Supplemental Data

Sleeping Beauty-engineered CAR T cells achieve anti-leukemic activity without severe toxicities

Chiara F. Magnani, Giuseppe Gaipa, Federico Lussana, Daniela Belotti, Giuseppe Gritti, Sara Napolitano, Giada Matera, Benedetta Cabiati, Chiara Buracchi, Gianmaria Borleri, Grazia Fazio, Silvia Zaninelli, Sarah Tettamanti, Stefania Cesana, Valentina Colombo, Michele Quaroni, Giovanni Cazzaniga, Attilio Rovelli, Ettore Biagi, Stefania Galimberti, Andrea Calabria, Fabrizio Benedicenti, Eugenio Montini, Silvia Ferrari, Martino Introna, Adriana Balduzzi, Maria Grazia Valsecchi, Giuseppe Dastoli, Alessandro Rambaldi, Andrea Biondi

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Supplemental Methods

cGMP compliant T-cell manufacture

CARCIK-CD19 cells was manufactured by a continuous process using fresh donor-derived PBMC source material as previously described (1). Most of the steps are closed and all open handling steps was conducted in a Class A GMP Biosafety cabinet. The manufacturing process is divided into five distinct steps: cell isolation, cell transduction, cell irradiation, transduced cell stimulation and expansion. The drug substance (DS) manufacturing process is summarized below and is briefly represented in Supplementary Figure 1B. Donor PBMCs was isolated by centrifugation over Ficoll-Hypaque gradient (Ge Healthcare). The donor-derived peripheral blood (PB) has been provided by the donor's center of origin, i.e. the transplant center for sibling/family donors or the registry for unrelated donors and has been tested for presence of infectious agents. From the total of PBMCs, a portion was collected for subsequent irradiation (60Gy of 137Cs γ-rays). The remaining major PBMC portion was resuspended in Amaxa P3 Primary Cell 4D-Nucleofector buffer (Lonza) with the GMP-grade SB DNA plasmids (CD19.CAR/pTMNDU3, 15 µg and pCMV-SB11, 5 µg, VGXI, GMP grade) and electroporated using Amaxa 4D-NucleofectorTM (Program EO-115). Irradiated autologous feeder PBMCs were added to the electroporated cells at "feeder cell: electroporated PBMCs" ratio 1,5:10 together with IFN-γ (1000 U/ml, Boehringer Ingelheim). The day after (day 1), cells were stimulated with OKT-3 (50 ng/ml, Takara) and IL-2 (300 U/ml, Novartis). Cells were then maintained in fresh medium with IL-2 (300 U/ml) and expanded every 3-4 days with a seeding density of $0.75x10^6$ cells/ml until the biological target values were reached. In process controls were performed during the expansion and differentiation. The target values to be reached are described in Supplemental Table 1. Phenotype characterization, identity, potency and purity was performed by multiparametric flow cytometry. Cells were sampled at different time points of the process and stained with specific fluorochrome-labeled

mAbs such as: CD8, CD56, CD3 (all BD), CD45RO, CD14, CD62L, CD15, CD19, CD45 (all BD Pharmingen), CD4 (IQP). Surface expression of CD19CAR was detected using Alexa Fluor 647-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch, West Grove, PA). Acquisition was performed using a FACS Canto II (BD, CA, USA) and analyzed using BD FACS Diva software. Each marker is expressed as the percentage of the nucleated-cell gate or of CD3+ cell subset. The cell viability test was performed by using trypan blue vital dye. Mycopalsma test was performed using the VenorGeM®Mycoplasma Detection kit (Sigma Aldrich, St. Louis, Missouri, USA). The test procedure was developed by Minerva Biolabs GmbH (Berlin, Deutschland) according to Eu Ph 2.6.7 – 2.6.21. Bacterial endotoxin test was performed according to Eu Ph 2.6. by addition of a solution containing endotoxin to a Limulus polyphemus (LAL) extract. The sensitivity of the reagent used in the test is 0.03 EU/ml, the sensitivity threshold used for cell culture supernatants and cell suspensions is 0.5 EU/ml (Endosafe, Charles River, MA, USA). TSB Growth Promotion test and TSB visual testing were followed by Sterility testing of cellular products performed by Eurofins Biolab (Vimodrone, MI, Italy).

Cytotoxicity

Cytotoxicity was evaluated by a 4-hour co-culture assay. Cell death and apoptosis were detected using GFP-Certified™ Apoptosis/Necrosis detection kit (Enzo Life Sciences, Farmingdale, NY), according to manufacturer's instructions. CD19+ REH acute lymphocytic leukemia (ALL) cell line (ATCC, Manassas, VA) was previously labeled with 5-(and 6)- Carboxyfluorescein diacetate succinimidyl ester (CFSE, 1 μM, eBioscience) and co-cultured with T cells at 5:1 effector:target (E:T) ratio. The final percentage of killed cells was determined using the following equation: (observed lysis – spontaneous lysis) x100/(100 – spontaneous lysis).

