Title: Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells.

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One Sentence Summary: Universal gene-edited CAR19 T cells eliminate infant leukemia.

Abstract:

Autologous T cells engineered to express chimeric antigen receptor against the B cell antigen CD19 (CAR19) are achieving remarkable leukemic remissions in early phase trials, but can be difficult to manufacture, especially in infants or heavily treated patients. We generated universal CAR19 T cells (UCART19) by lentiviral transduction of non-HLA matched donor cells and simultaneous transcription activator-like effector nuclease (TALEN) mediated gene-editing of T cell receptor alpha-chain and CD52 gene loci. Two infants with relapsed refractory CD19+ B cell acute lymphoblastic leukemia received lymphodepleting chemotherapy and anti-CD52 serotherapy, followed by a single dose infusion of UCART19 cells. Molecular remissions were achieved within 28 days in both infants, and UCART19 cells persisted until conditioning ahead of successful allogeneic stem cell transplantation. This bridge-to-transplantation strategy demonstrates the therapeutic potential of gene-editing technology.

Introduction

Despite extensive pre-clinical advances, to date there has been very limited application of DNA nucleases for therapeutic gene editing(*1*). Here we describe first-in-human use of TALEN (transcription activator-like effector nuclease) gene-edited T cells in two infants with refractory relapsed B cell acute lymphoblastic leukemia (B-ALL), and the successful induction of molecular remission ahead of allogeneic stem cell transplantation (allo-SCT).

Autologous T cells engineered to express chimeric antigen receptors (CARs) against leukemia antigens such as CD19 on B cells can be highly efficacious in refractory relapsed leukemia(*2-8*). In heavily treated cancer patients, and especially in infants, it is not always possible to manufacture an effective therapeutic product(*9*). In some circumstances, it may be possible to use donor T cells from HLA-matched allogeneic hematopoietic stem cell donors as part of a transplant strategy(*10, 11*), but the CAR T cell manufacturing process is still bespoke and time consuming. Access to CAR-T cell therapies is still relatively limited, and wider application requires specialized manufacturing facilities, expertise, and logistical support to collect, manipulate, and deliver cells. Alternative approaches using off-the-shelf therapies derived from non-matched donors are highly attractive, but must overcome criticaHLA barriers as well as homeostatic competition from the host T cell compartment. Crucially, HLA-disparate T cells must evade host-mediated immunity and deliver anti-leukemic effects without graft versus host disease (GVHD). Lymphodepletion of recipients reduces homeostatic T cell competition and minimizes the risk of host-mediated rejection of allogeneic cells(*12*). Immunosuppressive agents such as fludarabine and cyclophosphamide may be combined with antibody therapies such as alemtuzumab (anti-CD52) or antithymocyte globulin (ATG). Of note, serotherapy used during conditioning can persist for many weeks and can impact incoming donor lymphocyte infusions(*13*). Genetic disruption of CD52 expression through gene editing of T cells was designed to allow infused cells to evade the depletion effects of alemtuzumab, and with simultaneous editing of T cell receptor genes to reduce the risk of GVHD, we demonstrate the feasibility of generating a 'universal' CAR T cell bank. Here we report the successful application of such cells targeting CAR19 in two HLA mis-matched infants with relapsed refractory B-ALL.

Results

TALEN engineered CAR19 T cells

We used TALENs to disrupt the CD52 gene, the target antigen of alemtuzumab, in T cells transduced to express a chimeric antigen receptor against CD19 (CAR19) (Fig. 1A). This was designed to enhance survival in the presence of the anti-CD52 lympodepleting antibody. At the same time we used a second TALEN pair to target the constant region of the T cell receptor alpha chain (TRAC), thereby disrupting cell surface expression of TCRαβ. These manipulations were performed at high efficiency as a multiplex operation by electroporation of TALEN mRNA into lentiviral transduced CAR19 T cells(*14*). Residual TCRαβ T cells were stringently removed by CliniMacs magnetic bead depletion to minimize the risk of GVHD(*15*). The vector configuration also incorporated a 2A peptide-linked sort/suicide gene RQR8, comprised of a 136 amino acid cell surface protein which includes a CD34 epitope for cell enrichment and the CD20 target epitope for rituximab for in vivo depletion in case of adverse effects(*16*).

