.V. contributed to study design, identified study patients and provided expertise in medical care for study patients. R.H. and 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, analyzed data, wrote the manuscript and was chief investigator of the study.

Competing interests

S.G., A.M.K., L.M., G.W-K.C., 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, which has licensed the intellectual property and know-how 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.

Additional information

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Methods

Study design. We derived a CD19-specific scFv (CAT) from the CAT131E10 hybridoma. Single-chain variable fragments (scFvs) from the FMC63 and CAT13.1E10 hybridomas were generated, and their binding to CD19 was assessed. Chimeric antigen receptors in a 4-1BB-containing second-generation format were generated from the FMC63 and CAT scFvs. A preclinical 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 antitumor efficacy within a xenogeneic model of ALL, involving transfer of NALM6 tumor cells to immunodeficient mice. Next, we began a clinical study of CAT CAR T cells for the treatment of high-risk B lineage ALL in children and young adults.

Cell lines. Raji, K562 and 293 T cell lines were obtained from the American Type Culture Collection. SupT1 cells were purchased from the European Collection of Authenticated Cell Cultures and transduced with an SFG vector to express human CD19 (SupT1-CD19). From the transduced population of SupT1-CD19, single cells were selected by flow cytometry to generate a cell line. NALM6 expressing GFP and firefly luciferase were provided by H. Almasbak[25].

CAR, scFv and transfer vector engineering. The DNA sequence for CD19 scFvs was obtained from previous publications[26] or derived through rapid amplification of 5′ complementary DNA 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)-293 T 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[27]. Transgene expression was driven by the human PGK-1 (hPGK-1) promoter in all cases. To compare cell surface stability of CAR expression, CD19-41BBZ CARs[12] were tagged with a V5 tag and co-expressed with mCherry in a bicistronic lentiviral vector (Extended Data Fig. 2d). CAR expression could then be detected independently of CAR-antigen affinity through detection of the V5 tag, and compared against the transduction efficiency as assessed by mCherry. However, for all other preclinical comparisons, an untagged version of the CD19-41BBZ CAR was used (vector schematic shown in Supplementary Fig. 1a).

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^{-1}. 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. The three loops identified in the crystal structure were mutated by single-residue alanine scanning and tested for loss of binding by flow cytometry. For competitive scFv binding assays, FMC63 scFv was first covalently immobilized on a CM5 chip, then recombinant CD19 with a C-terminal polyhistidine tag was injected at a flow rate of 30 µl min^{-1}, 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 was used to monitor the unfolding of scFv-Fc constructs during exposure to a temperature gradient[28]. 22.5 µl of scFv-Fc in PBS was mixed with 2.5 µL of 10× SYPRO Orange solution (Life Technologies), diluted from 5000× stock in double-distilled H_2O. 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 minutes before exposure to a temperature gradient of 20–95 °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 T_m values were determined using first-order derivative curves of triplicate experiments with reference to a blank buffer as background.

T cell transduction for preclinical experiments. Lentiviral supernatants were generated by co-transfection of 293 T 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).

PBMCs were isolated by Ficoll density centrifugation of healthy donor blood, obtained under an ethically approved study protocol after obtaining informed consent from participants. 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 1×10^6 cells ml^{-1} 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 analysis 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-γ 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) and 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]. Natural killer cell depletion was performed before assays using CD56 magnetic bead depletion (Miltenyi) according to manufacturer's instructions. Specific lysis was calculated as:

$$\%\,\text{Lysis} = (\text{experimental lysis} - \text{spontaneous lysis}) / (\text{maximum lysis} - \text{spontaneous lysis}) \times 100$$

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 (Supplementary Fig. 4a–c). Effector and target cells were co-cultured at varying ratios for 24 h. Countbright beads (Thermo Fisher) were added, and cells were 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 culturing effector and irradiated target cells at a 1:1 ratio in triplicate in 96-well plates. After 48 h, the cells were pulsed with 1 µCi tritiated thymidine per well and processed as previously described[30]. Specific proliferation was calculated as: CPM (effectors+targets) – CPM effectors only – CPM targets only.

Cytokine production was analyzed by obtaining supernatants after 48 h of a 1:1 coculture of effector and target cells in triplicate wells using a CBA Human T_H1/T_H2/T_H17 cytokine kit (BD), according to manufacturer's protocols. Data were analyzed 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). NSG mice (female, aged 6–10 weeks) were obtained from Charles River Laboratory (Wilmington, MA) and raised under pathogen-free conditions. Mice were sublethally irradiated with 2.8 Gy at 1 day prior to intravenous injection with 1×10^6 F-Luc$^+$ GFP$^+$ NALM6 (CD19$^+$ acute lymphoblastic leukemia). Disease engraftment was assessed at day –1 by bioluminescent imaging (BLI), and photon emission from F-Luc$^+$ NALM-6 cells was quantified using Living Image software. Cohorts were randomized, and recipients with similar tumor burdens were distributed evenly across the groups prior to CAR T cell injection or non-transduced T cells as negative controls. Photon emission from NALM6 cells, expressed in photon per second per cm^2 per steradian, was quantified using Living Image software (Xenogen) as previously described[30]. The experimental schema is shown in Fig. 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 euthanized according to the Protection of Animals Act, after which BM and spleen were investigated for presence of disease and CAR$^+$ T cells.

Clinical study. The CARPALL study (NCT02443831; the clinical trial protocol can be found within the Supplementary Appendix) was a multicenter, non-randomized, open-label phase I A'Hern single-stage clinical study in which eligible UK patients were recruited via a national referral pathway. The study was conducted in three hospitals. Eligible patients were children and young adults (age ≤24 years) with high risk, relapsed CD19$^+$ hematological 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 authorization no. 20363/0361/001), the London – West London & GTAC Research Ethics Committee (REC ref no. 16/LO/0283) and the research and development department of each participating National Health Service trust. Written informed consent was obtained from patients or their caregivers 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 central polypurine tract, Rev response element, and mutated woodchuck hepatitis virus was 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 d in a WAVE bioreactor (GE healthcare). Dynabeads were magnetically removed on day 7 of manufacture, and the cell product either rested overnight or was cryopreserved the next day. Throughout manufacture, cells were cultured in X-VIVO15 medium (Lonza) supplemented with 5% human AB serum (Seralab). Exogenous cytokines were not routinely supplemented (13/14 products infused), except in 1 case where the level of peripheral lymphocytes was $<0.5\times10^9$ cells l^{-1} 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 (30 mg per m^2, days −7 to −3, total 150 mg per m^2) and cyclophosphamide (0.5 g per m^2, days −4 to −2, total 1.5 g per m^2). One patient (CPL-05) received 90 mg per m^2 of fludarabine because of prior leukoencephalopathy. CAR T cells were infused at a single time point, and the target cell dose was 1×10^6 CAR T cells per 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 0.5×10^6 cells per kg up to the target dose. Doses cryopreserved for each patient are given in Extended Data 5.

Endpoints. 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.[14]), as well as biological efficacy in terms of the proportion of patients achieving an MRD-negative BM remission. This was determined by a nationally accredited qPCR assay for leukemia-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 OS at 1 and 2 years post infusion.

OS 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. EFS 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 d ($n=2$), or morphological relapse after having CR 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. EFS was secondarily defined by more stringent criteria under 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 and 14 post CAR T cell infusion by an International Organization for Standardization-accredited method using cytometric bead array analysis of IL-2, IL-4, IL-6, IL-10 TNF-α and IFN-γ (BD Biosciences). The validated lower limit of this assay is 50 pg ml^{-1}. Cryopreserved serum samples from each time point were also analyzed for a panel of 30 cytokines using the more sensitive MAGPIX Reader and Human Cytokine Magnetic 30-Plex Panel Kit (IL1RA, FGF-Basic, MCP-1, G-CSF, IFN-γ, IL-12, IL-13, IL-7, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IFN-α, IL-15, IL-10, MIP-1α, IL-17, IL-8, EGF, HGF, VEGF, MIG, RANTES, Eotaxin, MIP-1β, IP-10, IL-2R) 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 were analyzed using Milliplex Analyst software.