Quantitative Real-time PCR analysis for absolute detection of transposase enzyme

Total RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized with SuperScript II Reverse Transcriptase in the presence of RNaseOUT Ribonuclease Inhibitor (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. cDNA samples (25ng RNA equivalent) were run in duplicate or triplicate, and levels of transposase transcript were determined as relative expression by normalizing to GUS Control Gene Standards (Quiagen). This method has been developed and GMP-grade validated from previous studies(2–4) at Laboratorio Tettamanti. Specific primers and probes were used:

GUS-1102-Fw GAAAATATGTGGTTGGAGAGCTCATT

GUS-1162-Rv CCGAGTGAAGATCCCCTTTTTA

Probe GUS 1142 FAM/TAMRA CCAGCACTCTCGTCGGTGACTGTTCA

SB11XRT-Fw AAGCCGAAGAACACCATCC

SB11XRT-Rv AGCACCCCCACAACATGA

Probe: # 87 FAM CTGACTTGCCAAAACT (Roche, Basel, Switzerland)

Study design

We conducted a open label, single arm, multicentric, dose escalation Phase I/II trial to determine the feasibility and safety of a single dose of allogeneic (donor-derived) T cells transduced with a transposon CD19 CAR and differentiated as CIK cells in adult and pediatric patients with relapsed or refractory (r/r) B-ALL, after HSCT. Dose limiting toxicity was defined as occurrence of grade IV CRS within 2 weeks of the cell infusion.

Additional trial information

Inclusion criteria

1. Children (1-17) and adults (18-75 years old);
2. Relapsed or refractory adult and pediatric B- ALL after HSCT;
3. Evidence of CD19 tumor expression in bone marrow (BM) and/or PB by flow cytometry;
4. BM with ≥ 5% lymphoblasts by morphologic assessment at screening;
5. No evidence of overall aGVHD > Grade I or chronic GVHD (cGVHD) greater than mild at time of enrollment and in the previous 30 days;
6. No longer taking immunosuppressive agents for at least 30 days prior to enrollment;
7. No evidence of concomitant life-threatening infectious disease;
8. Life expectancy > 60 days;
9. Lansky/Karnofsky scores > 60;
10. Absence of severe renal disease (creatinine > x 3 normal range for the age);
11. Absence of severe hepatic disease (direct bilirubin > 3 mg/dl or SGOT > 500);
12. Patient/guardian able to give informed consent.

Exclusion criteria

1. Patients with GVHD Grades II-IV;
2. Any cell therapy in the last 30 days;
3. Patient with concomitant life-threatening infectious disease;
4. Lansky/Karnofsky score <60;
5. Patients with hepatic or renal disease as specific above;
6. Pregnant or breast-feeding females;
7. Rapidly progressive disease that in the estimation of the investigator and sponsor would compromise ability to complete study therapy;
8. Subjects must have recovered from the acute side effects of their prior therapy, such that eligibility criteria are met;

9. HIV/HBV/HCV Infection: Seropositive for HIV antibody. Seropositive for hepatitis C or positive for Hepatitis B surface antigen (HBsAG);

10. Uncontrolled, symptomatic, intercurrent illness including but not limited to infection, congestive heart failure, unstable angina pectoris and cardiac arrhythmia;

11. Active Central Nervous System (CNS) involvement by malignancy, defined as CNS-3 per National Comprehensive Cancer Network (NCCN) guidelines. Note: Patients with history of CNS disease that has been effectively treated will be eligible;

12. Patient has received an investigational medicinal product within the last 30 days prior to screening;

13. Pregnant or nursing (lactating) women;

14. Women of child-bearing potential (defined as all women physiologically capable of becoming pregnant) and all male participants, unless they are using highly effective methods of contraception for a period of 1 year after the CARCIK-CD19 infusion.

Primary Objective(s)

1. To determine the Maximum Tolerated Dose (MTD) and the Recommended Phase 2 Dose (RP2D) of CARCIK-CD19 infusion.

2. To assess the safety of CARCIK-CD19 infusion.

Secondary Objectives

1. To evaluate activity of CARCIK-CD19 administration as Overall Remission Rate (ORR) which includes Complete Remission (CR) and CR with incomplete blood count recovery (CRi)

2. To evaluate the duration of remission (DOR).

3. To evaluate relapse-free survival (RFS), event-free survival (EFS) and overall survival (OS).

4. To characterize the *in vivo* cellular pharmacokinetic (PK) profile (levels, persistence, trafficking) of CARCIK-CD19 cells in target tissues (blood, bone marrow, and other tissues if

available).

5. To describe the levels of B and T cells (peripheral blood and bone marrow) prior to and following CARCIK-CD19 infusion for safety monitoring.

6. To describe the prevalence and incidence of immunogenicity to CARCIK-CD19.

Investigation therapy

Starting Dose.

The first starting Dose has been identified in $1x10^6$ cells/kg.

Dose escalation

According to the preclinical data with CARCIK-CD19 and the available published data on the use of CAR T and CIK cells, the dose escalation phase has been performed by four combinations of dose escalating levels:

- $1x10^6$/kg +/- 0.1;
- 3 $x10^6$/kg +/- 0,3;
- 7,5$x10^6$/kg +/- 0,8;
- 15$x10^6$/kg +/- 1,5;

The dose of CARCIK-CD19 referred to transduced cells and consist of a single infusion.

The number of cells referred to CIK CAR CD19 transduced cells, as evaluated at the time of freezing.

