Title:
Phase 1 clinical trial of CRISPR engineered CAR19 universal T cells for treatment of children with refractory B-cell leukemia

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One sentence summary: Development of a universal CRISPR-edited CAR-T cell therapy for pediatric B-ALL

Abstract

Genome editing of allogeneic T cells can provide “off-the-shelf” alternatives to autologous chimeric antigen receptor (CAR) T cell therapies. Disruption of T cell receptor alpha chain (TRAC) to prevent graft versus host disease (GVHD), and removal of CD52 (Cluster of differentiation) for a survival advantage in the presence of alemtuzumab, have previously been investigated using transcription activator-like effector nuclease (TALEN) mediated knockout. Here we deployed next generation Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 editing and linked CAR expression to multiplexed DNA editing of TRAC and CD52 through incorporation of self-duplicating CRISPR guide RNA expression cassettes within the 3’Long Terminal Repeat (LTR) of a CAR19 lentiviral vector. Three cell banks of TT52CAR19 T cells were generated and cryopreserved. A Phase 1, open label, non-randomised, clinical trial was conducted and treated six children with relapsed/refractory CD19-positive B-cell acute lymphoblastic leukaemia (B-ALL) (NCT04557436). Lymphodepletion included fludarabine, cyclophosphamide and alemtuzumab and was followed by a single infusion of 0.8-2.0x10^6 CAR19 T cells/kg with no immediate toxicities. Four of six patients infused with TT52CAR19 T cell exhibited cell expansion, achieved flow cytometric remission, and then proceeded to receive allogeneic stem cell transplantation. Two patients required biological intervention for grade II cytokine release syndrome (CRS), one patient developed transient grade IV neurotoxicity, and one patient developed skin GVHD which resolved following transplant conditioning. Other complications were within expectations and primary safety objectives were met. This study provides a demonstration of the feasibility, safety, and therapeutic potential of CRISPR engineered immunotherapy.

Introduction

While survival outcomes for children with standard-risk paediatric B cell acute lymphoblastic leukemia (B-ALL) are above 90% (*1, 2*), innovations are required for high-risk relapsed disease which has a poor prognosis.(*3*) Autologous chimeric antigen receptor T cells (CAR T cells) targeting CD19 on refractory and relapsed (R/R) B-ALL are now available as approved therapies (*4, 5*) as well as through non-commercial providers.(*6, 7*) However, logistical and manufacturing challenges, risks of disease progression and high costs of such personalized treatments are barriers to broader access and applications. Pre-manufactured CAR-T cells banks derived from non-matched donors, and suitable for timely infusion to multiple recipients, are being enabled by the incorporation of genome editing strategies into the manufacturing process.(*8*)

Here we report adoption and human application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9(*9, 10*) through the generation of universal donor anti-CD19 chimeric antigen receptor (CAR19) T cells for children with R/R B-ALL. The strategy builds on a previous approach with T cells modified using transcription activator-like effector nucleases (TALEN) to disrupt endogenous T cell receptor alpha chain (TRAC) and CD52 (Cluster of differentiation) genes in human leukocyte antigens (HLA) mis-matched allogeneic CAR19 T cells ahead of allogeneic stem cell transplantation (allo-SCT) (*11, 12*) Experience had indicated that rapid anti-leukemic effects, over a period of 1-4 weeks, could be exploited by a single infusion of pre-manufactured universal CAR19 T (UCART19) cells before they were rejected by the recovering host immune compartment. Preparative lymphodepletion including alemtuzumab, an anti-CD52 monoclonal antibody, provided edited $CD52^-$ CAR19 T cells with a survival advantage.

Here we now apply next generation CRISPR/Cas9 technology incorporated into a lentiviral configuration for coupled expression of multiplexed sgRNAs and CAR19 in an investigational T cell therapy, TT52CAR19. A trial was designed to exploit powerful anti-CD19 anti-leukemic effects within 28 days after infusion and then to proceed to allo-SCT for donor derived consolidation and rapid haematopoietic reconstitution. This time-limited 'bridge-to-transplant' application provided valuable safeguards against concerns of possible CRISPR/Cas9 toxicities by removing residual TT52CAR19 T cells with pre-transplant conditioning. This early experience of lentiviral CRISPR/Cas9 editing provides evidence of feasibility, safety, and therapeutic potential of engineered immunotherapy.

Results

Production & characterisation of TT52CAR19 T cell banks

A self-inactivating (SIN)-lentiviral vector stock for CAR19 transgene-coupled genome editing effects was generated for compliant T cell manufacture. The vector 3' Long Terminal Repeat (LTR) included multiplexed 'minimal' U6 and H1 pol III promoter-sgRNA cassettes which self-duplicated during reverse transcription,(*13*) and mediated editing effects at TRAC and CD52 target loci in the presence of Cas9 (Fig. 1A). Healthy donor peripheral blood lymphocytes (PBL) were activated and transduced using an automated CliniMacs Prodigy device and were returned to the chamber after off-device electroporation with Cas9 mRNA, and then expanded before magnetic bead mediated depletion of residual TCR$\alpha\beta$ T cells (Fig. 1A). Cell products from three volunteer donors were cryopreserved in vials of either 10 or 20 million cells for easy dose banded application across pediatric weight ranges.

Each production yielded a final product sufficient for several dozens of patients, depending on weight and dosing schedule. Flow cytometry analysis characterised CAR19 expression, knockout of TRAC and CD52, and subset markers (fig. S1). High CAR19 expression (86-98%) and depletion of TCR$\alpha\beta$, resulted in residual expression of 0.1-1.1% of TCR after automated magnetic bead and column processing (Fig. 1B). A similar process for the removal of residual CD52+ T cells was not applied, but rather it was anticipated that alemtuzumab would mediate in vivo depletion of any remaining positive cells. In joint consideration, carriage of TCR$\alpha\beta$ T cells and CAR19 expression determined dose-banding regimens across a wide range of pediatric weights to ensure thresholds for Graft Versus Host Disease (GVHD) were not breached and therapeutic dosing was comparable to the autologous setting and previous allogeneic strategies (table 1).

On-target editing effects were quantified by direct sequencing and bioinformatic analysis using Tracking of Indels by Decomposition (TIDE) and Interference of CRISPR Edits (ICE) software for signatures of non-homologous end joining (NHEJ) showing efficient disruption at both target sites (Fig. 1C and fig. S2). End of production cells were karyotype screened and examined for possible translocations from the TRAC locus by fluorescence in situ hybridization (FISH) (table S1) as well as digital droplet (dd) PCR assessment of the frequency of four predicted translocation events between the short arm of chromosome 1 and long arm of chromosome 14 (Fig. 1D and fig. S3). Vector copy number ranged from 2.94-4.57 copies/cell (table 1) and baseline vector integration sites were mapped

by ligation mediated PCR. The top 10 most frequent integration sites accounted for <1% of over 80,000 sequence reads (Fig. 1E).

Next generation sequencing was also applied to quantify non-homologous end-joining (NHEJ) editing signatures and confirm negligible activity at top-scoring sites of predicted sgRNA off-target activity (Fig. 1F and fig. S4). Western blot analysis for Cas9 confirmed the Cas9 protein was undetectable at the end of production (fig. S5). Functional assessments were undertaken in vitro to confirm CD19 specific cytotoxicity and cytokine responses (fig. S6 and fig. S7). TT52CAR19 T cells exhibited ≥50% lysis of ^{51}Cr labelled Daudi targets compared to untransduced (UnTD) or transduced non-edited controls (at E:T cell ratios 1.25:1 to 5:1) and produced higher concentrations of interleukin 2,4,6,10 and 17A, as well as Inteferon-γ and Tumor necrosis factor. Cells from each Good Manufacturing Practice (GMP) compliant batch exhibited their ability to inhibit CD19+ human leukemia in comparison to UnTD cells in immunodeficient mice over a 28 day period as confirmation of *in vivo* potency (fig. S8).