A bank of non-HLA matched, universal, CAR19 T cells (UCART19) was manufactured from a healthy female donor under GMP conditions (Fig. 1B). Expression of CAR19 was present on 85% of cells, and over 64% of

cells were depleted of both TCRαβ and CD52 on flow cytometric analysis. Further, after magnetic bead-mediated depletion of residual TCRαβ cells, only 0.7% of cells had detectable cell surface TCR (Fig. 1C). Additional subset analysis confirmed the presence of key naïve (CD45RA$^+$CD62L$^+$), central memory (CD45RA$^-$CD62L$^+$), and effector (CD45RA$^+$CD62L$^-$) T cells (Fig. 1C). RQR8 expression was detected on only 19.9% of cells, which was unexpectedly discordant from the high expression of CAR19, despite linked transcription and translation through a self-cleaving 2A peptide configuration. Cytogenetic analysis found that 5/100 metaphase spreads exhibited an abnormal karyotype, and a FISH probe targeting the TRAC locus detected rearrangements in around 4% of nuclei examined (fig. S1). This was consistent with previous studies that have detected translocations between TRAC and CD52 at frequencies between 10^{-4} & 10^{-2} by quantitative PCR(*14*). Next generation sequencing (NGS) detected on-target events indicative of non-homologous end joining (NHEJ; insertions, deletions, and indels) at a frequency of >70% for both TRAC and CD52 loci (Fig. 1D), and similar events at a frequency of <0.18% at each of fifteen in-silico predicted off-target TALEN sites (table S1). The batch was subjected to detailed release characterization, including screening for replication-competent lentivirus and potency assessments in a human:murine chimeric model. Therapy was provided for two infants under UK special licence arrangements after institutional ethical reviews. Both had relapsed refractory CD19$^+$CD52$^-$ B-ALL and had previously undergone haematopoietic stem cell transplantation (allo-SCT) but had subsequently relapsed and failed other experimental interventions.

Subject 1

In June 2015, an 11-month-old mixed race infant with high risk t(11;19) CD19$^+$ infant B-ALL had suffered bone marrow relapse 3 months after receiving fludarabine (150 mg/m^2), treosulphan (36 g/m^2), thiotepa (10 mg/kg), ATG (60 mg/kg) in combination with an 8/10 mismatched (single HLA-A/DQ alleles) allo-HSCT from an unrelated male donor. Her disease had progressed with 70% blasts in marrow despite treatment with blinatumomab, an experimental bi-specific T-cell engager (BITE) monoclonal antibody therapy(*17*). After one dose of vincristine and asparaginase and 7 days of dexamethasone, there were still blasts in her bone marrow, and she received cytoreduction with fludarabine (total dose 90 mg/m^2), cyclophosphamide (total dose 1.5 g/m^2), and alemtuzumab (total dose 1 mg/kg) (Fig. 2A). She received 4.6x10^6/kg CAR19$^+$TCR$^-$ T cells in a single dose without any immediate toxicity at a time when her bone marrow exhibited persisting disease (fig. S2), as measured by two MRD markers (Fig. 2B). There were no infusion-related toxicities and no evidence of cytokine release syndrome, with unperturbed concentrations of key serum cytokines (IFNγ, TNFα, IL1β, and IL6) measured at frequent intervals for 28 days (fig. S3). GCSF-dependent recovery of neutrophils was noted by day 30, but this was then followed by protracted multi-lineage cytopenia until a second allo-SCT (Fig. 2A). Although the patient was profoundly cytopenic, molecular chimerism studies in blood and marrow revealed an increasing presence of well-demarcated third party (UCART19) cells, reaching 27% in marrow by six weeks, as well as low level persistence of residual recipient cells (3-7%) (Fig. 2C). There was sustained cytogenetic and molecular remission from this time onwards. Vector copy number (VCN) was used to track UCART19 cells in blood and marrow (Fig. 2D), and when possible this was supported by flow cytometry using anti-F(ab) staining (fig. S4). The majority of UCART19 cells were CD4$^+$ in both blood and marrow (fig. S4A). Additional analysis at 8 weeks detected a greater proportion of CD3$^+$ than CD3$^-$ CAR19$^+$ T-cells, suggesting persistence and expansion of