Lymphodepletion

From 2 to 14 days before the CARCIK-CD19 infusion all patients have undergone lymphodepletion with Fludarabine (30 mg/m2 intravenously (i.v.) daily for 4 doses) and cyclophosphamide (500 mg/m2 i.v. daily for 2 doses starting with the first dose of fludarabine) before CARCIK-CD19 administration.

Salvage therapy

A cytoreductive therapy have been eventually administered under PI decision, based on the bulk of the disease and the general condition of the patients, in order to manage the period from inclusion into the study and lymphodepletion therapy administration. Any cytoreductive therapy should be terminated 10 days before lymphodepletion.

Toxicity assessment

CRS was treated according to "ASTCT Consensus Grading"(5) (Supplemental Table 6). The diagnosis of CRS was predominantly clinical. The onset of CRS was defined by the day on which the patient had persistent fever or myalgias not caused by another diagnosis for 24 hours. The grade of CRS was determined by the most severe event. The severity of the toxicity was defined by CTCAE (Common Terminology Criteria for Adverse Events) v5.0 classification. Fever was specified as body Temperature ≥38°C, with no other possible cause. If patients received antipyretics or anti-cytokines (i.e. tocilizumab) or steroids, fever was no longer required for the subsequent classification of CRS, and in this case, it was established based on hypotension and hypoxia. Low flow oxygen was defined as flow ≤ 6L / minute, while high flows were defined as oxygen administration at > 6 L / minute. First line treatment in case of fever was large-spectrum antibiotic therapy, according to the institutional policy of febrile neutropenia (after performing blood cultures and chest-x-ray). Fever was also controlled by the use of diclofenac. Hypotension was treated with i.v. fluid and/or vasopressors (dopamine and/or noradrenaline) and hypoxia with oxygen. In case of need of multiple vasopressors or rapid deterioration, Tocilizumab was administered (<30 kg = 12 mg/kg i.v.; >30 kg = 8 mg/kg i.v.). In absence of clinical improvement after 8-12 hours, a second dose of tocilizumab was considered. In case of absence of clinical response even to the second dose of tocilizumab, the use of methylprednisolone at 2 mg/kg was considered (6). CRS resolution was defined by the resolution of all signs and symptoms leading

to the diagnosis of CRS. Neurotoxicity (including these symptoms: aphasia, altered level of consciousness, impairment of cognitive skills, motor weakness, seizures, and cerebral edema) was treated according to ICE score (Immune effector cell-associated encephalopathy). If patients presented seizures, a brain CT-scan and EEG were performed. From grade 2 toxicity we used dexamethasone, in case of concomitant CRS in patients who did not respond to tocilizumab, in the absence of CRS in all patients.

Integration site retrieval and sequencing

Genomic DNA form Integration Site (IS) retrieval was obtained from the pellets of patients' transduced CARCIK-CD19 cells or whole peripheral blood using Qiagen Blood & Cell Culture DNA Midi Kit, following manufacturer's instructions. DNA was quantified with Invitrogen Qubit 2.0 Fluorometer with the dsDNA High sensitivity kit, following manufacturer's instructions.

DNA Sonication: 150-1500 ng of genomic DNA were suspended in a final volume of 50 µl in TE using AFA Fiber 8 microTUBE Strips positioned in the appropriate Rack (12 place 8 microTUBE Strip) and shaered using a Covaris E220 Ultrasonicator in order to obtain a Target Peak of 1000 bp, using the following conditions: Peak Incident Power: 175; Duty Factor: 2%; Cycles per Burst: 200; Treatment Time: 45 seconds; Temperature 7°C; Water level: 6; E220 Intensifier: Yes. The relatively large size of 1 kb was chosen because after PCR the resulting products are shorter since the position of the IRDR2 junction within the fragment and the shear site changes randomly.

End Repair/Adenylation and Ligation: The fragmented DNA was split in 3 parts to obtain 3 technical replicates and then subjected to End Repair/Adenylation and Ligation, using the NEB Next Ultra kit.

Purification of Ligation products: In order to avoid carryover of Linker cassette, that could lower the efficiency of the following Exponential PCR, the ligation products were purified with Ampure

XP beads using a Ligation product:Beads ratio of 1:0.7. The purified ligated DNA was then eluted from beads in 32 microliters of TrisHCl 10mM.

Exponential PCR set up: The whole ligation product was used as template for the Exponential PCR with IRDR_FB1 and FB-Linker-P7 primers. The PCR was performed using Qiagen Taq DNA Polymerase with the following thermal protocol: 95°C for 5 min, and 95°C for 1 min, 60°C for 45 sec, and 72°C for 90 sec, for 35 cycles, followed by a further 10 min incubation at 72°C.

Fusion-primer PCR: The purified amplification product was used as template for additional 10 cycles of amplification using barcoded Fusion-IRDR primers and P7 primer (5'-caagcagaagacggcatacga-3'). The PCR was performed using Qiagen Taq DNA Polymerase with the following conditions: 98°C for 30 sec, and 98°C for 10 sec, 58°C for 45 sec, and 72°C for 45 sec, for 10 cycles, followed by a further 5 min incubation at 72°C.

Quantification of Fusion-primer PCR products and library composition: Fusion-primer PCR products are the final products and can be directly sequenced on an Illumina sequencer. In order to sequence each sample with similar depth we quantified each Fusion-primer PCR by qPCR (Kapa Biosystems), following manufacturer instructions.