CAR T cell expansion and persistence were assessed in the peripheral blood (PB) on days 0, 2, 7, 14 and 28, monthly up to 6 months, 6 weekly to 1 year then 3 monthly up to 2 years post infusion. BM was assessed monthly for the first 6 months and then at the same intervals as for blood. 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 per µg DNA. Circulating CAR T cells in blood and BM 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) and 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 Fig. 9d). From this, the absolute CAR T cell count was established. Normal donor PBMCs 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 BM 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 BM mononuclear cells according to standardized 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 75-bp paired-end sequencing to a depth of at least 120×. Fastq files were trimmed using Trimommatic to remove adapter and low-quality sequencing (trimmed when a sliding window of 4 bases fell below a median Q-score of Q20). Bowtie-2 was used for alignment to the human genome (Genome Reference Consortium Human Build 38) and aligned data were then deduplicated using Picard tools before variant calling with Strelka2 (ref. [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 the 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 d in G-Rex 24-well plates. On day 14, untransduced and CAR-transduced lymphocytes were labeled with ^{51}Cr and co-cultured at different target:effector ratios (in triplicate) for 6 h. Supernatant was harvested and incubated overnight with scintillant (Optiphase, PerkinElmer) 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 AUC analysis of CAR T cell levels up to 28 d (AUC 0–28) was estimated by a trapezoidal algorithm and represented early CAR T cell expansion. C_{max} was the peak concentration of CAR T cells documented, T_{max} was the time in days from infusion to maximal CAR T cell concentration and T_{last} was the time from infusion to the last documented detection of CAR T cells. $T_{1/2}$ was the half-life of CAR T cell persistence over the contraction phase, as measured in patients with a minimum of three data points documented after T_{max}.

Statistical analysis. Unless otherwise stated, preclinical data are expressed as mean ± standard error, and 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 performed by two-way ANOVA with donor matching and Tukey post-test for multiple comparisons, or by two-tailed Student's *t*-test, as indicated in the 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 and/or CRi) at 28 d and at 3 months post infusion is reported along with exact binomial 95% CI. Time-to-event outcomes were summarized 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 datasets were generated or analyzed during the current study. Data supporting the findings of this study are available on request from the corresponding author.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The whole-exome sequencing data files from the CARPALL study are available in controlled-access format from the European Genome-phenome Archive (http://www.ebi.ac.uk/ega; accession no. EGAS00001003733). Sequencing data

requests will be reviewed by the Independent Data Monitoring Committee and Trial Management Group of the CARPALL study and may be subject to patient confidentiality. After approval, a data-access agreement with UCL will be required. All requests for raw and analyzed data and materials will be reviewed by UCL Business (UCLB) to verify whether the request is subject to any intellectual property or confidentiality obligations.

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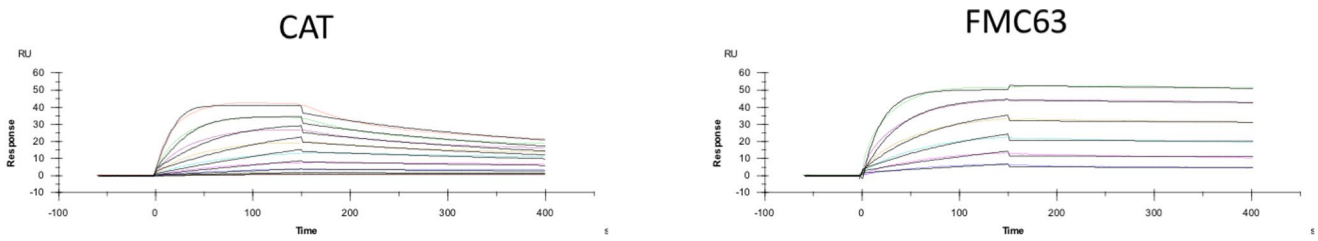
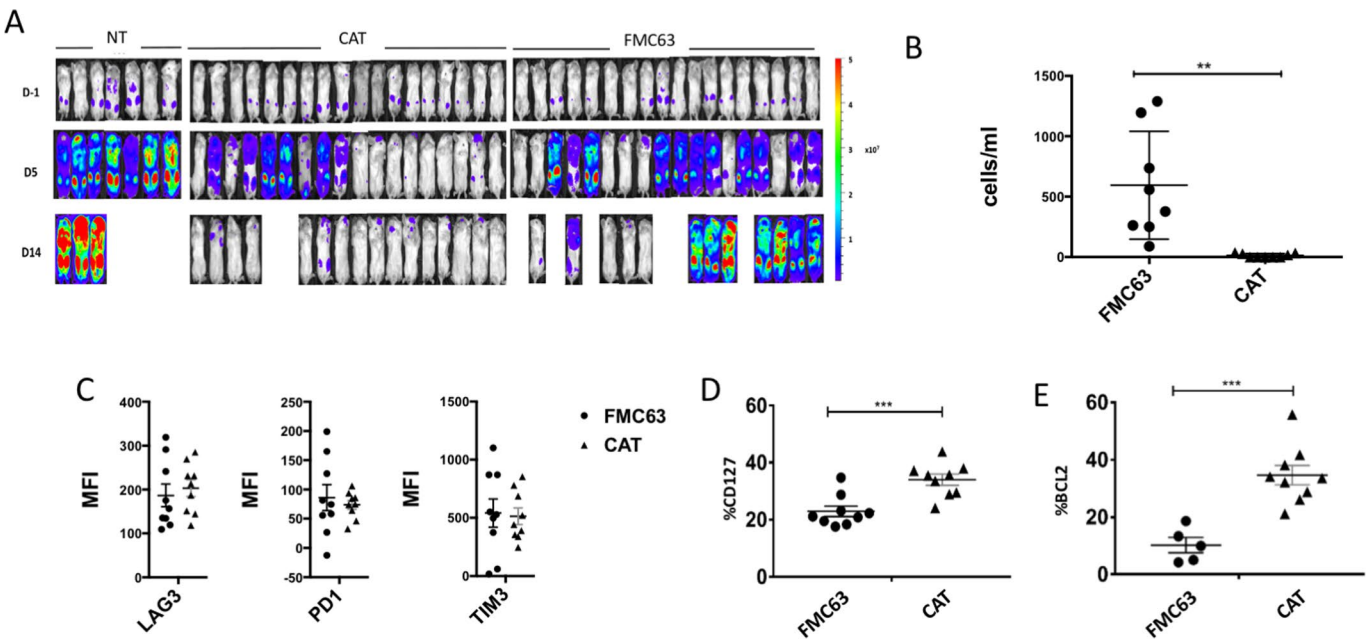
A



B

Clone	*ka* (1/Ms)	*kd* (1/s)	KD (M)
CAT	2.153E+5	0.003096	1.438E-8
FMC63	2.076E+5	6.810E-5	3.280E-10

Extended Data Fig. 1 | CD19 scFvs derived from different CD19 hybridomas show different kinetic binding properties. Anti-CD19 scFvs were fused to mouse IgG2a-Fc in a SFG-eBFP γ-retroviral expression vector and HEK293T cells were transfected to generate secreted scFvs, which were then purified on a protein A column. **a**, Surface plasmon resonance sensograms of binding kinetics between CAT and FMC63 scFv-Fcs to CD19. **b**, Tabulated kinetic and equilibrium dissociation constants of the CD19 binders. Experiments were repeated independently twice with similar results.



Extended Data Fig. 3 | In a xenograft NALM-6 model, CAT CAR T cells express similar levels of markers of activation and exhaustion (TIM3, LAG3, PD-1) to FMC63 CAR T cells, but a greater proportion express CD127 and Bcl-2 at the tumor site. Mice were injected with 1×106 GFP$^+$F-luc$^+$ NALM-6 cells 24 h after sublethal irradiation and 7 d prior to T cell injection. Disease engraftment was assessed at day –1. Cohorts were randomized, and recipients with similar tumor burdens were distributed evenly across the groups prior to CAR T cell injection or nontransduced T cells as negative control. **a**, Tumor growth was evaluated using the IVIS imaging system. **b**, Blood NALM-6 cell absolute numbers are reduced in the cohort that received CAT CAR transduced T cells ($n = 9$) compared to FMC63 ($n = 8$). Data are mean ± s.d.; $P = 0.001$; two-sided Student's t-test. **c**, There was no significant differences in median fluorescence intensity (MFI) of activation and exhaustion markers LAG-3, TIM-3 and PD-1, after gating on CAR$^+$ cells. Data are mean ± s.d.; $n = 9$. **d**, Proportion of CD127-positive cells in BM was determined by flow cytometry after gating on CAR$^+$ T cells. Data are mean ± s.d.; $n = 9$; $^{***}P = 0.0007$, two-sided Student's t-test. **e**, Proportion of Bcl-2-positive cells in BM. Data are mean ± s.d.; $n = 5$ (FMC63 CAR); $n = 9$ (CAT CAR); $^{***}P = 0.0004$, two-sided Student's t test. Experiments were repeated twice with similar results.