residual TCR$^+$ cells from the UCART19 infusion (fig. S4B). Flow cytometric sorting of these lymphopenic samples was technically challenging, and the purity of enrichment was limited to around 70% based on VNTR signatures (Fig. 2E). The frequency of NHEJ events in these populations was estimated after nested PCR amplification at the TRAC and CD52 loci to be 18-27% and 64-73%, respectively (Fig. 2E). This provided additional evidence of persistence of both TCR$^-$ and TCR$^+$ cells and was consistent with survival of gene-edited, CD52 negative cells after alemtuzumab exposure. Grade 2 skin GVHD was confirmed by histology at 9 weeks (fig. S5) and was initially managed with topical steroids, but because UCART19 cells were still detectable (by quantitative PCR for VCN) after rituximab (4x375 mg/m^2), systemic steroids were subsequently administered. The infant was then conditioned using ATG (total dose 4.5 mg/kg), fludarabine (total dose 120 mg/kg), cyclophosphamide (total dose 60 mg/kg), thiotepa (total dose 5 mg/kg), and TBI (4 Gy) ahead of a second TCRαβ cell-depleted allograft from the original mismatched unrelated donor to correct aplasia and accelerate reconstitution. By one month after eradication of UCART19 cells and transplantation, the child had full donor chimerism in blood and marrow, and was at home in complete remission. Molecular MRD markers have remained negative and the infant is well 16 months after UCART19 therapy, with full donor chimerism and normalized lymphocyte profiles.

Subject 2

A second female infant, aged 16 months of Caucasian parents was treated using a similar protocol in December 2015. She had been diagnosed at 4 weeks of age with high-risk congenital MLL-rearranged B-ALL, for which she had received a matched unrelated donor SCT after conditioning with treosulphan (total 30 g/m^2), fludaribine (total 150 mg/m^2), thiotepa (total 10 mg/kg), and ATG (total 10 mg/kg) in January 2015 while in molecular remission. Her leukemia relapsed in November 2015, with 78% blasts in marrow but no evidence of CNS disease. There had been no response to blinatumomab, and bone marrow after cytoreduction exhibited 3% blasts. Lymphodepletion consisted of fludarabine (total dose 150 mg/m^2), cyclophosphamide (total dose 120 mg/kg), and alemtuzumab (total dose 1 mg/kg) and was followed by single infusion of 4.0x10^6/kg CAR19$^+$ TCR$^-$ T cells (Fig. 3A). There were no acute infusion-related toxicities, and a transient erythematous rash was immediately responsive to topical steroids. Symptoms of irritability were investigated in the third week after infusion, but cranial CT imaging was unremarkable and cerebrospinal fluid (CSF) was clear with normal protein and glucose. Laboratory investigations confirmed the presence of UCART19 cells by flow cytometry, as well as chimerism and VCN in peripheral blood at two weeks and in marrow samples after 4 weeks during molecular remission (Fig. 3B, C, D). Of note, flow cytometric analysis found that all circulating UCART19 cells were CD3$^-$CD52$^-$ by this latter time point (Fig. 3E)

The infant had donor-derived neutrophil recovery (confirmed to be derived from the original transplant donor) and went on to have a successful second allogeneic SCT from the same (10/10) donor within ten weeks of UCART19 therapy. This was undertaken after rituximab (375 mg/m^2) therapy, followed immediately by conditioning with ATG (total dose 10 mg/kg), fludarabine (total dose 120 mg/kg), cyclophosphamide (total dose 120 mg/kg), and TBI (2 Gy). Subsequent marrow examinations have been MRD-negative with undetectable UCART19 cells, and the child remains clinically well at home, without evidence of GVHD, 10 months after therapy.

Discussion

This therapeutic application of TALEN-engineered cells highlights the feasibility and potency of gene-editing strategies for the delivery of anti-tumor immunity. The potential of CAR19 therapies has already been demonstrated in the autologous (2-9) and HLA-matched allogeneic settings (10, 11), but the infrastructure and expertise required to produce personalized cell products present challenges, and low T cell counts in heavily treated individuals may preclude autologous approaches. An alternative strategy, using pre-manufactured, 'off-the-shelf' engineered T-cells is highly attractive but must overcome HLA barriers in terms of both host-mediated rejection and donor-derived alloreactivity.

We used TALEN-mediated cell engineering in combination with lentiviral transduction to produce universally applicable CAR19 T cells, designed to target B cell leukemia, but >99% depleted of endogenous TCR and resistant to the effects of in vivo serotherapy directed against CD52. This allowed their application in a third party setting in two infants who had relapsed after conventional myeloablative allo-SCT. The manufacture of autologous CAR19 T cells was not feasible in these lymphopenic infants, and attempting a second allo-SCT in the setting of refractory relapse was considered futile.