Clinical trial protocol

An open label Phase 1 trial (NCT04557436) investigated TT52CAR19 in patients with refractory/relapsed (R/R) B-ALL aged between 6 months and 18 years at a single centre under approvals from UK agencies (fig. S9). Infants and children presenting with CD19+ B-ALL were eligible with morphological disease (>5% blasts) or with Minimal Residual Disease (MRD) $>10^{-4}$ (0.01%) (by either flow cytometry or quantitative PCR) if they had exhausted other treatment options, including autologous CAR19 therapy. The treatment aimed to ensure leukemic remission (MRD$<10^{-4}$) within 28 days, which was a primary endpoint for disease response, and toxicity using National Comprehensive Cancer Network (NCCN) criteria. Successful remission allowed patients to continue in the treatment scheme to receive allo-SCT as part of standard-of-care therapy (fig. S10).

After consent and screening patients received a tailored cytoreduction (debulking), before the initiation of the treatment phase with standardised lymphodepletion comprising fludarabine 150 mg/m^2, cyclophosphamide 120 mg/kg and alemtuzumab 1.0 mg/kg over a seven-day period. Following lymphodepletion, a single dose of TT52CAR19 (CAR19 dose range allowed 0.8-2.0 x 10^6/kg) was then infused on day 0 (D0) and children were monitored in hospital for at least 28 days for toxicities and pending count recovery. After day +28 (D28), in the follow-up period patients in remission were then re-conditioned for allo-SCT using age-appropriate regimens, comprising chemotherapy, anti-thymocyte globulin (ATG) and radiotherapy dependent on donor-type, infection status and history of previous transplantation. This step simultaneously ensured depletion of residual TT52CAR19 cells and provided a rapid haematological recovery. Throughout the treatment and follow-up phases local antimicrobial

prophylaxis guidelines were followed, including anti-viral, anti-fungal, and anti-pneumocystis prophylaxis (fig. S10).

TT52CAR19 expansion and anti-leukemic effects in patients

Results are reported for the first eight children (P1-8, aged 11 months to 11 years) who were enrolled and consented for screening by the 31st of October, 2021. Six eligible children received debulking therapy comprising inotuzumab (*n*=2), steroids/vincristine (*n*=3) or high dose cytarabine (*n*=1) ahead of standardised lymphodepletion (table 2). In all patients >99% of leukemic blasts were CD19+ and CD52 was exhibited in P5 and P6, albeit only partially. Absence of pre-existing anti-HLA antibodies was confirmed in all patients at initial screening. All subjects achieved lymphodepleting serum concentrations of alemtuzumab above 0.1 µg/ml for at least fourteen days (Fig. 2A). Infused TT52CAR19 were detected using digital droplet PCR (ddPCR) for vector proviral copy and cell numbers peaked after 7-14 days in four children with detection of allogenic cells corroborated by molecular chimerism studies, which also quantified transplant donor chimerism from previous allo-SCT (Fig. 2B, Fig. 2C).

In P7 and P8, flow cytometric analysis detected the presence of $TCR^{-}CD52^{-}$CAR19 T cells in peripheral blood whereas no $TCR^{+}CAR19^{+}$ T cells were detected (Fig. 2D). Serial serum cytokine measurements detected elevation of IL-6 in five out of six subjects during this period (Fig. 2E) and variable increases in ferritin across the six patients (Fig. 2F). By day 28, circulating TT52CAR19 cells had dissipated in all patients, although low amounts were still detectable in P5 and P7, that eventually cleared during allo-SCT. Bone marrow examination after 28 days found four subjects (P2, P5, P7, P8) in morphological complete remission (CR) who were also MRD negative by flow cytometry, although P8 had borderline MRD detectable by PCR (Fig. 3).

Two subjects (P3, P6) did not exhibit TT52CAR19 expansion and experienced progressive CD19+ disease by day 28 and were not eligible for transplantation (table 2). One infant (P7) exhibited skin GVHD after day 28 which was confirmed by skin biopsy and required topical steroid therapy, but it remains uncertain if this was mediated by T cells derived from his first transplant donor or by TT52CAR19 T cells. The rash resolved promptly once conditioning for his second allo-SCT commenced. Patient P2 developed grade 2 skin GVHD following allo-SCT and required topical and systemic steroids for 3 weeks with subsequent resolution. No TT52CAR19 cells were detectable in the peripheral blood (Fig. 2B) or in the bone marrow (fig. S11) after transplant and GVHD was attributed to the allo-SCT donor. Follow-up data to the 31st of May, 2022, at least 1 month after SCT for all subjects achieving remission and undergoing transplant, is provided (Fig. 3 and Fig. 4).

Toxicities: CRS and ICANs, Cytopenia and Infections

Overall toxicities were similar to previous experiences with CAR19 therapies,(*14*) and were manageable with only one patient requiring intensive care support. Cytokine release syndrome (CRS), manifesting with fever and rise in ferritin or serum IL-6 occurred in all patients within 2-7 days (Fig. 2D and Fig. 2E), and required additional interventions in P7 and P8 for grade 2 severity. Both received fluid bolus support for hypotension and were treated with tocilizumab. P7 received two doses of tocilizumab for the treatment of grade 2 CRS and developed Grade 4 immune effector cell-associated neurotoxicity syndrome (ICANS) manifesting as generalised seizures, which required intensive care and anti-epileptic treatment.

There was no known predisposition and this complication successfully resolved within 72 hours following treatment with siltuximab and dexamethasone 0.2 mg/kg/day. Cytopenia was manifested in all subjects, five requiring treatment with granulocyte colony stimulating factor (GCSF) from day 14 for neutropenia (neutrophils < $0.5x10^9$/L), with two patients (P3, P5) remaining neutropenic at day 28 despite GCSF therapy (Fig. 4). Latent viral reactivations were pre-emptively treated with antivirals in P2 (Cytomegalovirus, Epstein-Barr virus), P3 (Adenovirus), P6 (Adenovirus, Cytomegalovirus), P8 (Cytomegalovirus) and surveillance before or during the 28-day treatment period had detected asymptomatic respiratory viruses included SARS-Cov-2 (P2, P5) and rhinovirus (P7). Symptomatic viral infections requiring specific treatment occurred in P8, who developed upper airways Respiratory Syncytial virus infection and BK-virus-associated haemorrhagic cystitis (Table 3).

Haematopoietic stem cell transplantation

Allo-SCT was offered as standard of care to children in CR on bone marrow assessments at day 28 post infusion. Three infants, under the age of 2 years, who had undergone previous allo-SCT, received low dose radiotherapy (2G) combined with anti-thymocyte globulin (ATG), Fludarabine and Cyclophosphamide ahead of a second graft from the same donor (Fig. 4). P8, underwent a first transplant from a matched sibling donor in the context of active viral complications and received 14G total-body irradiation (TBI) and Etoposide conditioning. Post-transplantation TT52CAR19 was not detectable in any patient (Fig. 2B and Fig S12). Transplant complications included GVHD (P2), EBV reactivation requiring therapy with Rituximab (P2), and BK-virus-associated haemorrhagic cystitis that required selective embolization (P8). Bone marrow assessment 1 month after allo-SCT revealed positive molecular MRD in two subjects (P5 and P8). Although CD19 expression was confirmed in P8 after relapse, flow cytometry did not detect the original CD19+ leukemia-associated immune phenotype in P5, despite a high burden of disease revealed by the molecular assay ($5.6x10^{-1}$). P5 received palliative care and further molecular characterisation of CD19 expression was not possible.