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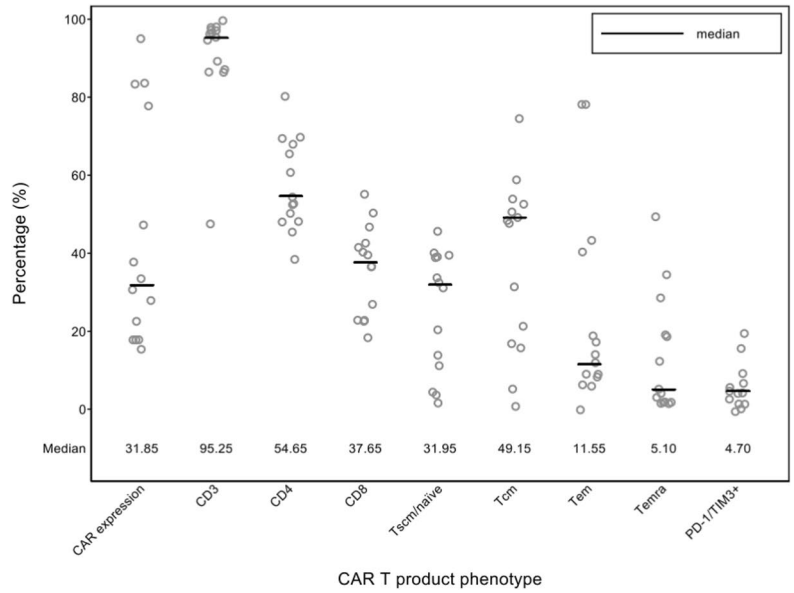


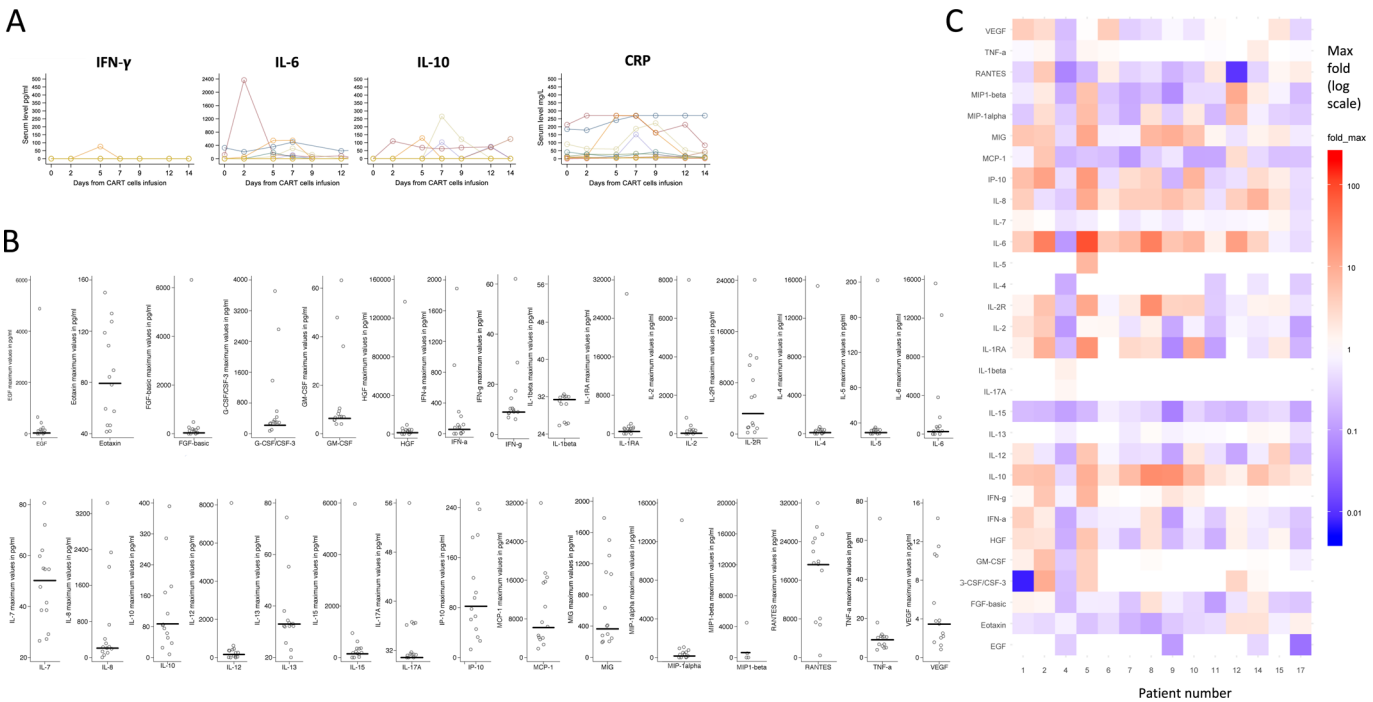
B

Patient	Dose CAR T cells cryopreserved ($x10^6$/kg)
CPL-01	1.1
CPL-02	1.2
CPL-04	1.2
CPL-05	1.2
CPL-06	1.2
CPL-07	1.0
CPL-08	1.2
CPL-09	1.2
CPL-10	1.2
CPL-11	0.9
CPL-12	1.2
CPL-14	0.9
CPL-15	1.2
CPL-17	1.2
Median	1.2

Extended Data Fig. 5 | CAR T cell product phenotype and dose. The CAR T cell product generated was assessed by flow cytometry for CAR T cell expression, and the percentage of viable CD3, CD4 and CD8 cells. In addition, viable $CAR^+CD45^+CD3^+$ lymphocytes were stained for CD45RA and CCR7 to determine the proportion of CAR T cells in different T memory subsets ($CD45RA^+CCR7^+$ = T stem cell memory/naive; $CD45RA^-CCR7^+$ = T central memory; $CD45RA^-CCR7^-$ = T effector memory; $CD45RA^+CCR7^-$ = T effector memory re-expressing RA). Finally, viable $CAR^+CD45^+CD3^+$ lymphocytes were stained for the activation and exhaustion markers PD-1 and TIM-3, and the proportion of CAR T cells expressing both of these markers was assessed. $n = 14$ products, except for exhaustion marker analysis in which $n = 13$ evaluable products. Lines represent median values. A target cryopreserved cell dose was 1.2×10^6 CAR T cells/kg, giving an infused dose of 10^6/kg, accounting for 20% cell loss during thawing.

	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%)

Extended Data Fig. 6 | 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 <5 B cells per µl blood post CAR T cell infusion. Hypogammaglobulinemia was defined as <3 g IgG per L blood.



Extended Data Fig. 7 | Serum cytokine and CRP measurements. Serum levels of IFN-γ, IL-6, IL-10 as well as CRP (**a**) as assessed by cytometric bead array during the first 14 d post CAR T cell infusion in all 14 patients; the *y* axis denotes serum level in pg/ml for cytokines and mg/L for CRP. Serum samples from 14 patients were also assessed by a 30-cytokine panel on a MagPix-Luminex platform. Maximal absolute values of analytes are given in **b**; lines represent median values. Maximal fold change relative to day 0 (that is, pre-CAR T cell infusion) is depicted in **c**, where red represents an increase and blue a decrease from baseline values.

nature research

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Last updated by author(s): 2019/07/03

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	FACs data were acquired with the use of FACS DIVA V8.0.1 (BD Biosciences), bioluminescence data were acquired and analysed with the use of Living Image v4.3.1 (Xenogen) software, clinical data were collected with the use of MACRO v4.9.1. xPONENT® software (v4.0, Millipore) was used for plexed cytokine acquisition
Data analysis	STATA 15.1 and Graphpad prism version 7 were used for statistical analyses, BIAevaluation software Version 2.0 (GE Healthcare) was used for affinity analyses as stated in the methods, Milliplex Analyst v5.1 and FCAP Array Software v3.0 were used to analyse cytokine analysis