Each Fusion-primer PCR product was diluted 10,000 times in TrisHCl 10mM and quantified in triplicate on a 384-plate. The qPCR plate was run on a Thermofisher Viia7 Real-Time PCR System.

Library quantification: The libraries were quantified by qPCR using the Kapa Biosystems Library quantification kit following manufacturer instructions. The library was diluted 10000, 100000 and 1000000 and each dilution was quantified in triplicate using on a Viia7 Real-Time PCR System.

Sequencing: The sequencing libraries were denatured in 0.1N NaOH, diluted to a final concentration of 13 pM, mixed with Illumina Phix sequencing control at 30% and sequenced on a MiSeq sequencer (2x250 paired end sequencing cycles).

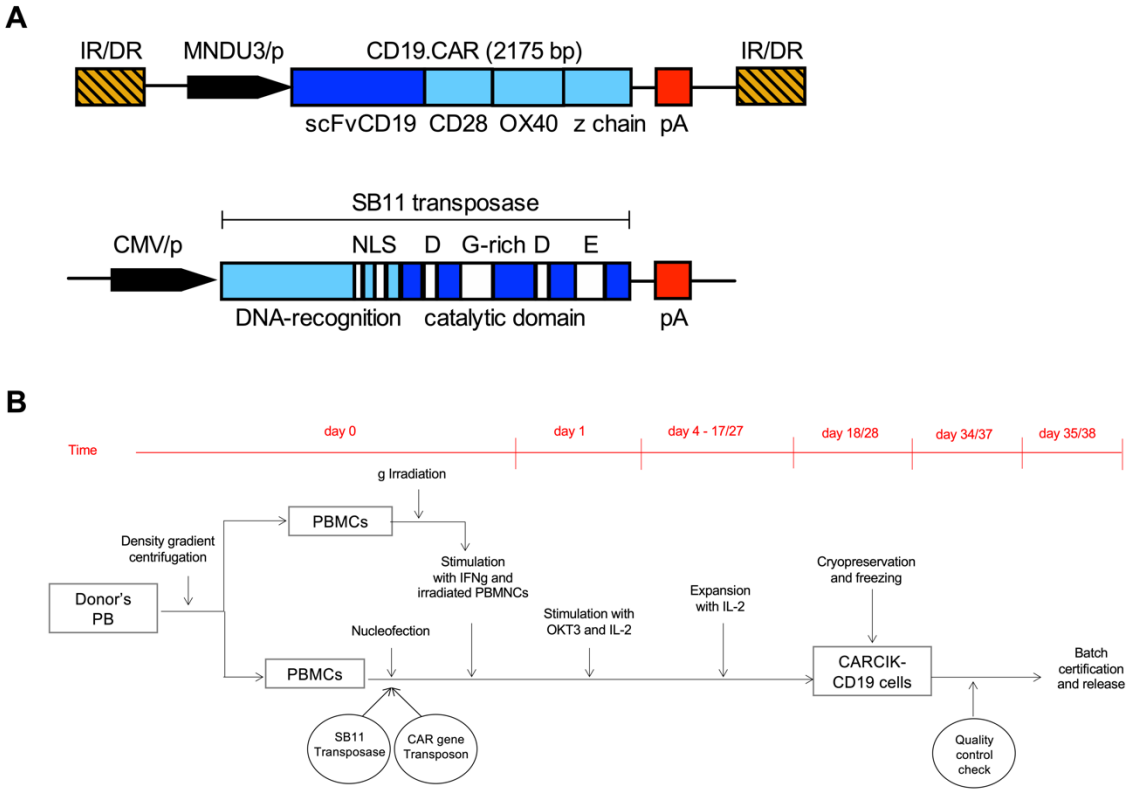
PCR and Fusion Primer sequences for integration site retrieval

Linkers: Linker cassettes are generated by annealing a long and short oligo and delivered as ready-to-use 15 micromolar. The long oligo contains the 8-nucleotide barcode for sample identification and the 12-nucleotide random sequence used as unique molecular identifier.