Two children P2 and P7 who remain in ongoing remission >12 months and >3 m after allo-SCT, respectively, continue to be monitored.

Discussion

Emerging therapeutic applications of CRISPR/Cas9 have been reported for a small number of human studies providing important reassurances surrounding potential risks of aberrant nuclease activity.(*15-17*) Early therapies have included ex-vivo CRISPR/Cas9 modified tumour infiltrating lymphocytes (TILs) disrupting the checkpoint molecule Programmed cell death protein 1 (PD1),(*18*) and a Phase 1 study of T cells expressing recombinant antigen specific T cell receptors (TCR) for anti-cancer therapy after editing of endogenous T cell receptor and PD1 genes.(*19*) In both cases there were modest knockout effects through non-homologous end joining (NHEJ) repair of CRISPR directed DNA breakage without any overt indications of genotoxicity. Similar CRISPR strategies have also been applied in haematopoietic stem cells (HSC) to disrupt BCL11A (B-cell lymphoma/leukemia 11A) for the treatment of hemoglobinopathies,(*20*) and for disruption of CCR5 (C-C chemokine receptor type 5), a critical HIV-1 coreceptor, in a subject with human immunodeficiency virus (HIV) and lymphoblastic leukemia.(*21*) Direct in human therapy using lipid nanoparticles encapsulating Cas9 mRNA and sgRNA for knockout of transthyretin in amyloidosis has also been reported (*22*), this technique has also been used for the retinal disorder Leber's amaurosis.(*23*) Our study adds to an emerging landscape of the feasibility, safety, and therapeutic potential of CRISPR/Cas9 genome editing.

Autologous, patient personalized, CAR-T cell therapies are now available as licenced medicines for certain B cell malignancies, albeit with quite complex logistical and infrastructure requirements. Genome-edited allogeneic "universal" donor CAR T cells suitable for multiple recipients offer the prospect of reduced costs and wider accessibility for patients. Moreover, healthy donor derived T cells are expected to exhibit superior fitness compared to patient derived T cells, especially in the context of multiple lines of chemotherapy or previous stem cell transplantation. The risk of inadvertent carriage and transduction of leukemic blasts, as previously described (*24*), is also prevented by using healthy donor derived T cells. Additionally, readily available 'off the shelf' CAR T cells should address delays associated with manufacturing and release of autologous products. Several competing allogeneic strategies, using a variety of genome editing platforms, are currently in early-stage clinical phase testing. We have previously reported the first application of TALENs for disruption of TCR and CD52 and successful therapy with UCART19 against paediatric B-ALL, as well as subsequent multicentre trials in children and adults with B-ALL.(*12*) There are ongoing trials in non-Hodgkin B cell lymphoma,(*8*) where pre-release assessments of the cell banks after TALEN editing had identified chromosomal

abnormalities in up to 5% of metaphase spreads using FISH and karyotyping. Chromosomal aberrations after CRISPR/Cas9 modification of T cells engineered with recombinant TCRs has also been reported (*19*). We found that for TT52CAR19, 'on-target' translocations between chromosomes 14q (TRAC) and 1p (CD52), using ddPCR, across predictable sites, were detected at a frequency of up to 1%, although no abnormality was detected by FISH or karyotyping. In addition, deep sequencing of potential sites of guide directed 'off target' sites for both TRAC and CD52 was undertaken. Previously, we had described the application of Digenome-seq analysis to interrogate sites and had uncovered only low amounts of NHEJ at possible off-target sites in manufactured cells.(*25*) For the clinical study, the top six scoring sites were all found to harbour negligible signatures of NHEJ, and alongside data relating to vector integration sites, the sites may only become of relevance in the event of clonal predominance or suspicion of genotoxic adverse events. There have been two reports of lentiviral induced T cell clonal expansion, both in the CAR setting,(*26, 27*) and both were viewed as supporting leukemic clearance. More concerning, have been two cases of T cell derived lymphoma following transformation after piggyBac transposon mediated CAR19 delivery. Although mechanistic insights are incomplete, contributing factors may have related to transposase biology, high numbers of integrants and their insertion sites, or the manufacturing process.(*28, 29*) Crucially, the strategy reported here aimed to achieve leukemic clearance within a period of 28 days, with subsequent clearance of engineered cells, and thereby reducing risk against possible longer term transformational consequences.

Preparative lymphodepletion, most commonly comprising treatment with fludarabine and cyclophosphamide, has become a mainstay for autologous CAR therapies, and may also have a direct impact against disease burden.(*30*) Lymphodepletion promotes homeostatic expansion of infused cells, but in the context of allogeneic CAR T cells, there is an additional hurdle of having to overcome host cellular immunity. This could be addressed by providing more intense lymphodepletion, but would have to be balanced against increased likelihood of protracted cytopenia and the risk of problematic viral reactivations. Experience from allo-SCT has established a role for anti-CD52 serotherapy to augment preparative lymphodepletion and the risk profile of alemtuzumab is well established.(*31, 32*) Alemtuzumab pharmacokinetics can be highly variable in children and lytic concentrations may extend for several weeks after dosing.(*33*)TT52CAR19 T cells were edited to remove CD52 and thus acquired an in vivo survival advantage after infusion during which period alemtuzumab had ongoing inhibitory effects against CD52+ host T and NK cells. Of note, CD52 expression was tested on leukemic blasts in all patients, and four out of six patients were CD52 negative whereas the other two had only partial CD52expression, suggesting that it is unlikely that alemtuzumab contributed to any disease debulking prior to TT52CAR19 infusion. All six subjects

achieved lymphodepleting concentrations of alemtuzumab, where one subject with partial CD52 expression (P6) exhibited accelerated clearance. Reciprocally, early recovery of host lymphocytes, mainly NK cells, was documented in this patient and may explain their reduced expansion of TT52CAR19 cells. Whereas both P3 and P6 had previously received autologous CAR19 T cell therapy, human anti-murine antibodies (HAMA) were not detected, and lymphodepletion should have addressed any pre-existing cell mediated immunity to murine components of the CAR19 receptor. Overall, expected adverse events of lymphodepletion were clinically relevant but manageable and included neutropenia, which responded to GCSF, and viral reactivations, which were managed with antiviral drug therapy and immunoglobulin replacement therapy. As with our previous experience with UCART19, viral infections were a common consequence of enhanced lymphodepletion. In this cohort a similar pattern was observed to previous UCART19 therapy,(*12*) with 4/6 patients treated for viral complications. Alternatives to intense lymphodepletion are being investigated through further cell engineering steps, and strategies to address host mediated immune rejection have involved direct disruption of HLA molecules, with a particular focus on removal of HLA class I expression. CRISPR Therapeutics have reported preliminary data of a trial (NCT04035434) where universal donor CAR19 T cells, were generated using Adeno-associated virus (AAV) to incorporate a CAR19 cassette into the TRAC locus and were also disrupted at the HLA class I associated B_2M gene locus with CRISPR/Cas9. The company has treated adults with relapsed refractory diffuse large B-cell lymphoma and reported a 38% remission rate without GvHD and limited complications. Whether removal of class I HLA is sufficient to prevent host mediated rejection or if additional measures are warranted to also address 'missing self' responses and tackle class II HLA mediated immunity are a critical area for further investigation. Once addressed, the solutions may ultimately allow allogeneic cells to be used with less intense lymphodepletion.