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The whole exome sequencing data files from the CARPALL study are available in controlled-access format from the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega; accession number EGAS00001003733). Sequencing data requests will be reviewed by the Independent Data Monitoring Committee and Trial Management Group of the CARPALL study and may be subject to patient confidentiality. After approval, a data access agreement with UCL will be required. All requests for raw and analyzed data and materials will be reviewed by UCL Business (UCLB) to verify if the request is subject to any intellectual property or confidentiality obligations.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The response rate with CD19CAR T cells is expected to be at least 50% (data from the 3 major US studies found 60-80%), when it is normally 20% or less with conventional chemotherapy in this high risk cohort. A trial of 15 patients has 80% power to detect a difference of 20 vs 50%, with one-sided 7% statistical significance (A'Hern phase II sample size; 'Sample size tables for clinical studies' software, by Machin et al). If at least 6 patients have a response, this would be evidence of efficacy for CD19CAR T-cells.
Data exclusions	There were no data exclusions
Replication	All data were representative of 2 experiments or the data were pooled from at least 2 experiments. All attempts at replication were successful
Randomization	Randomisation was not incorporated into the design of this study as this was a Phase 1/2 first in Man study focusing on toxicity and biological endpoints. Further, since the majority of patients had relapsed post transplant, there are no effective curative approaches available with which to randomize against.
Blinding	Investigators were blinded in the assessment of tumour burden for in vivo tumour model experiments and the determination of tumour burden by bioluminescence. There was no blinding carried out in the clinical study as it was a single cohort study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	The following antibodies were used for phenotypic analysis of CAR T cells in accordance with manufacturer's instructions or as indicated: Anti-CAT CAR Idiotype antibody (bespoke product, Evitria 1/40) CD2 APC (Mnaufacturer Miltenyi, cat no. 130-098-579, clone LT2, 1/100), CD3 PerCPCy5.5 (Biolegend 300430 UCHT1, 1/200), CD4 PE-Vio770 (Miltenyi, 130-100-454, M-T466, 1/50) , CD8 PE or FITC (Biolegend 300908 and 300906 respectively, HIT8A, 1/100), CD19 BV605 (Biolegend, 115539, HIB19 1/100), CD19 PE (Biolegend, 392506, 4G7, 1/100), CD45RA BV605 (Biolegend, 304134, HI100, 1/100), CCR7 APC (Biolegend, 353214, G043H7, 1/100), CD107a FITC (BD), CD223 APC-eFluor 780 (LAG-3, eBioscience), CD279 BV421 (PD1 Biolegend 1/100), CD366 or TIM3 BV711(Biolegend 345024 F38-2E2, 1/100), CD95 BV711 (BD 563132, DX2, 1/50), CD127 BV421 (Biolegend 351310, A019D5, 1/10), CD107A FITC (BD, 555800, H4A3) IFN-gamma APC (502512, 4S.B3), TNF-alpha BV421 (502932, MAb11), IL-2 BV605 (500332, MQ1-17H12, Biolegend), Anti-Rabbit Goat F(ab')2 FITC (Jackson Immunoresearch, 1/100), Anti-Rabbit IgG BV421 (Biolegend, 1/100), Anti rat IgG PE (Biolegend 405406 Poly4054, 1/200). For clinical assays e.g. CAR T cell persistence in patient samples, manufacturer's guidelines were followed. anti-His monoclonal antibody (GE Life sciences, 27-4710-01) was used in accordance with manufacturer's guidelines for competitive scFv binding assays
Validation	Primary antibodies were validated and titrated with appropriate positive and negative controls in order to determine the optimal stain concentration for each test. Serum cytokine measurements are validated to UK ISO standards, CAR T cell detection with the CAT CAR anti-idiotype antibody were validated against healthy donor controls and pure populations of CAR T cells in accordance with standard operating protocols in UK NEQAS and ISO accredited laboratories

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	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
Authentication	Cell line authentication was not carried out
Mycoplasma contamination	All cell lines tested negative for mycoplasma
Commonly misidentified lines (See ICLAC register)	RAJI cells directly obtained from ATCC were utilized as target cells for a discrete number of in vitro experiments, alongside other CD19-expressing target cell lines e.g. NALM6 and engineered SupT1 cells which are not on the misidentified register and gave results as expected.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	NOD-SCID-γ–(NSG,female, aged 6-10 weeks) were obtained from Charles River Laboratory (Wilmington, MA)
Wild animals	These were not utilized in this study
Field-collected samples	These were not utilized in this study
Ethics oversight	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)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics	1. Children and young adults (age 24 years or younger) with high risk/relapsed CD19+ haematological malignancy 2. Within the cohort of 14 treated patients, 13 were male (ALL being more common in boys than girls) 3. Agreement to have a pregnancy test, use adequate contraception (if applicable) 4. Written informed consent
Recruitment	Participants were recruited via a national referral pathway involving a national multi-disciplinary team contributed to by representatives from all UK treating centers. As a result, there was multi-party oversight of the referrals process, reducing bias from e.g. self referrals or referral from any single center
Ethics oversight	Research Ethics Committee (REC)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	NCT02443831
Study protocol	http://www.ctc.ucl.ac.uk/TrialDetails.aspx?Trial=116&term=carpall
Data collection	3 hospitals, registration from 03may2016 to 12jun2018; The end of the trial will be 10 years after the last patient has received the CD19CAR T-cells (or the last patient last visit if this occurs earlier).
Outcomes	1) Toxicity evaluation following CD19CAR T-cell infusion; 2) Proportion of patients achieving molecular remission at 1 month post-CD19CAR T-cell infusion; 3) Proportion of patients in molecular remission without further therapy at 2 years; 4) Persistence and frequency of circulating CD19CAR transduced T-cells in the peripheral blood; 5)Incidence and duration of hypogammaglobulinaemia; 6) Relapse rate, Disease-Free Survival and Overall Survival at 1 and 2 years after immunotherapy with CD19CAR transduced T-cells

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Samples were either derived from healthy donors or study patients. Red cells were lysed using Ammonium-Chloride-Potassium (ACK) Lysing Buffer, water lysis method or removed by ficoll density gradient centrifugation. Up to 1x10^6 cells were stained in 100ul of staining buffer containing PBS or PBS/FCS 2%. Cells were washed 1-2 times prior to analysis. For samples stained in BD Trucount tubes, to obtain absolute cell numbers, cells were stained according to manufacturer's guidelines.
Instrument	Samples were run on BD Fortessa, BD LSRII or BD FACSCanto II instruments
Software	BD FACSDiva Software Version 8.0.1, Flowjo, Treestar Version X
Cell population abundance	N/A
Gating strategy	For experiments defining proportions or characteristics of CAR T cells, samples were pre-gated on viable CAR+ CD45+ CD3+ cells falling within a lymphocyte morphological gate after doublet exclusion. Where necessary, fluorescence minus one (FMO) or negative / healthy donor controls were used to set threshold gates for expression

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

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SUPPLEMENTARY INFORMATION

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In the format provided by the authors and unedited.

Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR

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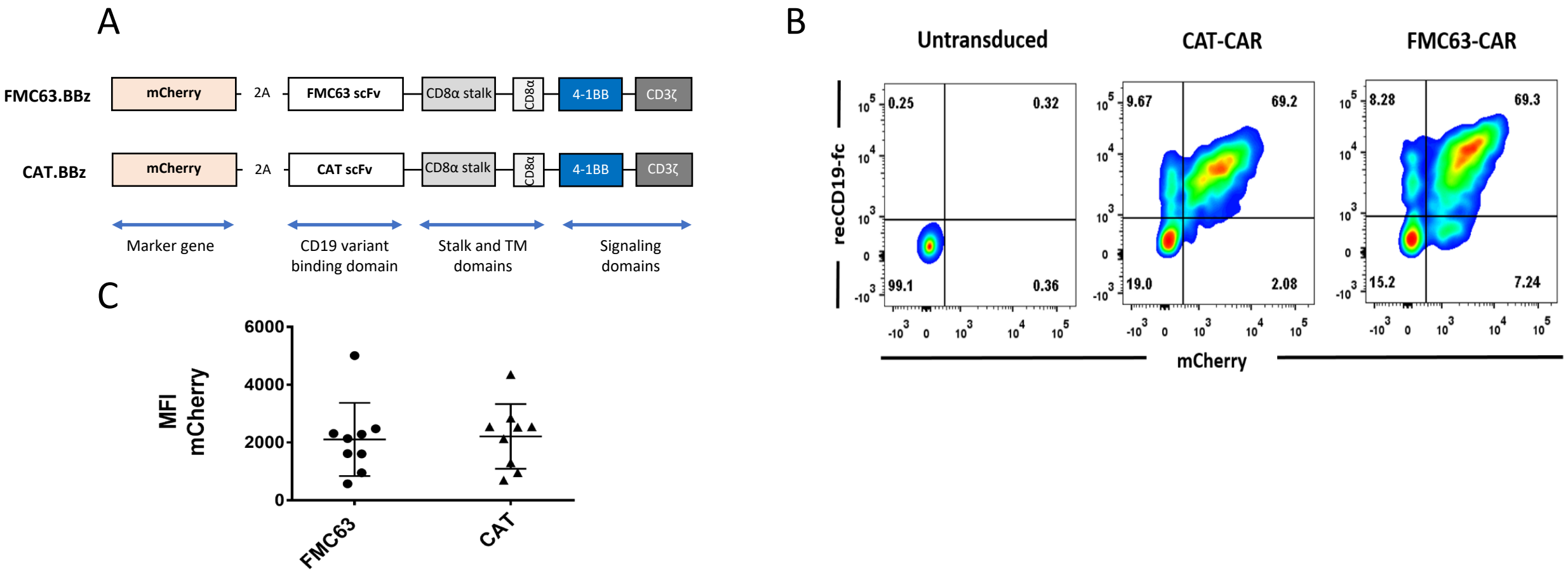
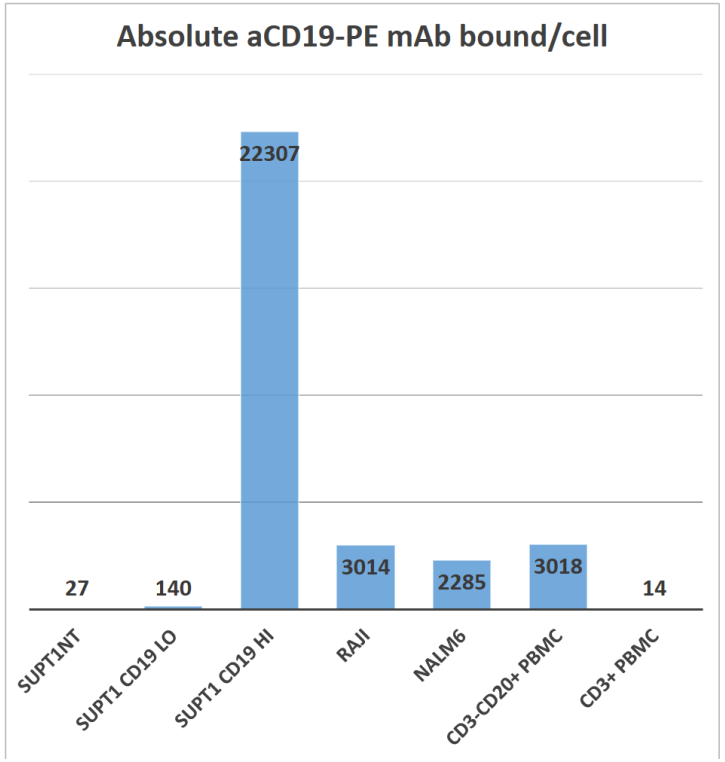
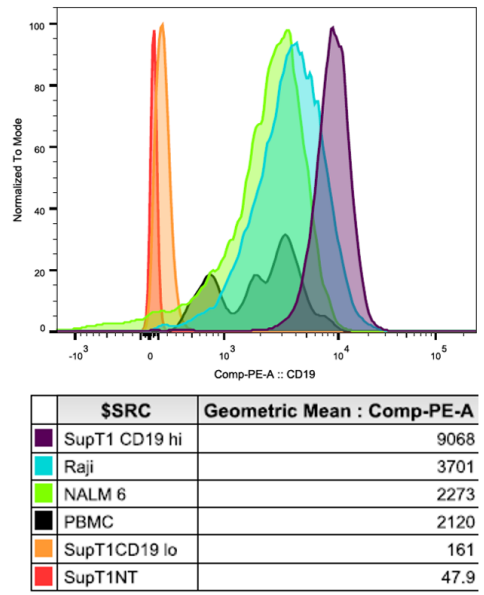
Supplementary Figure 1

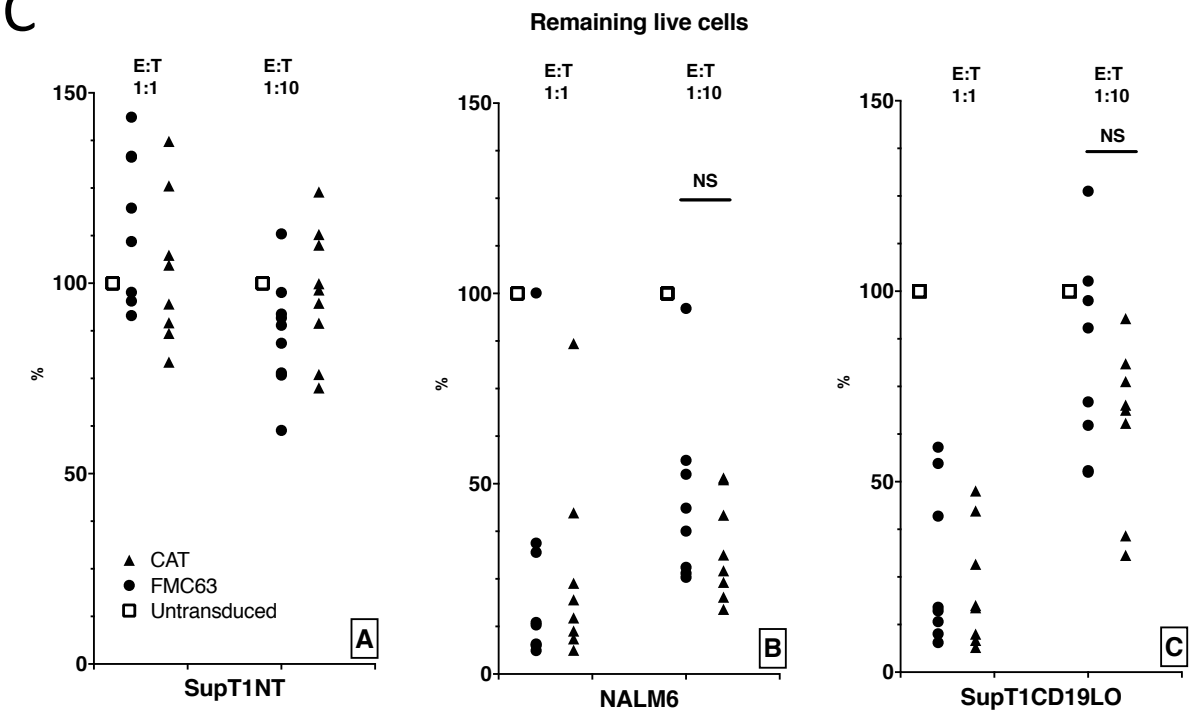


Supplementary Figure 1. Expression of Chimeric Antigen Receptor is identified by transgene expression and binding to recombinant CD19 soluble protein.
(A) Schematic representation of CD19 CARs. Both CAR constructs contain an aCD19 scFv, from either the FMC63 or CAT hybridoma, a CD8 transmembrane domain, a CD137 (4-1BB) costimulatory domain, and a CD3 ζ- signalling domain co-expressed with mCherry as a marker of transduction (B) Transduction efficiency of activated CD3/CD28 bead-expanded human T cells transduced with bi-cistronic lentiviral vectors shown in (A). The panel shows detection of CAR using mCherry transgene expression (x-axis) and a recombinant CD19 protein with an Fc tag construct (y-axis). Data are representative of >20 independent experiments. Similar levels of CAR expression as measured by median fluorescence intensity of mCherry were observed (MFI FMC63 2476, MFI CAT 2132 in representative FACS plot), but a lower MFI for rCD19 in CAT CAR, in accordance with its lower affinity for CD19 (MFI FMC63 7680, MFI CAT 4831); (C) Transgene expression, as measured by MFI of mCherry was determined 7 days-post transduction with bicistronic vectors expressing aCD19 CAR (FMC63 or CAT CAR) and mCherry. Data shown as mean ± SD. No significant difference was noted, *n=9*.