Host preparation involved lymphodepletion with a combination of immunosuppressive chemotherapy and alemtuzumab (anti-CD52) and, in both cases, the absence of CD52 expression on the leukemic blasts suggests that the disease was not directly targeted by the antibody. Reduction of disease burden during lymphodepletion may have accounted for the absence of subsequent cytokine release syndrome(18), but previous relapses after myeloablative chemotherapy, allogeneic SCT, and BITE therapy with blinatumomab underlined the aggressive nature of the leukemia in these infants. The inoculated cell dose of 5×10^7 T cells used in both cases had been cryopreserved in pre-filled vials manufactured for a proposed Phase 1 dose-escalation study, and for these infants provided $4-5\times10^6$ CAR19 T cells/kg . After processing through a TCRαβ depletion column, less than 1% of T cells exhibited TCRαβ expression, but nonetheless carriage of such a population was recognized to still constitute a risk for possible GVHD. In haploidentical transplantation using TCRαβ depleted stem cell grafts there is often a threshold effect for GVHD above 5×10^4/kg mismatched T cells(19), and our usual practice is to aim to infuse $<1\times10^4$/kg in such a setting. However, in these refractory infants, we prioritized the maximum CAR19+ T cell dosing available in each pre-filled vial and accepted the risk that residual TCRαβ carriage may breach thresholds for GVHD. In both patients, UCART19 cells could be detected by PCR as early as two weeks after infusion in both subjects, although detailed quantification in blood and bone marrow was only possible after 4 weeks in the first patient. Highly sensitive molecular MRD studies showed disease remission, and this was confirmed by flow analysis and cytogenetic studies on serial marrow samples. In P2, flow cytometry revealed the presence of CAR19^{+}CD3^{-} T cells, and these were all CD52^{-}; however, in P1, analysis of bone marrow by 8 weeks revealed the majority of CAR19^{+} cells were CD3^{+}. Molecular analysis of TALEN target loci in CAR^{+} cells isolated by flow cytometric sorting revealed a high frequency of NHEJ events at the CD52 site, consistent with survival of CD52-negative T cells after alemtuzumab. A reduced NHEJ event frequency was detected at the TCR locus, and this was in keeping with the emergence of CD3^{+} T cells in both bone marrow and peripheral blood. In subject 1, almost all UCART19 cells isolated from the bone marrow and peripheral blood were CD4^{+}, and although it is unclear how these cells contributed to GVHD, strikingly similar findings were recently

reported in murine allogeneic CAR19 experiments, where GVHD developed in the presence of CD19^{+} leukemia (*20*). There had been transient recovery of neutrophils at 3-4 weeks supported by GCSF, and this was followed by a second period of cytopenia and subsequent manifestations of GVHD in the skin. Of note, MRD was undetectable by the time GVHD developed, but we speculate that residual TCR^{+} cells capable of allo-recognition survived and may have been driven by constitutive signaling via the 41BB domain of the CAR and were then able to expand in the absence of homeostatic competition in this lymphodepleted host. It is possible that in subject 1, GVHD contributed to anti-leukemic activity, but did not manifest clinically for several weeks after the induction of molecular remission. The manifestations of GVHD were limited to skin and marrow, in contrast to typically multi-system and usually fulminant complications of transfusion-associated GVHD, which can occasionally be mediated by mismatched allogeneic lymphocytes transferred in blood transfusions to immunodeficient hosts (*21*).

In subject 2, there were only transient features suggestive of mild skin GVHD that reversed promptly with topical steroids and an episode of unexplained irritability within the first three weeks after infusion. There were no active abnormalities of CSF or brain imaging, but transient UCART19 mediated neurotoxicty may have occurred and resolved without intervention. Subject 2 shared three HLA class 1 alleles with the UCART19 batch used, whereas subject 1 was mismatched at all loci, and this may have been a driver for greater alloreactivity for residual TCR^{+} UCART19 cells in the first subject.