TT52CAR19 displayed a manageable safety profile as only one subject experienced a high-grade toxicity attributed to the infused allogeneic CAR-T cells. These early safety observations are encouraging and build on the emerging data on allogeneic CAR-T cells, warranting further investigations in larger cohort of patients. Limitations in this phase 1 setting include the small number of patients treated with TT52CAR19, the open label design without control groups, lack of comparisons with autologous CAR19, and a relatively short follow-up period to date. Additionally, as two patients failed to show expansion of TT52CAR19 T cells after infusion, it was not possible to identify a causative mechanism for this occurrence and identification of risk factors for rejections of allogeneic CAR-T cells remains problematic. Lastly, planned long-term follow-up of patients receiving TT52CAR19 will be needed to provide further information regarding final outcome, including the role of allo-SCT.

Allo-SCT was pre-agreed for children achieving remission and had two major objectives; to promote heathy donor derived reconstitution (in preference to autologous recovery) and to ensure removal of any persisting TT52CAR19 T cells. Preparative conditioning was not specifically designed to confer additional anti-leukemic effects but rather was tailored to account for donor source, graft composition, and previous transplant history. Three younger infants undergoing second procedures from the same allogeneic donors received identical regimens comprising single fraction low dose TBI and ATG for lymphodepletion in combination with fludarabine and cyclophosphamide. An older child with active virus-related complications received 14G TBI and Etoposide for a first allogeneic transplant, in the context of molecular MRD at the limit of quantification. It is unlikely that minimal intensity conditioning protocols adopted for second allo-SCTs contributed to additional anti-leukemic effects, but rather facilitated accelerated donor derived multilineage reconstitution. Overall, this small cohort of patients with high-risk refractory disease faced a poor prognosis given that historical data suggested 3-year event-free survival of 15% and overall survival of 20% in children with relapse after first allo-SCT.(*34*) Although the primary endpoint of this Phase I trial was safety, salvage of 2/6 patients treated under this approach is encouraging and further data from larger cohorts and their long-term outcomes will be required. The role of consolidative allo-SCT is still being debated in patients who have undergone CAR therapy(*35*), but in this first-in-human application of a genome-edited investigational product, transplant provided the safeguard of strictly limiting the period of time the engineered cells were allowed to persist. As safety information accumulates across studies, it may be feasible to defer or avoid entirely subsequent allo-SCT in patients once infused cells have delivered anti-leukemic activity. It also seems likely that additional engineering steps will be adopted for multiplexed removal of HLA proteins and modification of checkpoint or regulatory pathways. In the first instance, this Phase 1 study provides encouraging early-stage evidence of feasibility, safety, and therapeutic potential CRISPR/Cas9 editing.

Materials & Methods

Study Design

This was a phase 1 clinical trial (NCT04557436), investigating the safety and feasibility of first -in - human allogeneic cell therapy (TT52CAR19) in a cohort of children with refractory or relapsed B-ALL ahead of allo-SCT. The study was a single-center, non-blinded, non-randomised, non-controlled trial, without dose-escalation, but with residual TCRαβ cells capped at $<5.5x10^4$/kg.

All participants' legal guardians provided written informed consent upon enrolment. Eligibility inclusion criteria included diagnosis of R/R B-ALL expressing CD19; minimal residual disease > 10^{-4} by

flow or PCR; age 6 months to 18 years; weight > 6 kg; Eastern Cooperative Oncology Group score < 2; suitable transplant donor identified and available. Exclusion criteria included rapidly progressing disease; presence of CD19 negative disease; uncontrollable central nervous system (CNS) leukemic involvement (CNS grade 3 according to National Comprehensive Cancer Network); absence of suitable transplant donor; uncontrolled active viral, fungal and bacterial infections; ongoing GVHD requiring systemic therapy; ongoing treatment with steroids; presence of anti-HLA antibodies against TT52CAR19 batches; risk of pregnancy or non-compliance with contraception; lactating females; patients unwilling to undergo follow-up for 15 years; known hypersensitivity to any of the test materials or related compounds; intrathecal chemotherapy within two weeks of starting lymphodepletion; prior CRS/ICANS grade 3 or higher in patients treated with autologous CAR-T cell therapy.

A Bryant and Day 2-stage design was applied with stopping rules for enrolment based on lack of response (in 3/3 patients or 5/6 patients or 7/9 patients) or severe adverse reactions such as GVHD (≥ grade 2), ICANS (≥ grade 3) and CRS (≥ grade 3) (in 2/3 patients or 3/6 patients or 4/9 patients). Trial oversight was provided by an independent Data Monitoring and Safety Committee and adverse events were documented according to the Common Terminology Criteria for Adverse Events, version 5. Patient characteristics, baseline data and procedures undertaken are listed in table 2.

Manufacture of TT52CAR19

Human lymphocytes were collected under ethical approval by steady state leukapheresis from volunteers identified independently by the Nolan transplant registry and tissue typed at St Barts Hospital National Health System (NHS) Trust. All were healthy adult donors and were screened for HIV1,2, Hepatitis B/C/E, Herpes viruses (VZV, CMV, EBV), *treponema pallidum*., *toxoplasma gondii*. and HTLV-1.

Plasmids for lentiviral vector TT52CAR19 were produced by Plasmid Factory and virus stock manufactured at the Rayne Institute, King's College London and then release tested including for sterility, impurities and replication competent lentivirus by Bioreliance, as previously described(*36*) The CAR19 construct included a scFv derived from 4g7 and had been previously investigated in human studies.(*11*)

Capped, polyadenylated, and uridine modified Cas9 mRNA was produced by Trilink . A CliniMACS prodigy device with modified T-cell transduction (TCT) program was employed,(*37*) and cells were activated with TransAct anti-CD3/CD28 and cultured in TexMACS GMP medium supplemented with 3% human AB serum and 20ng/ml Human Recombinant IL-2 . Electroporation used a customised Lonza 4D device. Depletion of residual TCRαβ T cells used an anti-biotin bead kit.

Phenotype, Function and Molecular Characterization

Flow cytometry

Flow cytometry was undertaken using FacsCanto II (Becton, Dickinson BD Biosciences) at Great Ormond Street Hospital NHS trust, with additional characterisation using a BD LSRII (Becton, Dickinson BD Biosciences), and analysis using FlowJo v10 (TreeStar Inc.) (fig. S12).

Determination of vector copy number (VCN)

CAR19 transgene in transduced cells was assessed by measuring VCN on genomic DNA at the end of manufacture of TT52CAR19, and from patient blood or bone marrow samples at Great Ormond Street Hospital NHS trust. Vector copies were determined by qPCR or digital droplet PCR (ddPCR) targeting HIV-psi or human albumin sequences.(*11*)

Karyotype and Fluorescence In Situ Hybridization (FISH)

TT52CAR19 cells were cultured with Colcemid overnight in order arrest cells in metaphase. Karyotype using Giemsa-Banding (G-Banding) and fluorescence in situ hybridization (FISH) analysis was performed at Great Ormond Street Hospital NHS trust; the latter used a dual colour, break-apart FISH probe (Cytocell TCRAD LPH 047-S; 14q11, red/green fusion) to interrogate interphase nuclei.