Supplementary Figure 2







Supplementary Figure 2. 24-hr Cytotoxicity assay by flow cytometry at 1:1 and 1:10 E:T ratio to investigate CAR T cell functional avidity

Flow-cytometric quantification of CD19 expression levels on target cells using the PE-QuantiBRITE kit. Cell lines assessed included SupT1NT, a T lymphoblastic cell line which does not express CD19, SupT1 cells transduced to express varying densities of CD19, including SupT1CD19LO cells and SupT1CD19HI, as well as Raji, NALM-6 cell lines and peripheral blood B cells which express CD19 at intermediate levels. Absolute CD19 PE mAb bound/cell SupT1NT 27, SupT1CD19LO 140, NALM-6 2285 depicted as bar chart (A) and as distribution of CD19 expression (B, x-axis = CD19). (C) CAR+ T cells were incubated at 1:1 and 1:10 E:T ratios with non-irradiated target cells (SupT1NT, SupT1CD19LO and NALM6) for 24 hours. The remaining live cell fraction was calculated relative to the live cell fraction in the well co-cultured with non-transduced T cells. At a 1:1 ratio against NALM-6 the mean % remaining live fraction ± SEM was: CAT 26.77 ± 9.47, FMC63 26.82 ± 11.17, *n=8*, *p=0.84*. Against SupT1CD19LO the mean % remaining live fraction ± SEM was: CAT 21.08 ± 5.02, FMC63 28.57± 6.621,*n=8*, *p=0.16*. At a 1:10 ratio against NALM 6 the mean % remaining live fraction ± SEM was: CAT 38.61 ±7.007, FMC63 51.23 ±9.161,*n=8*, *p=0.16*. Against SupT1CD19LO the mean % remaining live fraction ± SEM was: CAT 67.98± 7.29, FMC63 84.23 ± 8.429,*n=8*, *p=0.13*. Comparisons were analysed using two-tailed Wilcoxon matched-pairs signed rank test (non-parametric paired t-test)

Inclusion criteria

1. Children and young adults (age 24 years or younger) with high risk/relapsed CD19+ haematological malignancy:
    a) Resistant disease (>25% blasts) at end of UKALL 2011 or equivalent induction
    b) ALL with persistent high level MRD at 2nd time point of frontline national protocol (currently > 5 x 10-3 at week 14 UKALL2011 or equivalent)
    c) High risk infant ALL (age < 6 months at diagnosis with MLL gene rearrangement and either presenting white cell count > 300 x 109/L or poor steroid early response (i.e. circulating blast count >1x109/L following 7 day steroid pre-phase of Interfant 06)
    d) Intermediate risk infant ALL with MRD > 10-3 at end of Interfant06 induction
    e) Very early (< 18 months from diagnosis) bone marrow or extramedullary relapse of acute lymphoblastic leukaemia (ALL)
    f) Early (within 6 months of finishing therapy) bone marrow, or combined extramedullary relapse of ALL with bone marrow minimal residual disease (MRD) > 10-3 at end of re-induction
    g) Any on therapy relapse of ALL in patients age 16-24
    h) Any relapse of infant ALL
    i) ALL post ≥ 2nd relapse
    j) Any refractory relapse of ALL
    k) ALL with MRD >10-4 prior to planned stem cell transplant
    l) Any relapse of ALL eligible for stem cell transplant but no available HLA matched donor or other contraindication to transplant
    m) Any relapse of ALL after stem cell transplant
    n) Any relapse of Burkitt's or other CD19+ lymphoma
2. Agreement to have a pregnancy test, use adequate contraception (if applicable)
3. Written informed consent

Exclusion Criteria for registration

1. CD19 negative disease
2. Active hepatitis B, C or HIV infection
3. Oxygen saturation ≤ 90% on air
4. Bilirubin > 3 x upper limit of normal
5. Creatinine > 3 x upper limit of normal
6. Women who are pregnant or lactating
7. Stem Cell Transplant patients only: active significant acute GVHD (overall Grade ≥ II, Seattle criteria) or moderate/severe chronic GVHD (NIH consensus criteria) requiring systemic steroids
8. Inability to tolerate leucapheresis
9. Karnofsky (age ≥ 10 years) or Lansky (age < 10) score ≤ 50%
10. Pre-existing significant neurological disorder (other than CNS involvement of underlying haematological malignancy)

Exclusion criteria for CD19CAR T-cell infusion

1. Severe intercurrent infection at the time of scheduled CD19CAR T-cell infusion
2. Requirement for supplementary oxygen or active pulmonary infiltrates at the time of scheduled CD19CAR T-cell infusion
3. Allogeneic transplant recipients with active significant acute GVHD overall grade >2 or moderate/severe chronic GVHD requiring systemic steroids at the time of scheduled CD19CAR T-cell infusion

Supplementary Table 1. Study inclusion criteria

Baseline characteristics	N (%) N=14
Age, in years	
N	14 (100%)
Median (range)	9.24 (1.35 to 19.28)
Sex	
Female	1 (7%)
Male	13 (93%)
ALL cytogenetics at diagnosis	
Normal	1 (7%)
t(9,22)	1 (7%)
MLL rearrangement	2 (14%)
Other abnormal	9 (64%)
ND	1 (7%)
Status at registration	
1st relapse	1 (7%)
2nd relapse	8 (57%)
>2nd relapse	5 (36%)
Very early relapse	5 (36%)
Relapse post SCT	10 (71%)
CNS disease at relapse	
1^{st} relapse	5 (36%)
2^{nd} relapse	3 (21%)
Post SCT relapse	6 (43%)
Lines of treatment	
Median (range)	4 (2-7)
Prior inotuzumab	2 (14%)
Prior blinatumomab	1 (7%)
Tumour burden prior to lymphodepletion	
Morphological disease	
> 5 % blasts	4 (21%)
≤ 5% blasts	10 (79%)
MRD by IgH PCR/flow cytometry	
$> 10^{-5}$ to $\leq 10^{-4}$	1 (10%)
$> 10^{-4}$ to $\leq 10^{-3}$	3 (30%)
$> 10^{-3}$ to $\leq 10^{-2}$	1 (10%)
$> 10^{-2}$	1 (10%)
Negative	4 (40%)
CNS status at registration	
CNS I	12 (86%)
CNS II-III	2 (14%)

Supplementary Table 2. Summary of Clinical Characteristics of Infused Patients

BASELINE CHARACTERISTICS				BM STATUS AT LYMPHODEPLETION		BM STATUS AT INFUSION		CNS STATUS	CRS								MAX GRADE NEUROTOXICITY	BEST OUTCOME BY D90	OUTCOME AT LAST FOLLOW UP	DURATION OF F/U (DAYS)	B CELL APLASIA AT LAST F/U	CAR T CELL DETECTION AT LAST F/U (QPCR)
PATIENT	AGE (YRS)	NO OF RELAPSES	PRIOR ALLO SCT	% BLASTS	MRD BY IGH PCR	% BLASTS	MRD BY IGH PCR		MAX CRS GRADE BY LEE CRITERIA	MAX GRADE BY UPENN CRITERIA	HYPOTENSION DUE TO CRS REQUIRING MULTIPLE FLUID BOLUSES	OXYGEN REQUIREMENT (<40% FIO2)	COAGULOPATHY REQUIRING SUPPORT	VENTILATORY SUPPORT	INOTROPES	SOURCE OF SEPSIS DURING CRS						
CPL-01	1.4	2	Yes	1 (FISH)	ND	ND	ND	Ongoing CNS disease	2	2	No	No	No	No	No	Fungal chest infection	None	Stable disease	Death due to progressive (CD19 +) disease	28	Y	Yes
CPL-02	15.8	2	Yes	17	>10-2	88	ND	Clear	2	3	Yes	No	No	No	No	Fungal chest infection	None	Molecular CR/CRi	CD19 - relapse	189	Y	Yes
CPL-04	17.2	2	Yes	3	Negative	1	Negative	Ongoing CNS disease	1	1	N/A	N/A	N/A	No	No	Device related infection	Grade 2 Dysarthria, Dysphasia, Nystagmus	Molecular CR/CRi	Ongoing molecular CR	689	Y	Yes
CPL-05	11.3	2	Yes	81 (flow)	>10-2	40	ND	Clear	2	3	Yes	Yes	Yes	No	No	None	Grade 2 Encephalopathy	Molecular CR/CRi	CD19- relapse	91	Y	Yes
CPL-06	7.9	>2	Yes	3	> 10-4 to 10-3	0	> 10-4 to 10-3	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Ataxia	Molecular CR/CRi	Ongoing molecular CR	728	Y	Yes
CPL-07	7.3	2	Yes	0	> 10-3 to 10-2	0	> 10-3 to 10-2	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	CD19- relapse	401	N/A	Yes
CPL-08	4.3	1 (very early)	No	50 (flow)	>10-2	1 (flow)	>10-2	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Dysarthria	Molecular CR/CRi	CD19- relapse	366	Y	Yes
CPL-09	6.9	>2	No	35	10-5 to 10-4	58	10-5 to 10-4	Clear	2	3	No	Yes	No	No	No	None	None	Molecular CR/CRi	CD19- relapse	362	N/A	Yes
CPL-10	10.6	2	Yes	0.5	> 10-4 to 10-3	0	10-5 to 10-4	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Tremor	Molecular CR/CRi	CD19+ relapse	359	Y	No
CPL-11	7.4	2	No	0	Negative	0	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	Ongoing molecular CR	357	Y	Yes
CPL-12	19.3	>2	Yes	1	Negative	ND	ND	Clear	0	0	N/A	N/A	N/A	No	No	N/A	Grade 4 encephalopathy	Molecular CR/CRi	Death in CR	119	Y	No
CPL-14	13.5	>2	Yes	1	Negative	ND	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 2 parasthesiae	Molecular CR/CRi	Ongoing molecular CR	273	Y	Yes
CPL-15	3.3	>2	Yes	0.01 (flow)	> 10-4 to 10-3	0	10-5 to 10-4	Clear	1	1	N/A	N/A	N/A	No	No	None	None	SD	Alive with (CD19+) disease	28	No	No
CPL-17	13.1	2	No	1	10-5 to 10-4	0	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	Ongoing molecular CR	119	Y	No

Supplementary Table 3. Characteristics and outcomes of CARPALL study patients.