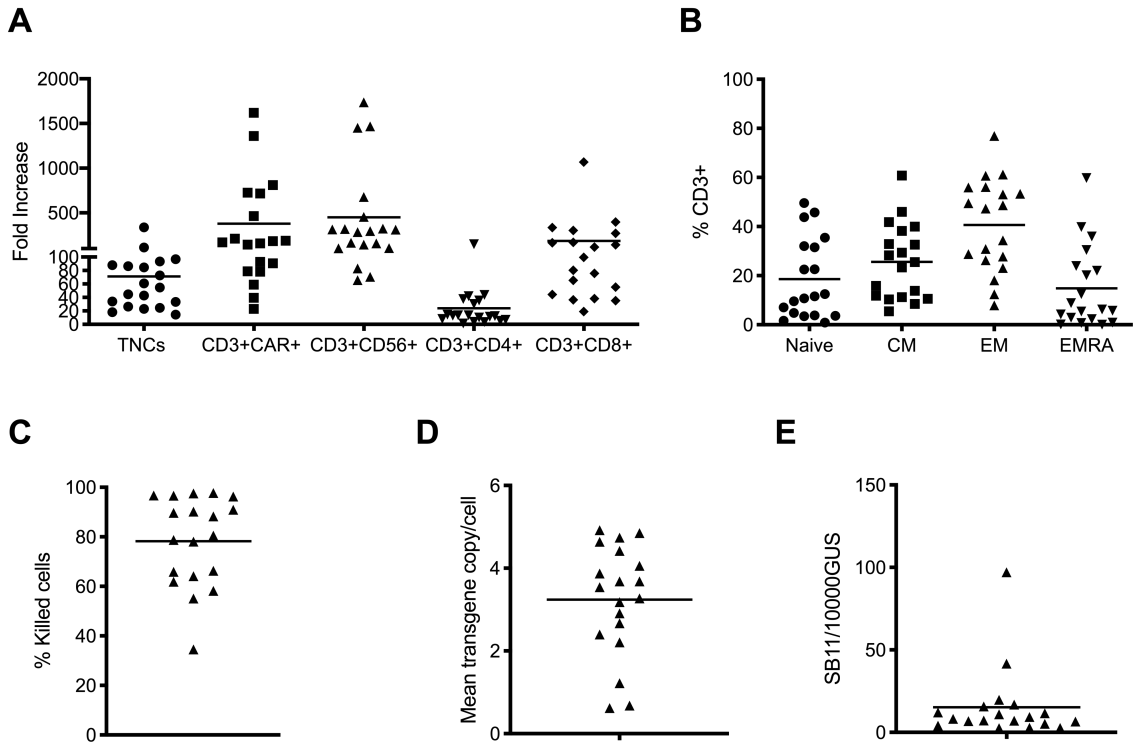
Linker name	Long oligo sequence	Short oligo sequence	Barcode
LC2	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNAACAACCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	AACAACCA
LC6	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACTCACCGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	AACTCACC
LC10	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAAGGTACAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	AAGGTACA
LC14	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNACACAGAAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	ACACAGAA
LC18	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNACATTGGCGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	ACATTGGC
LC22	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNACGTATCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	ACGTATCA
LC26	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAGATGTACGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	AGATGTAC
LC30	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAGGCTAACGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	AGGCTAAC
LC34	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNATAGCGACGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	ATAGCGAC
LC38	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNATTGAGGAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	ATTGAGGA
LC42	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCAAGGAGCGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CAAGGAGC
LC46	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCAGATCTGGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CAGATCTG
LC50	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCCAGTTCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CCAGTTCA
LC54	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCCGTGAGAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CCGTGAGA
LC58	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCGAACTTAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CGAACTTA
LC62	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCGCTGATCGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CGCTGATC
LC66	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNCTGAGCCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	CTGAGCCA
LC70	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNGAATCTGAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	GAATCTGA
LC74	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNGAGTTAGCGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	GAGTTAGC
LC78	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNGCCACATAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	GCCACATA
LC82	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNGGAGAACAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	GGAGAACA
LC86	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNGTCTGTCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	GTCTGTCA
LC90	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNTCCGTCTAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	TCCGTCTA
LC94	GACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNTGGCTTCAGTCACCGTGTCGTCAATCC*T	/5Phos/GGATTGACGACACGGTGAC/3C6/	TGGCTTCA

Primer Name	Primer sequence
IRDR_FB1	AGACAGGGAATCTTTACTCGGA
FB-Linker-P7	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
P7	CAAGCAGAAGACGGCATACGAGAT

IRDR Fusion Primer Name	Primer sequence	Barcode
FB-P5-Rd1-IRDR.1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAAACATCGGGCTAAGGTGTATGTAAACTTCCG	AAACATCG
FB-P5-Rd1-IRDR.3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAACCGAGAGGCTAAGGTGTATGTAAACTTCCG	AACCGAGA
FB-P5-Rd1-IRDR.5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAACGTGATGGCTAAGGTGTATGTAAACTTCCG	AACGTGAT
FB-P5-Rd1-IRDR.7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAAGACGGAGGCTAAGGTGTATGTAAACTTCCG	AAGACGGA
FB-P5-Rd1-IRDR.9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAAGGACACGGCTAAGGTGTATGTAAACTTCCG	AAGGACAC
FB-P5-Rd1-IRDR.11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNAATCCGTCGGCTAAGGTGTATGTAAACTTCCG	AATCCGTC
FB-P5-Rd1-IRDR.13	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACAAGCTAGGCTAAGGTGTATGTAAACTTCCG	ACAAGCTA
FB-P5-Rd1-IRDR.15	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACACGACCGGCTAAGGTGTATGTAAACTTCCG	ACACGACC
FB-P5-Rd1-IRDR.17	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACAGCAGAGGCTAAGGTGTATGTAAACTTCCG	ACAGCAGA
FB-P5-Rd1-IRDR.19	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACCACTGTGGCTAAGGTGTATGTAAACTTCCG	ACCACTGT
FB-P5-Rd1-IRDR.21	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACGCTCGAGGCTAAGGTGTATGTAAACTTCCG	ACGCTCGA
FB-P5-Rd1-IRDR.23	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNNACTATGCAGGCTAAGGTGTATGTAAACTTCCG	ACTATGCA