Quantification of on- and off-target editing effects and translocations

PCR amplicons of target genomic DNA were Sanger sequenced and NHEJ events were quantified and analysed using web-based Tracking of Indels by Decomposition (TIDE) & Inference of CRISPR Edits (ICE) protocols (https://tide.nki.nl/ / https://ice.synthego.com/#/). On-target and off-target sites informed by previous Digenome-seq studies(*25, 38*) were captured using Phusion polymerase (New England BioLabs), and the products amplified using TruSeq HT Dual Index primers. Libraries were then subjected to paired-end Next Generation Sequencing (NGS) using MiniSeq (Illumina) as previously described (*39*)Demultiplexed fastq files were uploaded to Galaxy for quality checks, trimming and alignment. Non-homologous end joining (NHEJ) signatures were anlyzed using Pindel and figures were created in R. ddPCR was used to amplify and quantify predicted translocations between chromosomes 14q (TRAC) and 1p (CD52) loci (fig. S3) and analyed using Quantasoft (BioRad)

Vector integration site analysis

Lentiviral integration sites (IS) were mapped by Shearing Extension Primer Tag Selection Ligation-Mediated PCR (S-EPTS/LM PCR) (GeneWerk GmbH) as previously described (*36*) and the top 10 most frequent loci tabulated.

In vitro studies

In vitro cytotoxic function of TT52CAR19 cells was quantified by co-culture with ^{51}Cr loaded CD19+Daudi cells for 4 hours at 37°C) at increasing effector-to-target (E:T) ratios and release quantified using a microplate scintillation counter. Cytokine release was quantified after overnight co-culture of TT52CAR19 cells with CD19+Daudi cells at a 1:1 ratio using a TH1/TH2/TH17 human bead array kit (Becton Dickinson Biosciences).(*25*)

In vivo studies

In vivo function was assessed in NOD/SCID/γc (NSG) mice inoculated IV with $0.5x10^6$ CD19+ Daudi tumor cells also expressing enhanced green fluorescence protein (EGFP) and luciferase by tail vein injection, followed by effectors, transduced non-edited or non-transduced controls after 4 days. Serial bioluminescence imaging using an IVIS Lumina III In Vivo Imaging System (PerkinElmer, Living Image® version 4.5.5) was used to track leukemia inhibition for up to 5 weeks.(*25*)

Statistical analysis

Statistical analyses were performed with Prism software (GraphPad) using unpaired *t* test to compare means where indicated and ANOVA with Tukey multiple comparison post-hoc for area under the curve comparisons. Values from 3 or more samples are presented as median with interquartile range or as mean with SEM. $P < 0.05$ was considered significant.

List of supplementary materials

Figures S1 to S12
tables S1 & S2
Data File S1
TT52 CRISPR CAR group list of authors and contributors

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Author contributions: PV, KR, AV and WQ were the principal investigators and chief investigators. WQ and PV designed the study; AV, KR, GO and WQ screened, enrolled, and treated patients. GO, CG and WQ wrote the first draft of the manuscript. CG performed flow cytometry and SAG performed molecular assay for TT52CAR19 persistence. SA performed molecular assays for disease monitoring. AK and JC helped to set up the study, to organise and deliver the treatment to the patients and ensured that patients were followed according to the study protocol. CG, AE, RP and WQ developed the vector configuration and genome editing strategy and performed the pre-clinical experiments. FS and HZ manufactured the TT52CAR19 batches and performed longitudinal stability tests. SG performed the molecular characterization of the final product. CG, GO and WQ analyzed the data and created the figures of this manuscript. All authors reviewed and approved the manuscript.

Competing interests: WQ/CG/RP/SG/AE; UCLB has filed intellectual property in relation to Therapeutic cells (WO/2018/115887; PCT/GB2017/053862) and U6 minimal promoter (WO/2020/183197; PCT/GB2020/050651). WQ interests unrelated to the work; Tessa Therapeutics, Wugen, Novartis, Kite, Autolus & Virocell.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. Lentiviral plasmids generated in this study are available upon request from the corresponding author upon providing a completed material transfer agreement. Translocation quantification analysis was done on Quantasoft (BioRad) and in R using twoddPCR as previously described(*39, 40*). Raw NGS files were deposited in the NIH BioProject database (BioProject ID PRJNA862892).

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The TT52 CRISPR-CAR Group

In addition to the TT52 CRISPR-CAR Group members who are authors (Christos Georgiadis, Giorgio Ottaviano, Soragia Athina Gkazi, Farhatullah Syed, Hong Zhan, Annie Etuk, Roland Preece, Agnieszka Kubat, Waseem Qasim, Jan Chu, Stuart Adams, Paul Veys, Ajay Vora, and Kanchan Rao), the following TT52 CRISPR-CAR Group members are collaborators who have contributed to study design, data analysis, and interpretation:

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Figure legends

Figure 1: Manufacturing and characterization of TT52CAR19 T cells of Good Manufacturing Practice (GMP) compliant cell bank 2 (GMP2). (A) Map of TT52CAR19 transfer vector and delivery system. Self-inactivating (SIN) lentiviral configuration (comprising Rev Response Element, (RRE) and central polypurine tract, (cPPT) elements for efficient gene transfer) showing coupling of terminal TRAC (TT) and CD52 (52) CRISPR guide RNAs, including non-homologous scaffolds, under the control of mini H1 and U6 promoters in the deleted unique (ΔU3) region, proximal to repeat (R) elements of the 3' long terminal repeat (3'LTR), with expression of a CAR19 transgene, under the control of a human PGK (Phosphoglycerate kinase) promoter. Coupling CAR expression with RNA guides allowed selective genome editing of transduced cells after providing Cas9 mRNA through electroporation (EP). A schematic timeline for manufacturing is provided: healthy donor peripheral blood mononuclear cells (PBMC) were activated and transduced on the following day with lentiviral vector. After two days in culture, cells were electroporated for delivery of Cas9 mRNA. On day (D)11 cells underwent automated TCRαβ T cell depletion. After this last step, a final cell product was harvested and cryopreserved. **(B)** CAR19 expression (>97%), residual TCR (<1%) and CD52 negativity (>70%) in peripheral blood lymphocyte (PBL) PBL-TT52CAR19 T cells was verified by flow cytometry at day 0 (D0) and end of production (D12) timepoints against non-transduced (UnTD) or transduced but non-edited controls. **(C)** Quantification of on-target editing by direct sequencing and bioinformatic analysis of Indels following NHEJ repair corroborated multiplexed knockout. **(D)** Digital droplet PCR (ddPCR) captured low frequency translocation events (blue dots) C1-C4 arising between the edited TRAC and CD52 loci **(E)** Ligation mediated PCR (LMPCR) detection and quantification of vector integration sites where the top 10 most frequent sites comprised <0.1% of integrants. **(F)** Circos plots and reported frequencies (table) from next generation sequencing of NHEJ editing signatures at the two on-target sites, TRAC-01 (solid red line marking locus in outer yellow circle), CD52-01 (solid red line marking inner yellow circle) and at predicted off-target sites TRAC-02-TRAC-07 (red arrows marking outer yellow circle) and CD52-02-CD52-07 (red arrows marking inner yellow circle).