FISH – Fluorescence in situ hybridization, N/D not done, N/A not applicable, CR complete remission, CRi complete remission with incomplete hematological recovery, SD stable disease

Adverse events	Any time point N (%) N=14	Within 60 days N (%) N=14	After 60 days N (%) N=12
Any AE of any grade	14 (100%)	14 (100%)	9 (75%)
Related to ATIMP	14 (100%)	14 (100%)	7 (58%)
Any grade 3-5 AE	14 (100%)	14 (100%)	9 (75%)
Related to ATIMP	11 (79%)	11 (79%)	7 (58%)

Supplementary Table 4. Summary of adverse events by severity and relation to CAR T cell infusion

Patient	CPL-01	CPL-02	CPL-04	CPL-05	CPL-06	CPL-07	CPL-08	CPL-09	CPL-10	CPL-11	CPL-12	CPL-14	CPL-15	CPL-17
EGF	10	35.92	46.44	63.4	6.32	5.8	6.68	5.8	5.8	5.8	5.8	5.8	5.8	8.8
Eotaxin	41.16	78.04	45.48	78.48	120.2	57.76	105.88	205.2	51.76	57.76	137.2	151.12	48.72	80.16
FGF-basic	28.48	25.64	6320	28.48	40.12	33.64	43.56	28.64	33.64	408.4	23.64	314.64	43.56	534.2
G-CSF/CSF-3	35188	2705.72	1289.2	442.84	121.2	218.56	218.56	218.56	218.56	218.56	698.84	294.08	218.56	218.56
GM-CSF	10	35.92	46.44	63.4	6.32	5.8	6.68	5.8	5.8	5.8	5.8	5.8	5.8	8.8
HGF	2013.2	1389.12	133320	2086	321.2	976.56	371.72	2196.48	574.4	4064	493.12	7288	655.56	9068
IFN-a	59.36	17.92	1842	20	17.92	52.72	68.12	280	52.72	126.72	25.68	907.88	52.72	472
IFN-g	7.52	28.56	13.04	62.2	7.52	7.88	8.96	16.16	12.12	7.88	7.88	8.96	7.88	8
IL-10	110.8	183.28	159.16	392.48	30.2	80.04	299.2	383.52	87.04	30.16	19.8	87.04	43.8	48.96
IL-12	30.36	26.2	8100	71.96	107.64	247	223.6	102.96	206	155.84	123.4	438.6	310.4	592.2
IL-13	32.96	32.96	73	32.96	32.96	32.96	32.96	27.36	27.36	31.12	19.96	38.56	31.12	101.04
IL-15	583.08	351.28	5824	140.56	36.84	93.04	93.04	385.04	70.72	103.08	191	1000.12	53.12	458.88
IL-17A	458.88	36.44	36.44	36.44	36.44	30.84	30.84	30.84	30.84	30.84	30.84	30.84	30.84	30.84
IL-1beta	26.24	26.24	57.16	26.24	26.24	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4
IL-1RA	173.84	992.28	28368	2180.8	115.36	495.52	246.96	1031.32	744.12	2765.16	68.32	1392.64	186.8	1046.12
IL-2	22.6	42.44	7896	12.6	12.6	20.16	22.88	91.12	22.88	76.12	12.64	898.92	17.52	419.32
IL-2R	8240	11828	11736	24112	519.64	736.96	5164	5456	11440	833	978.2	1221.48	953.92	881.28
IL-4	109.64	109.64	15004	109.64	109.64	107.2	107.2	107.2	107.2	358.32	107.2	312.8	107.2	349.2
IL-5	27.8	27.8	27.8	88.6	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96
IL-6	927.88	12280	15228	1772.76	38.52	87.36	372	497.56	136.44	33.44	433.76	38.36	29.56	47.48
IL-7	71.6	29.04	60.68	41	27.28	53.56	53.56	46.88	4.32	53.56	40.32	60.28	53.56	117.56
IL-8	3435.6	2330	346	2022.84	44.32	184.12	233.48	659.44	154	134.64	335.64	165.16	43.6	90.08
IP-10	102.24	236.68	121.8	247.32	16	74.44	89.6	191.16	199.4	22.6	39.72	63	41	57.4
MCP-1	15228	16544	31584	10612	2217.76	3745.04	6568	15068	4652	6080	18408	4840	2114.52	5060
MIG	397.08	1062.72	646.4	1310.08	216.8	175.72	983.72	1463.08	1843.92	209.68	261.8	297.08	192.6	332.64
MIP-1alpha	302.56	124.64	13844	112.08	61.44	101.32	130.92	174.56	130.92	793.68	244.96	1082.4	71.6	987.72
MIP1-beta	431.04	776.36	18676	648.12	154.48	335.84	723.44	1581.84	188.6	689.6	1581.84	4680	335.84	2720.92
RANTES	19108	17960	18328	19992	24204	17552	26288	13608	28868	24944	2718.88	27188	19328	31688
TNF-a	5.24	4.84	69.32	5.04	4.84	9	9	9	9	9.92	9	18.52	9	9.92
VEGF	3.36	2.48	10.16	0.84	5.84	10.48	14.36	2.08	4.24	1.92	1.72	1.72	3.56	10.96

Supplementary Table 5. Maximum serum cytokine levels (in pg/ml) noted between day 0 (pre) and day 14 post CAR T cell infusion

Adverse events	Maximum grade reported N=14	
	Grade 3-5	
	All AEs	Related to ATIMP
Abnormal laboratory parameters		
Anemia	5 (36%)	2 (14%)
Lymphocyte count decreased	8 (57%)	5 (36%)
Neutrophil count decreased	13 (93%)	9 (64%)
Platelet count decreased	5 (36%)	4 (29%)
White blood cell decreased	7 (50%)	3 (21%)
Alanine aminotransferase increased	2 (14%)	
Blood bilirubin increased	2 (14%)	1 (7%)
Bone marrow hypocellular	1 (7%)	1 (7%)
Hypernatremia	1 (7%)	
Hypokalemia	1 (7%)	
Blood and lymphatic system disorders		
Febrile neutropenia	8 (57%)	7 (50%)
Cardiac disorders		
Supraventricular tachycardia	1 (7%)	
Hypotension	3 (21%)	3 (21%)
Electrocardiogram QT corrected interval prolonged	1 (7%)	
General disorders and administration site conditions		
Fatigue	1 (7%)	
Fever	4 (29%)	4 (29%)
Infections and infestations		
Device related infection	2 (14%)	1 (7%)
Lung infection	2 (14%)	1 (7%)
Periorbital infection	1 (7%)	1 (7%)
Sepsis	2 (14%)	1 (7%)
Upper respiratory infection	1 (7%)	
Other infections	1 (7%)	
Injury, poisoning and procedural complications		
Generalized muscle weakness	1 (7%)	
Nervous system disorders		
Dysarthria	1 (7%)	
Encephalopathy	1 (7%)	1 (7%)
Paresthesia	1 (7%)	
Somnolence	1 (7%)	
Respiratory, thoracic and mediastinal disorders		
Bronchopulmonary hemorrhage	1 (7%)	1 (7%)
Hypoxia	2 (14%)	2 (14%)
Skin and subcutaneous tissue disorders		
Rash maculo-papular	1 (7%)	1 (7%)
Any adverse event		
Any adverse event	**14 (100%)**	**11 (79%)**

Supplementary Table 6. Summary of severe (grade 3-5) adverse events by system and relation to CAR T cell infusion