There was a theoretical concern that TALEN-induced translocations (or other effects, including off-target DNA scission) could cause genotoxicity or other adverse effects, but a time-limited application of UCART19 ahead of allo-SCT provided an important safeguard for this first clinical use. UCART19 cells carry RQR8,(*16*) a sort-suicide gene moiety comprising CD34/CD20 epitopes transcriptionally linked to CAR19, but unexpected uncoupling from CAR19 may have resulted in reduced sensitivity to CD20-targeting rituximab. This phenomenon needs to be investigated further, but a strategy incorporating pre-transplant conditioning ensured that UCART19 were eliminated completely. Of note, conversion to full donor chimerism by allo-SCT once molecular remission is achieved could have clear advantages in reducing the risk of relapse and the evolution of CD19^{-} escape variants or clonally related malignancies in other lineages, as described recently after CAR19 therapy (*22*). An additional advantage of this approach is the avoidance of long-term depletion of healthy B cells, as encountered in autologous studies (*23*), which would otherwise necessitate the use of long-term immunoglobulin replacement therapy.

This application of an emerging technology has provided a demonstration of the potential of gene-editing strategies for engineered cell therapies, albeit with a clinical experience limited to two infants. Important caveats include concerns relating to residual risks of GVHD, carriage of translocations and other genetic changes by the edited cells, and the use of lympodepleting agents and their attendant risks of viral reactivation and other complications. Additional limitations applicable to CAR therapies more broadly, including cytokine release phenomena, neurotoxicity, and the possibility of disease relapse through mutational escape of target antigens, must also be considered and will need to be investigated in structured clinical trials. Phase 1 trials for UCART19 cell therapy in both children (NCT02808442) and adults (NCT02746952) are now underway, with weight-targeted dose-banding arrangements and stringently capped carriage of residual TCR$\alpha\beta$ T cells to $<5 \times 10^4$/kg. As experience with gene-editing tools develops, further applications may involve targeting a variety of genetic loci involved in immune recognition and checkpoint pathways.

Materials and Methods

Study Design

Named patient, open-label, non-blinded, compassionate therapy was undertaken in two infants ahead of planned Phase 1 clinical trials.

Regulation, ethics, and consent

Treatment was provided by the UK National Health Service under the MHRA specials licence number MS 17328, and with the approval of institutional Drug & Therapeutics Board, after consultation of Great Ormond Street Hospital ethics committee and with informed parental consent. Cellectis SA gave their permission for use of UCART19 cells under these arrangements.

GMP cell manufacturing

A third generation self-inactivating lentiviral configuration encoding the 4g7 CAR19 (CD19scFv-41BB-CD3ζ), HIV central polypurine tract (CPPT), Rev Responsive Element (RRE), and mutated Woodchuck post regulatory element (WPRE) was manufactured and released as previously described (*24, 25*). Healthy donor peripheral blood mononuclear cells (PBMNC) were supplied cryopreserved (HemaCare) and were thawed and then activated with anti-CD3, anti-CD28 Expact beads (Miltenyi Biotec) in X-Vivo 15 medium (Cambrex) supplemented 5% human AB serum and 100 ng/ml interleukin-2 (IL2) (Miltenyi Biotec). After a single round of lentiviral transduction, the beads were removed by CliniMacs magnetic depletion, and the cells were subjected to multiplex gene editing by electroporation of high grade TALEN mRNA (Eufets) targeting TRAC and CD52. Cells were suspended in BTXpress cytoporation media-T in a customised GMP compliant electroporation chamber for use with an Agile Pulse device (BTX Harvard Apparatus). Thereafter, cells were transferred to a Wave Bioreactor 2/10 (GE Healthcare) and expanded for up to ten days before being depleted of residual TCR expressing cells by CliniMACS TCRαβ depletion (Miltenyi Biotec). The resulting cell product was cryopreserved in dose aliquots of 50 million cells.

Flow cytometry

Flow cytometry (BD LSRII) and cell sorting (FACSAria III) were performed using routine T cell panels (BD Biosciences Multitest 6 color BTNK antibodies) with the addition of goat anti-mouse IgG F(ab)2 (Jackson Immunoresearch) for CAR19 detection and mouse anti-human Qbend10 (Miltenyi Biotec) for human CD34 staining of RQR8.