Figure 2: Clinical response and TT52CAR19 tracking. (A) Measurement of serum alemtuzumab concentration within the first 28 days after TT52CAR19 infusion (D0) in patients. Lymphodepleting threshold is noted by the dotted line. **(B)** Expansion of infused TT52CAR19 measured by vector copies kinetics in peripheral blood mononuclear cells (PBMC) of patients using ddPCR up to three months (M3) post allogeneic stem cell transplantation (Allo-SCT), denoted by the arrowhead. **(C)** Longitudinal monitoring of chimerism for signals of TT52CAR19 and transplant donor or recipient in PBMC. (**D**)Phenotypic profiling of PBMC from P7 and P8 by flow cytometry (day +21) measuring PBLTT52CAR19+TCRαβ-CD52- T cells. **(E)** Serum concentration of IL-6 and **(F)** ferritin after infusion, (biomarkers of cytokine release syndrome, in six patients receiving TT52CAR19.

Figure 3: Disease monitoring and outcome. Monitoring of minimal residual disease (MRD) (log scale) at Screening; Day 0 (D0); Day +28 (D28); 1 month (M1) post- AlloSCT by molecular (qPCR) minimal residual disease with immunoglobulin gene rearrangement signatures (MRD-PCR, grey) and by leukemia-associated immune phenotype with flow cytometry (MRD-LAIP, blue). Arrowheads indicate TT52CAR19 therapy and allo-SCT.

Figure 4: Peripheral blood count kinetics and toxicities in patients. Blood absolute lymphocyte (blue) and neutrophil (orange) counts during treatment and follow-up (log scale). Arrowhead indicates timing of TT52CAR19 infusion and day 0 of allo-SCT. Timing of onset and duration of GVHD (pink), cytokine release syndrome (CRS, red) and immune effector cell-associated neurotoxicity syndrome (ICANS, grey)

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are represented as rectangles with maximum grading (from I to IV) within brackets. Arrows represents timing of treatment with tocilizumab (TCZ, purple) and siltuximab (STX, brown).

Tables

Table I: Release specifications and data from TT52CAR19 cell banks generated from three different healthy donor volunteers. All three Good Manufacturing Practice (GMP) compliant batches (GMP1, GMP2 and GMP3) were tested to meet the release criteria (first column) according to good manufacturing practice and quality assurance. Minimal target criteria are shown in the second column. High CAR19 expression and low residual TCR expression were achieved using the coupled CRISPR-CAR vector system. Abbreviations: TCR, T cell receptor; RCL, replication competent lentivirus.

	Target	TT52CAR19 GMP1	TT52CAR19 GMP2	TT52CAR19 GMP3
Viability	≥65% (Pre-cryopreservation)	99.6%	99.7%	99.8%
CAR19 Transduction	≥50% CD45+CAR+ cells	86.1%	93.7%	98.4%
TCR depletion (final)	≤3% CD45+TCRαβ+ cells	0.7%	1.1%	0.1%
Vector copy number	≤7 copies /cell	3.98 copies /cell	2.94 copies /cell	4.57 copies /cell
Sterility (culture)	No Growth	No Growth	No Growth	No Growth
Sterility (Gram stain)	No Organisms Seen	No Organisms Seen	No Organisms Seen	No Organisms Seen
Mycoplasma	None Detected	None Detected	None Detected	None Detected
Endotoxin	≤2.00 EU/ml	<1.00 EU/ml	<1.00 EU/ml	<1.00 EU/ml
RCL	No RCL detected	No RCL detected	No RCL detected	No RCL detected

Table II: Patient characteristics and TT52CAR19 treatment outcomes. Of six children infused, all had previously received autologous CAR19 therapy or had undergone a first Allo-SCT. Number in brackets (*n*) represent the months between previous treatment and trial inclusion. *P8 had previously received two infusions of autologous CAR19 T cells, in brackets months between latest infusion and trial inclusion.

Abbreviations: BiTE, Bispecific T cell engager (blinatumomab); HSCT, hematopoietic stem cell transplantation; CAR19, chimeric antigen receptor T cell therapy; CR, complete remission; InO, inotuzumab ozogamicin; Pre-LD, pre-lymphodepletion; Mol MRD, molecular minimal residual disease; Vinc, vincristine; Dex, dexamethasone; Pred, prednisolone; HD-ARAc, high-dose cytarabine; n/a, not assessable. A&W, Alive and well.

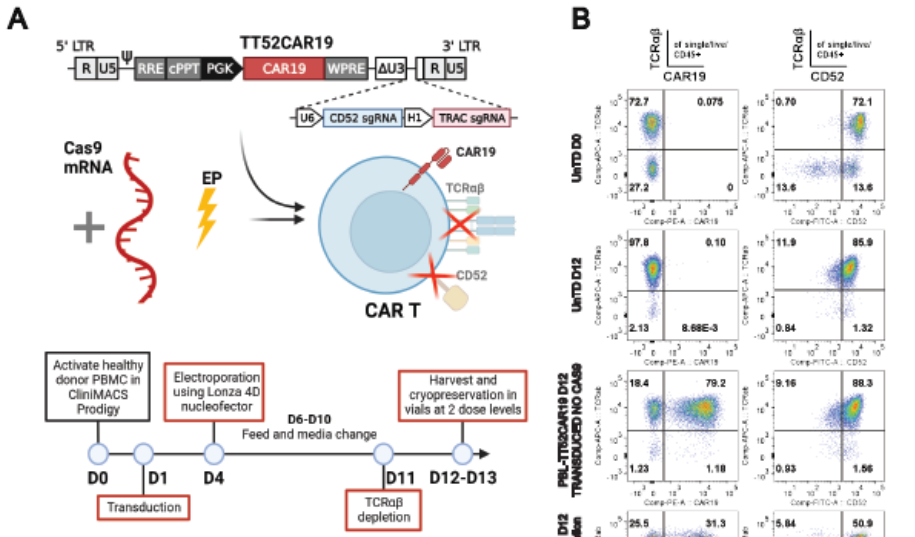
	P2	P3	P5	P6	P7	P8
Age (years)	2,5	2,1	1,2	3,1	1,4	11,7
Disease	Infant ALL	Infant ALL	Infant ALL	Infant ALL	Infant ALL	pB-ALL
Genetics	MLL	MLL	MLL	MLL	MLL	ETV6-RUNX1
Relapse (n)	1	2	3	2	1	3
Previous SCT	Y (20m)	Y (17m)	Y (4m)	Y (27m)	Y (5m)	N
Previous CAR19	N	Y (4m)	N	Y (20m)	N	Y (14m)*
Previous BiTE	N	N	Y (5m)	N	N	Y (2m)
Extramedullary disease	N	N	N	Y	N	N
Debulking	InO	InO	Vinc/Dex	Vinc/Dex	Vinc/Pred	HD-ARAc
Disease burden pre-LD	3.2%	21%	66%	0.63%	3.2%	58%
Disease burden D0	1.8%	n/a	n/a	$2.8x10^{-5}$	3%	0.26%
TT52CAR9 cell bank	GMP2	GMP2	GMP2	GMP2	GMP3	GMP3
Cell dose (10^6 cells/kg)	1.3	1.0	1.0	1.4	1.0	1.3
D+28 MRD	Flow & PCR NEG	Flow POS	Flow & PCR NEG	Flow POS	Flow & PCR NEG	Flow NEG Mol $1.4x10^{-4}$
Outcome	CR	Refractory	Relapse post SCT	Refractory	CR	Relapse post SCT
Status	A&W 12m post SCT	Died	Died	Died	A&W 3m post SCT	Died