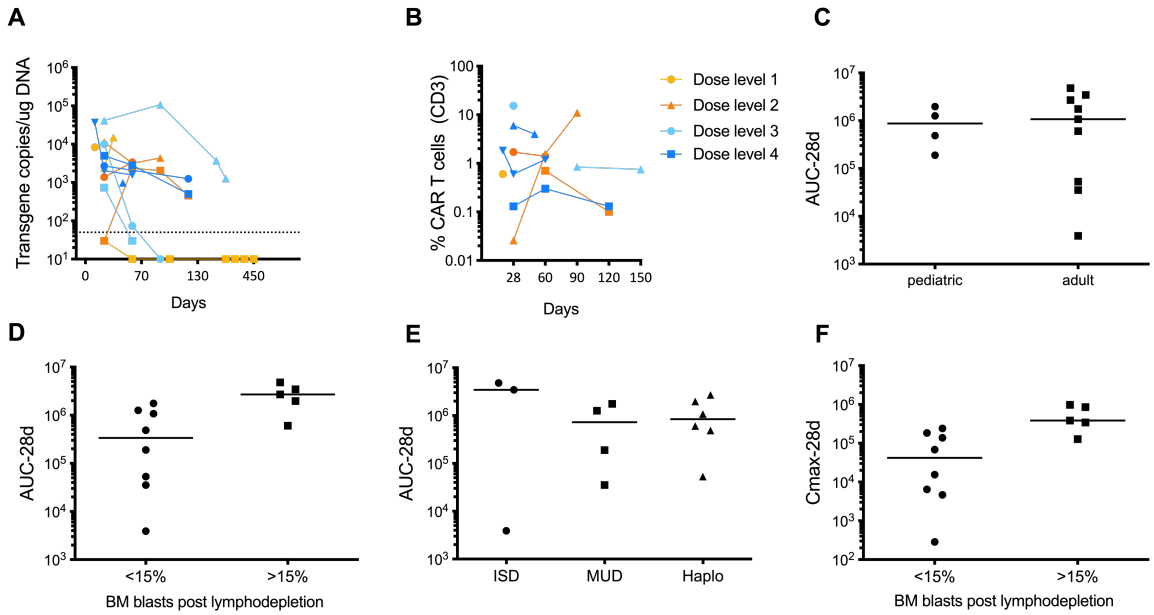


Supplemental Figure 1.
Vector design and manufacturing Process (A) Depiction of the GMP SB CD19.CAR/pTMNDU3 (MNZDU3) and pCMV-SB11 (MNZSB11) vectors used in this study, expressing the FMC63 scFv CD28:OX40:zeta CAR and the SB11 transposase, respectively. IR/DR, SB inverted repeats/directed repeats; MNDU3/p, the constitutive promoter from the U3 region of the MND retrovirus; scFv, single chain fragment variable; pA, poyadenylation signal from bovine growth hormone; CMV/p, CMV promoter; NLS, nuclear localization signal; Glycine-rich motif; D segment; E segment; DDE domani, which catalyzes transposition. (B) Schematic representation of CARCIK-CD19 manufacturing process. PB, peripheral blood; PBMC, peripheral blood mononuclear cells.