Patient	%MRD at CD19neg relapse	Nucleotide position (chromosome 16)	Location of mutation in CD19 gene	Mutation type	Effect	VAF at diagnosis	VAF CD19 neg relapse
CPL02	99%	g.28932553C>CG	Exon 2	INS	Frameshift (LOF)	0	0.18
		g.28933331C>CA	Exon 4	INS	Frameshift (LOF)	0	0.14
CPL05	100%	g.28932411T>C	Exon 2	SNV	Missense	0	0.37
		g. 28932413G>A	Exon 2	SNV	Stop gain (LOF)	0	0.28
		g.28932439T>C	Exon 2	SNV	Missense	0	0.03
		g.28933043G>T	Exon 3	SNV	Missense	0	0.30
		g.28936557G>A	Exon 7	SNV	Synonymous	0	0.04
CPL07	20%	g.28933075C>G	Exon 3	SNV	Missense	0.42	0.85
CPL08	50%	g.28933074T>TGTAAG	Exon 3	INS	Frameshift (LOF)	0	0.15
CPL09	6%	g.28933086G>A	Exon 3	SNV	Synonymous	0	0.05
		g.28938895G>A	Exon 14	SNV	Missense	0	0.03

Supplementary Table 7. Summary of mutations associated with CD19- relapse

nature research

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Last updated by author(s): 2019/07/03

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	FACs data were acquired with the use of FACS DIVA V8.0.1 (BD Biosciences), bioluminescence data were acquired and analysed with the use of Living Image v4.3.1 (Xenogen) software, clinical data were collected with the use of MACRO v4.9.1. xPONENT® software (v4.0, Millipore) was used for plexed cytokine acquisition
Data analysis	STATA 15.1 and Graphpad prism version 7 were used for statistical analyses, BIAevaluation software Version 2.0 (GE Healthcare) was used for affinity analyses as stated in the methods, Milliplex Analyst v5.1 and FCAP Array Software v3.0 were used to analyse cytokine analysis

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The whole exome sequencing data files from the CARPALL study are available in controlled-access format from the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega; accession number EGAS00001003733). Sequencing data requests will be reviewed by the Independent Data Monitoring Committee and Trial Management Group of the CARPALL study and may be subject to patient confidentiality. After approval, a data access agreement with UCL will be required. All requests for raw and analyzed data and materials will be reviewed by UCL Business (UCLB) to verify if the request is subject to any intellectual property or confidentiality obligations.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The response rate with CD19CAR T cells is expected to be at least 50% (data from the 3 major US studies found 60-80%), when it is normally 20% or less with conventional chemotherapy in this high risk cohort. A trial of 15 patients has 80% power to detect a difference of 20 vs 50%, with one-sided 7% statistical significance (A'Hern phase II sample size; 'Sample size tables for clinical studies' software, by Machin et al). If at least 6 patients have a response, this would be evidence of efficacy for CD19CAR T-cells.
Data exclusions	There were no data exclusions
Replication	All data were representative of 2 experiments or the data were pooled from at least 2 experiments. All attempts at replication were successful
Randomization	Randomisation was not incorporated into the design of this study as this was a Phase 1/2 first in Man study focusing on toxicity and biological endpoints. Further, since the majority of patients had relapsed post transplant, there are no effective curative approaches available with which to randomize against.
Blinding	Investigators were blinded in the assessment of tumour burden for in vivo tumour model experiments and the determination of tumour burden by bioluminescence. There was no blinding carried out in the clinical study as it was a single cohort study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	The following antibodies were used for phenotypic analysis of CAR T cells in accordance with manufacturer's instructions or as indicated: Anti-CAT CAR Idiotype antibody (bespoke product, Evitria 1/40) CD2 APC (Mnaufacturer Miltenyi, cat no. 130-098-579, clone LT2, 1/100), CD3 PerCPCy5.5 (Biolegend 300430 UCHT1, 1/200), CD4 PE-Vio770 (Miltenyi, 130-100-454, M-T466, 1/50) , CD8 PE or FITC (Biolegend 300908 and 300906 respectively, HIT8A, 1/100), CD19 BV605 (Biolegend, 115539, HIB19 1/100), CD19 PE (Biolegend, 392506, 4G7, 1/100), CD45RA BV605 (Biolegend, 304134, HI100, 1/100), CCR7 APC (Biolegend, 353214, G043H7, 1/100), CD107a FITC (BD), CD223 APC-eFluor 780 (LAG-3, eBioscience), CD279 BV421 (PD1 Biolegend 1/100), CD366 or TIM3 BV711(Biolegend 345024 F38-2E2, 1/100), CD95 BV711 (BD 563132, DX2, 1/50), CD127 BV421 (Biolegend 351310, A019D5, 1/10), CD107A FITC (BD, 555800, H4A3) IFN-gamma APC (502512, 4S.B3), TNF-alpha BV421 (502932, MAb11), IL-2 BV605 (500332, MQ1-17H12, Biolegend), Anti-Rabbit Goat F(ab')2 FITC (Jackson Immunoresearch, 1/100), Anti-Rabbit IgG BV421 (Biolegend, 1/100), Anti rat IgG PE (Biolegend 405406 Poly4054, 1/200). For clinical assays e.g. CAR T cell persistence in patient samples, manufacturer's guidelines were followed. anti-His monoclonal antibody (GE Life sciences, 27-4710-01) was used in accordance with manufacturer's guidelines for competitive scFv binding assays
Validation	Primary antibodies were validated and titrated with appropriate positive and negative controls in order to determine the optimal stain concentration for each test. Serum cytokine measurements are validated to UK ISO standards, CAR T cell detection with the CAT CAR anti-idiotype antibody were validated against healthy donor controls and pure populations of CAR T cells in accordance with standard operating protocols in UK NEQAS and ISO accredited laboratories

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	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
Authentication	Cell line authentication was not carried out
Mycoplasma contamination	All cell lines tested negative for mycoplasma
Commonly misidentified lines (See ICLAC register)	RAJI cells directly obtained from ATCC were utilized as target cells for a discrete number of in vitro experiments, alongside other CD19-expressing target cell lines e.g. NALM6 and engineered SupT1 cells which are not on the misidentified register and gave results as expected.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	NOD-SCID-γ–(NSG,female, aged 6-10 weeks) were obtained from Charles River Laboratory (Wilmington, MA)
Wild animals	These were not utilized in this study
Field-collected samples	These were not utilized in this study
Ethics oversight	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)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics	1. Children and young adults (age 24 years or younger) with high risk/relapsed CD19+ haematological malignancy 2. Within the cohort of 14 treated patients, 13 were male (ALL being more common in boys than girls) 3. Agreement to have a pregnancy test, use adequate contraception (if applicable) 4. Written informed consent
Recruitment	Participants were recruited via a national referral pathway involving a national multi-disciplinary team contributed to by representatives from all UK treating centers. As a result, there was multi-party oversight of the referrals process, reducing bias from e.g. self referrals or referral from any single center
Ethics oversight	Research Ethics Committee (REC)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	NCT02443831
Study protocol	http://www.ctc.ucl.ac.uk/TrialDetails.aspx?Trial=116&term=carpall
Data collection	3 hospitals, registration from 03may2016 to 12jun2018; The end of the trial will be 10 years after the last patient has received the CD19CAR T-cells (or the last patient last visit if this occurs earlier).
Outcomes	1) Toxicity evaluation following CD19CAR T-cell infusion; 2) Proportion of patients achieving molecular remission at 1 month post-CD19CAR T-cell infusion; 3) Proportion of patients in molecular remission without further therapy at 2 years; 4) Persistence and frequency of circulating CD19CAR transduced T-cells in the peripheral blood; 5)Incidence and duration of hypogammaglobulinaemia; 6) Relapse rate, Disease-Free Survival and Overall Survival at 1 and 2 years after immunotherapy with CD19CAR transduced T-cells

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Samples were either derived from healthy donors or study patients. Red cells were lysed using Ammonium-Chloride-Potassium (ACK) Lysing Buffer, water lysis method or removed by ficoll density gradient centrifugation. Up to 1x10^6 cells were stained in 100ul of staining buffer containing PBS or PBS/FCS 2%. Cells were washed 1-2 times prior to analysis. For samples stained in BD Trucount tubes, to obtain absolute cell numbers, cells were stained according to manufacturer's guidelines.
Instrument	Samples were run on BD Fortessa, BD LSRII or BD FACSCanto II instruments
Software	BD FACSDiva Software Version 8.0.1, Flowjo, Treestar Version X
Cell population abundance	N/A
Gating strategy	For experiments defining proportions or characteristics of CAR T cells, samples were pre-gated on viable CAR+ CD45+ CD3+ cells falling within a lymphocyte morphological gate after doublet exclusion. Where necessary, fluorescence minus one (FMO) or negative / healthy donor controls were used to set threshold gates for expression

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.