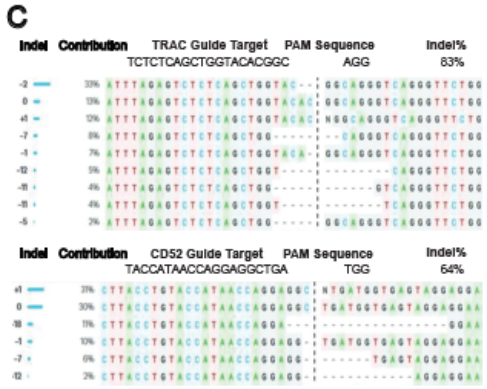
Table III: Adverse events following lymphodepletion and TT52CAR19 infusion. Neutropenia at day +28 (D28) was defined as absolute neutrophils count <1.5x10^9/L or supported by granulocyte colony stimulating factor (G-CSF). Thrombocytopenia at D28 was defined platelet count <150x10^9/L or requiring platelets transfusion in the last 5 days. Skin GVHD confirmed on biopsy occurred in one patient (P7) after day D28, and resolved after Allo-SCT. Another patient (P2) developed grade 2 GVHD after her second HSCT and required systemic steroids for three weeks. One dose limiting toxicity occurred in one patient (P7) with severe neurotoxicity (grade 4) and resolved without sequelae. Grading of adverse events was attributed according to Common Terminology Criteria of Adverse Events (CTCAE v5.0), with the exception of GvHD (graded according to CIBMTR criteria), CRS and ICANS (graded according to ASTCT consensus criteria).

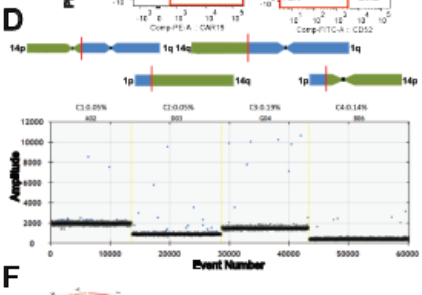
Abbreviations: CRS, cytokine release syndrome; GVHD, graft versus host disease; ICANS, immune effector cell-associated neurotoxicity syndrome.

	Any grade	Grade I-II	Grade III	Grade IV	Grade V
CRS	6	6	0	0	0
ICANS	2	1	0	1	0
GVHD	2	2	0	0	0
Infections					
Viral	8	1	7	0	0
Bacterial	2	0	2	0	0
Fungal	0	0	0	0	0
Thrombocytopenia (d+28)	5	1	0	4	0
Neutropenia (d+28)	6	0	1	5	0
Lymphopenia (d+28)	6	1	2	3	0

Figure 1







E

Top 10 IS Gene Names, Frequencies and Location

Rank	Gene Name	Frequency [%]	Location
Top1	LOC728175	0.051	4+184306101
Top2	FBXO34	0.044	14-55311249
Top3	UMC1	0.041	5+176965536
Top4	CMTR1	0.039	6+37470107
Top5	CLDN10	0.039	13-95425373
Top6	HSPBP1	0.038	19-55270895
Top7	PAXBP1	0.038	21+32738033
Top8	TSR1	0.037	17-2332908
Top9	TMEM259	0.035	19-1016849
Top10	FYN	0.034	6+111878067
#All Other mapp. IS	21938	99.604	

Seq Count 10 Strongest	335
Seq Count all other mapp. IS	84290
Total Seq Counts Used	84625

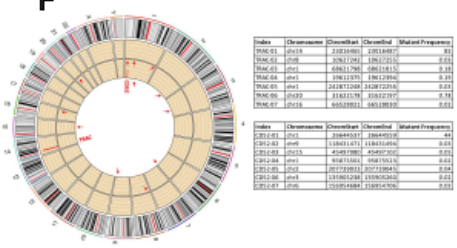


Figure 2

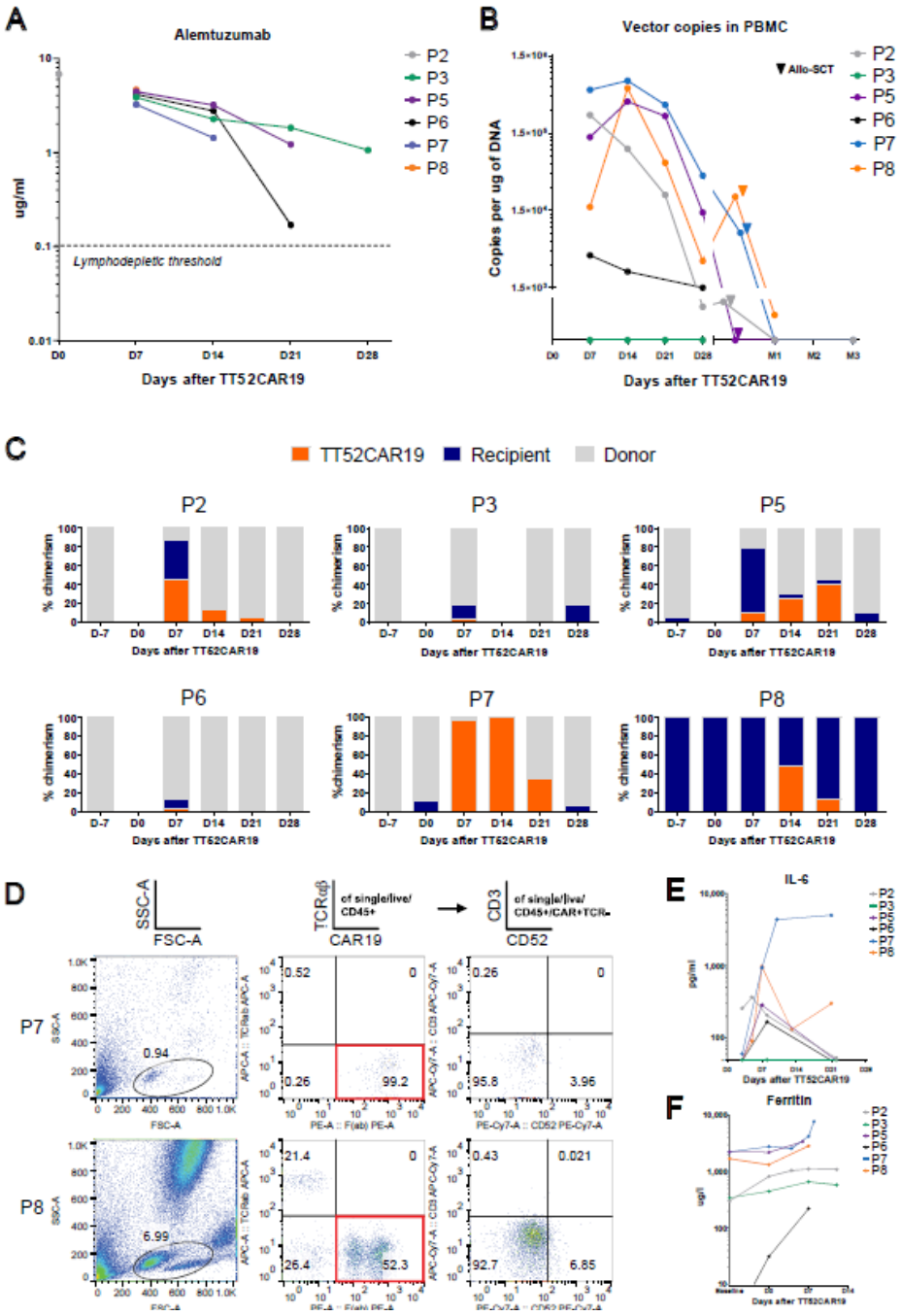

A
Alemtuzumab
ug/ml
Lymphodepletic threshold
Days after TT52CAR19
P2
P3
P5
P6
P7
P8
B
Vector copies in PBMC
Copies per ug of DNA
Allo-SCT
Days after TT52CAR19
C
TT52CAR19
Recipient
Donor
P2
P3
P5
P6
P7
P8
% chimerism
Days after TT62CAR19
D
SSC-A
FSC-A
TCRαβ
CD45+
CAR19
CD3
CD45+/CAR+TCR-
CD52
P7
P8
0.94
0.52
0
0.26
99.2
0.26
0
95.8
3.96
6.99
21.4
0
26.4
52.3
0.43
0.021
92.7
6.85
E
IL-6
pg/ml
Days after TT52CAR19
F
Ferritin
ug/l
Days after TT52CAR19