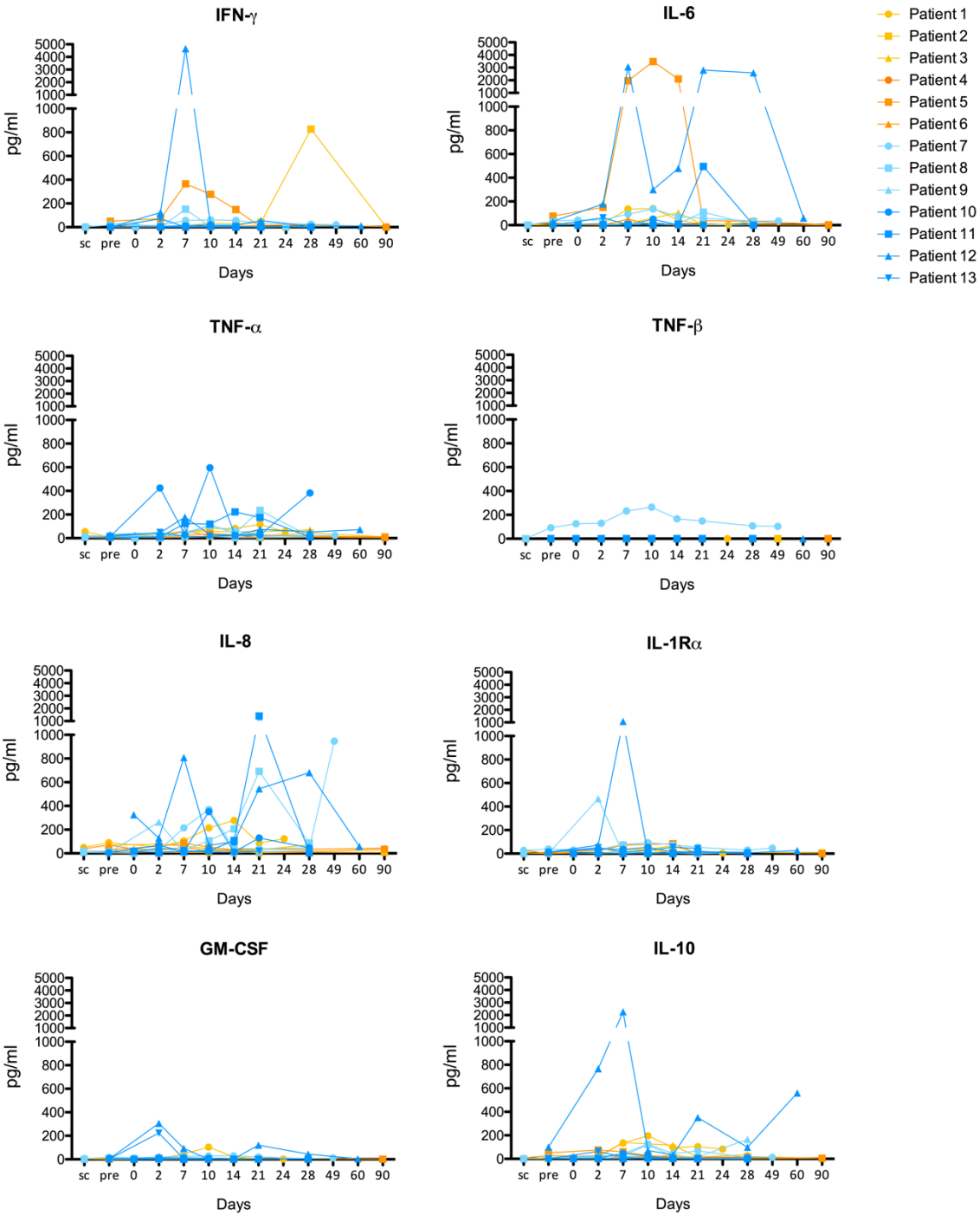


Supplemental Figure 2.

Fold increase and characterization of medicinal products (A) Fold increase of TNCs and of four different cellular subsets in 19 manufactured batches (calculated as day harvest /day 0 for TNC, CD3+CD56+, CD3+CD4+, CD3+CD8+ and as day harvest /day 7 for CD3+CAR+). TNC, total number of nucleated cells. (B) Expression of Naive, Central Memory (CM), Effector Memory (EM) and Effector Memory Terminally Differentiated (EMRA) cells as percentage of CD3+ T cells (n=19). (C) Potency of CARCIK-CD19 cells expressed as cytotoxic activity of the final products toward REH CD19+ target cells at 5:1 E:T ratio (n=19). (D) CAR transgene copy number per cell of final products as assessed by qPCR (n=19). (E) Transposase expression in final products by qRT-PCR, as number of transposase molecules normalized to 10^4 GUS copies (n=19). Mean are shown as horizontal line.



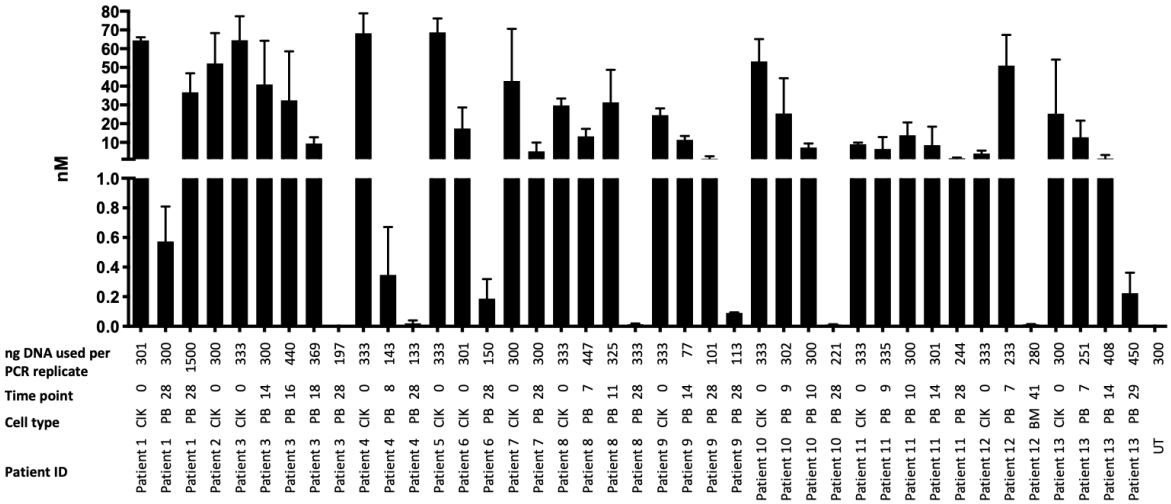
Supplemental Figure 3.
Engraftment of CAR T cells in BM and PB.
Transgene copy number per μg (A), percentage of CAR+ T cells within the CD3+ T cell subsets (B) at different intervals after CARCIK-CD19 infusion in the BM of patients treated at different dose levels. Each symbol and colour codify an individual patient sample (n=13). (C) AUC-28d according to the bulk disease, > or < 15% tumor burden in BM after lymphodepletion. (D) AUC-28d according to the type of the HSCT source for CAR T cell manufacturing. (E) Cmax-28d according to the bulk disease, > or < 15% tumor burden in BM after lymphodepletion (n=13). AUC, area under the curve (transgene copies/μg DNA); 28d, measurement from time 0 to 28; HLA-id, HLA identical donors; MUD, match unrelated donor; Haplo, haploidentical donor; Cmax, maximum concentration.