Chimerism, minimal residual disease, and vector copy number

Vector copy number (VCN) was determined by real-time quantitative PCR using 100 ng of DNA as a template for probe/primers specific to the *ps*i lentiviral sequence and human albumin for normalization. A reference standard was

generated by serial dilution of HT1080 fibrosarcoma encoding a single copy of the integrated vector. All reactions used Absolute qPCR ROX Mix (Thermo Scientific) and were run in a CFX96 Touch Real-Time PCR Detection System (BioRAD) under the following conditions: 10 min at 95C, then 40 cycles of 15 sec at 95C, and 1 min at 60C. MRD was tracked using markers for the immunoglobulin heavy chain IGHV6-1*01/IGHD1-1*01/IGHJ3*02 and IGHD3-10*01/IGHJ6*02 over 50 cycles under the following conditions: 2 min at 50C, then 10 min at 95C, 50 cycles of 15 sec at 95C, and 1 min at 58-68C. Results were reported as the relative frequency of leukemic cells with a threshold of $>10^{-4}$ (0.01%) used to define molecular relapse.

The extent of donor engraftment was assessed using the previously described method of analysis (*26*). Briefly, DNA was extracted from bone marrow or peripheral blood samples using Qiagen DNA mini spin columns (Qiagen), and 10 ng was used to amplify short variable number tandem repeats (vntr) with the Powerplex 16 system (Promega). PCR products were run on a 3500 xL Genetic Analyzer, and results were analyzed using GeneMapper Software 5 (Applied Biosystems).

Analysis of gene editing signatures

Samples were acquired on day 3 of manufacture (pre-nuclease) and at the end of production (post-nuclease), and DNA was extracted using a Qiagen EZ1 tissue kit. Target regions were amplified using fusion primers containing MiSeq adapter sequences fused to target sequences, and unique barcode sequences were then added to each amplicon. A pooled DNA library was sequenced on a single MiSeq flow cell as a 2x301 paired end sequencing run. Demultiplexed datasets were aligned against corresponding reference sequences and analyzed by a proprietary pipeline for read cleanup, trimming, and discard of short reads, followed by assembly of read pairs and then Needleman-Wunsch alignment of sequences against template. Insertions, deletions, and indels were quantified as events characteristic of NHEJ and their frequency calculated for each target site.

For analysis of UCART19 T cells recovered by sorting of blood and bone marrow, a nested PCR protocol was used to amplify molecular events (indels) generated in T cells treated with TRAC and CD52 TALENs. First and second PCR were performed with Herculase II Fusion DNA 15 Polymerases kit (Agilent Technologies) using 30 and 20 cycles of amplification, respectively, at 55C. The samples were then purified using the Agencourt AMPure XP system and subjected to deep sequencing analysis, and the frequency of NHEJ events was quantified as before (Illumina method, ICM)(*14*).

The oligonucleotides used for the first PCR were CD52 forward ATCTTGTACGATCTAGCCTGC, CD52 reverse AGGTTTCTCTCAGGGCAGCC, TRAC forward CATTTCAGGTTTCCTTGAGTGG, and TRAC reverse CAGATTTGTTGCTCCAGGCC; and for the second PCR CD52 forward AAGACTCGGCAGCATCTCCACAGATCTGCAGAAAGGAAGC, CD52 reverse GCGATCGTCACTGTTCTCCACCTGTTGGAGTCCATCTGCTG, TRAC forward

AAGACTCGGCAGCATCTCCAATCACTGGCATCTGGACTCCA, and TRAC reverse GCGATCGTCACTGTTCTCCACCTCATGTCTAGCACAGTTT.

Statistics

Full data are presented for the two subjects, and testing of statistical significance was not applicable.

Supplementary materials

Figure S1. Karyotype analysis and FISH

Figure S2. Cytokine studies

Figure S3. Bone marrow studies.

Figure S4. Flow cytometry for UCART19 in subject 1

Figure S5. Skin biopsy in subject 1

Table S1. Molecular characterization of gene editing using NGS across the TRAC, CD52 and 15 possible off-target loci

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Figures:
Figure 1. Manufacture of gene-edited universal CAR19 T cells (UCART19). A. Self-inactivating third generation lentiviral vector with deleted U3 long terminal repeat (LTR) elements and encoding a CAR19 (4g7) transgene linked by a self-cleaving peptide to RQR8, a sort/suicide molecule incorporating epitopes targeted by anti-CD34 (Qbend10) and anti-CD20 (rituximab) antibodies. The vector used an internal EF1α promoter and included a Rev-responsive element (RRE), central polypurine tract (CPPT), and mutated Woodchuck Post Regulatory element (WPRE).