Figure 3

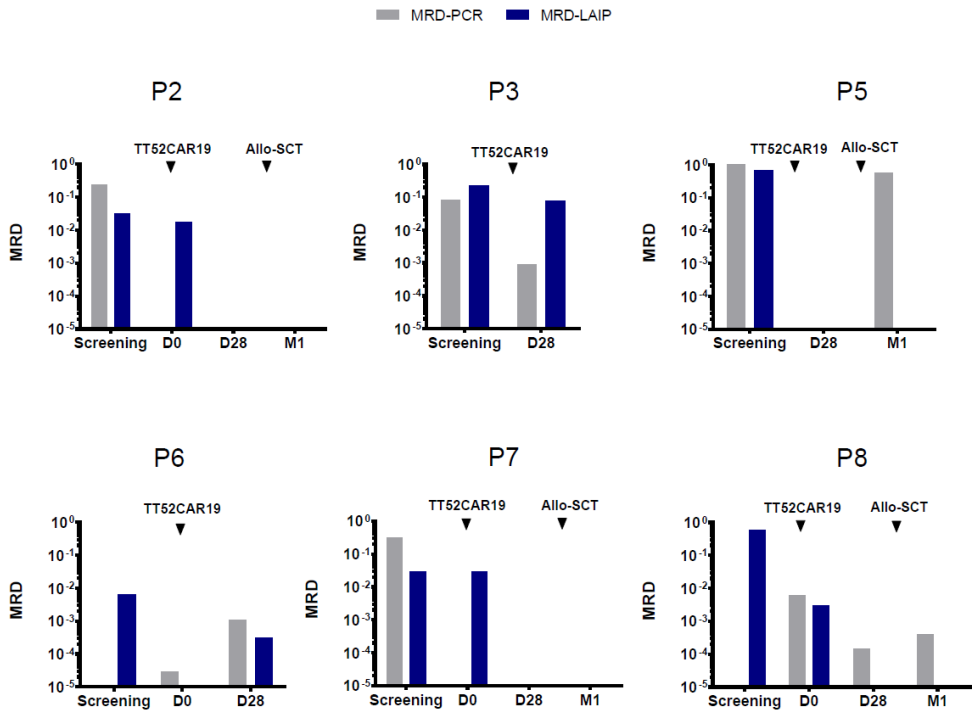
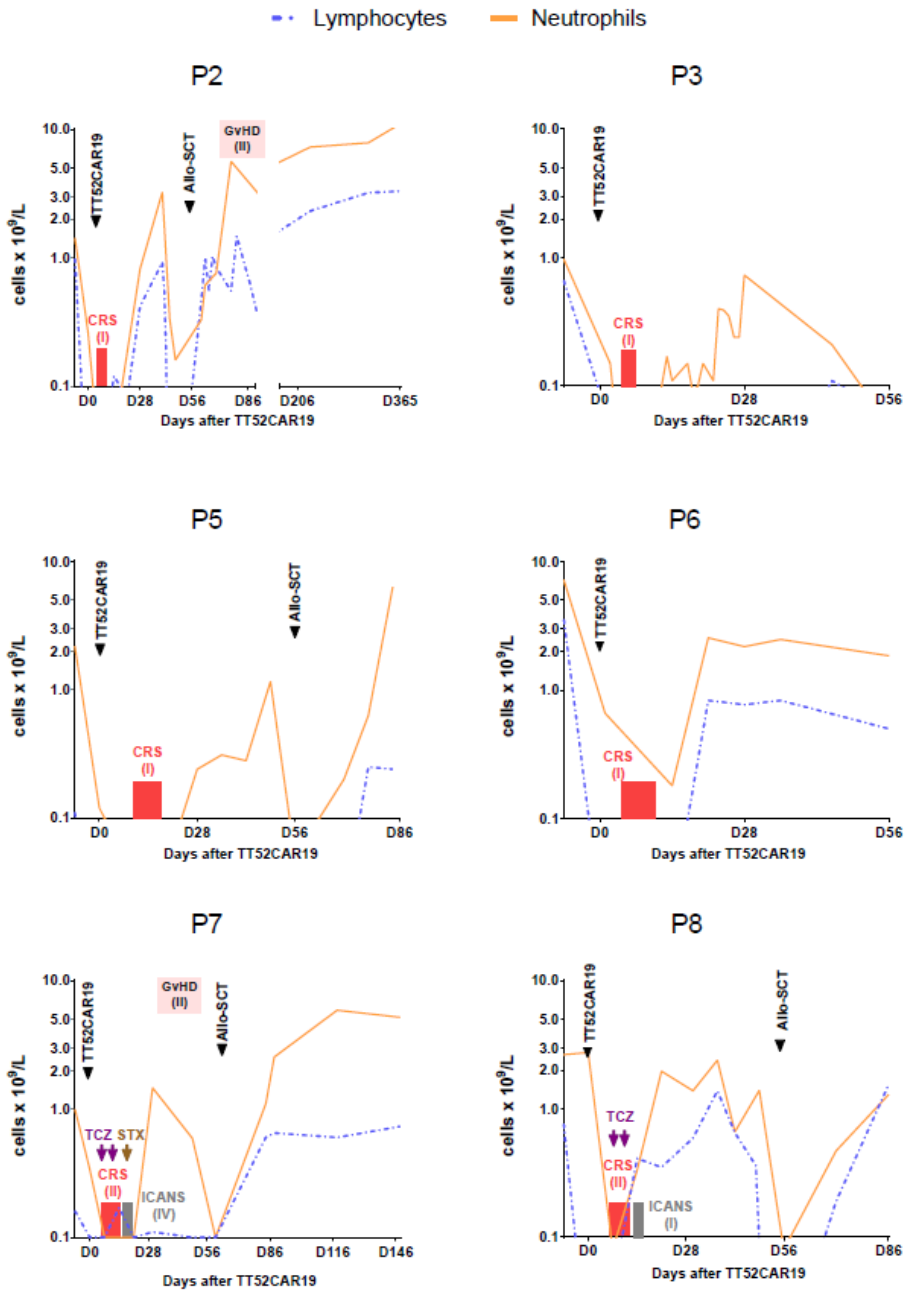

MRD-PCR
MRD-LAIP
P2
P3
P5
P6
P7
P8
TT52CAR19
Allo-SCT
MRD
Screening
D0
D28
M1

Figure 4


Lymphocytes
Neutrophils
P2
P3
P5
P6
P7
P8
cells x 10⁹/L
Days after TT52CAR19
TT52CAR19
Allo-SCT
GvHD (II)
CRS (I)
CRS (II)
TCZ
STX
ICANS (IV)
ICANS (I)
D0
D28
D56
D86
D116
D146
D206
D365