B. Cryopreserved healthy volunteer donor (non-HLA matched) non-mobilized leukapheresis harvest was the source of peripheral blood mononuclear cells (PBMNC). Cells were thawed and activated with anti-CD3/CD28 beads, transduced by a single round of lentiviral vector exposure and then electroporated with TALEN mRNA targeting the TRAC and CD52 loci. Thereafter, engineered cells were expanded in a Wave Bioreactor 2/10, before stringent

CliniMACS depletion of residual TCRαβ T-cells. The final product was cryopreserved in fixed dose aliquots and the cell bank characterized in detail for sterility, potency, and replication-competent virus.

C. We found over 85% expression of CAR19 by staining with anti-F(ab)2 antibody. This was notably higher than the expression of RQR8, detectable by anti-CD34 staining. We confirmed stringent depletion of TCRαβ, with less than 1% of T cells expressing CD3 and over 64% also exhibiting a CD52-negative phenotype. The CD8 subset was well represented, and the presence of 'naïve-like' phenotypes was confirmed by staining for markers CD45RA and CD62L.

D. Next generation sequencing of TRAC and CD52 target site sequences found a high frequency (>70%) of events consistent with NHEJ repair of double stranded DNA scission.

Figure 2. UCART19 therapy and response in subject 1. A. Lymphodepletion was administered to subject 1 over a period of seven days before UCART19 infusion and was comprised of fludarabine (F), cyclophosphamide (C), and alemtuzumab (A). This was sufficient to induce neutropenia, with transient GCSF-dependent recovery of neutrophils by 30 days followed by protracted multi-lineage cytopenia until second allo-SCT after 12 weeks. The second allo-SCT was preceded by 4 Gy total body irradiation (TBI), thiotepa (T), F, C, and ATG conditioning. GVHD of skin was treated with 1 mg/kg prednisolone and 4 doses of rituximab (375 mg/m^2).

B. The extent of residual disease was monitored by qPCR for two immunoglobulin gene rearrangement signatures (VH6/JH3 and DH3/JH6) and had remained $>10^{-3}$ throughout previous interventions, including first allo-SCT. Within 28 days of UCART19 therapy, and at serial time points thereafter for 12 months, MRD was reported negative at $<10^{-5}$.

C. Analysis detected cells of three origins: allo-SCT donor, recipient, and UCART19 cells, with the latter reaching above 25% in bone marrow by Day 77. Full donor chimerism was restored by second allo-SCT and remained stable thereafter.

D. Lentiviral vector copy number (VCN) per cell in PBMNC and bone marrow was quantified. The signal was lost after second allo-SCT, 12 weeks after infusion.

E. CAR19$^+$ T cells detected by flow cytometry 8 weeks after UCART19 infusion (fig. S4) were enriched by flow-based sorting using anti-F(ab)2 staining and subjected to molecular characterization alongside the original cell product. Chimerism studies of sorted PBMNC and BM indicated that the purity of the enrichment was around 60%, reflecting the technical limitations of analyzing samples from a lymphopenic infant. Nonetheless, NGS interrogation after nested PCR amplification of CD52 target sites confirmed a high frequency of NHEJ signatures for CD52, indicating post-alemtuzumab survival of gene-edited cells. Less frequent event signatures at the TRAC locus were consistent with flow cytometric detection of CD3$^+$ T cells.

Figure 3

Outline of UCART19 therapy in a second infant. A. Fludarabine (F), cyclophosphamide (C), and alemtuzumab (A) were administered to subject 2, followed by UCART19 cells. Transient skin rash suggestive of GVHD responded rapidly to topical steroids, and irritability was self-limiting, resolving without intervention. Stable

neutrophil recovery was established by 28 days. Second allo-SCT followed a single dose of rituximab (Rtx, 375 mg/m^2), 4 Gy TBI, F, C, and ATG conditioning 10 weeks after UCART19.

B. PCR quantification was positive ($>10^{-3}$) since relapse after first allo-SCT and became negative ($<10^{-5}$) only after UCART19 therapy.

C. Chimerism studies detected the original allo-SCT donor and UCART19 cells until second allo-SCT.

D. VCN in PBMNC confirmed the presence of UCART19 cells, peaking in the third week after infusion, and persisting at low levels until conditioning ahead of second allo-SCT at 10 weeks after infusion.

E. Almost all circulating T cells that expressed CAR19 were CD52$^-$ and CD3$^-$ on flow cytometric analysis.