Supplemental Figure 4.

Circulating cytokines in patient sera after CAR T cell infusion.

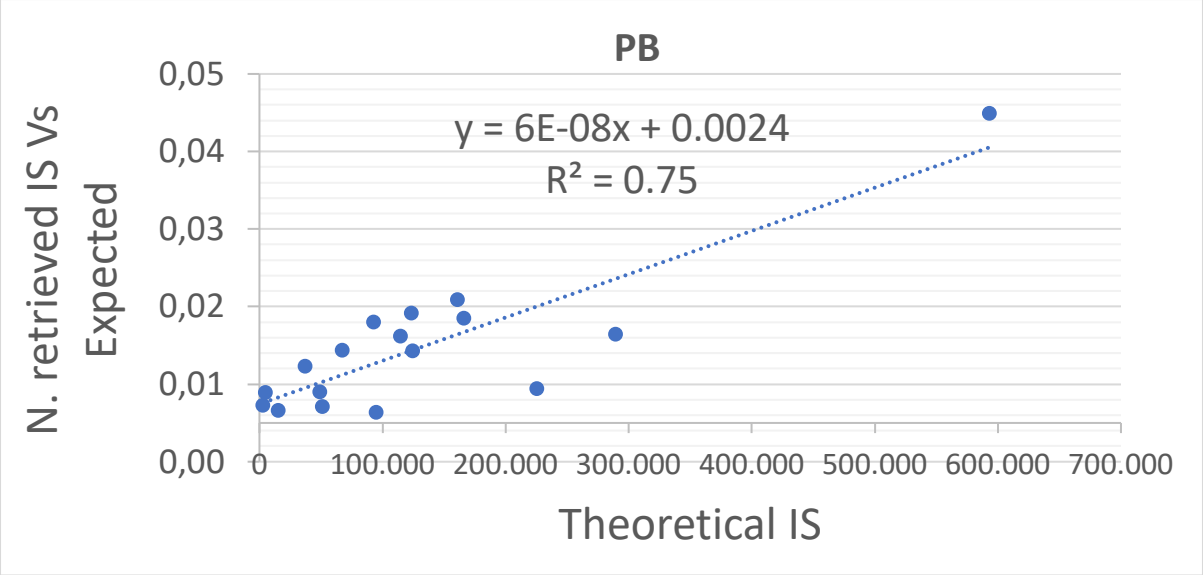
Serum IFN-γ, IL-6, and TNF-α, TNF-β, IL-8, IL-1Rα, GM-CSF, IL-10 concentrations at different intervals of time after CARCIK-CD19 infusion in patients treated at different dose levels. Each symbol and colour codify an individual patient sample (n=13).



Supplemental Figure 5.

Quantification of PCR products by qPCR.

The amount of amplified product in each PCR reaction was quantified by qPCR using the Kapa Biosystems Library quantification kit with Fusion-primer PCRs oligonucleotides on a Viia7 Real-Time PCR System. Quantification is expressed in nM (Y axis). Each column represents the average of 3 PCR replicates (standard deviations are shown) (n=41 samples).



Supplemental Figure 6.
Correlation between retrieved IS and theoretical IS present in in vivo samples.
For each sample the theoretical IS analyzed for each PCR reaction were calculated considering the number of equivalent genomes extrapolated by the total ng of DNA used and the vector copy number per cell calculated by qPCR.

Species assembly: hg19

CAR-CIK

GO Biological Process

Term Name	-log10 Binomial P-Value
alpha-beta T cell differentiation	113.54
alpha-beta T cell activation	108.33
regulation of defense response to virus	93.59
T cell activation involved in immune response	76.36
response to type I interferon	74.23
CD4-positive, alpha-beta T cell differentiation	73.23
CD4-positive, alpha-beta T cell activation	72.50
type I interferon signaling pathway	72.14
positive T cell selection	67.69
T cell differentiation involved in immune response	66.30
alpha-beta T cell differentiation involved in immune response	65.42
regulation of antigen receptor-mediated signaling pathway	63.22
execution phase of apoptosis	60.92
T-helper cell differentiation	59.40
interferon-gamma-mediated signaling pathway	56.19
T cell costimulation	54.65
lymphocyte costimulation	52.02
activated T cell proliferation	48.92
regulation of defense response to virus by virus	47.37
T cell proliferation	46.24

GO Cellular Component

Term Name	-log10 Binomial P-Value
nucleosome	35.72
cytoplasmic stress granule	30.66
proteasome core complex	20.97
I-kappaB/NF-kappaB complex	20.95
proteasome core complex, alpha-subunit complex	20.26
guanyl-nucleotide exchange factor complex	19.30
CD95 death-inducing signaling complex	17.18
TOR complex	15.28
telomerase holoenzyme complex	14.66
TORC2 complex	14.39
death-inducing signaling complex	13.98
ripoptosome	13.18
Piccolo NuA4 histone acetyltransferase complex	13.03
macrophage migration inhibitory factor receptor complex	12.70
nuclear nucleosome	12.68
Mre11 complex	11.77
integrin alpha4-beta7 complex	11.62
INO80-type complex	11.59
cytoplasmic side of endoplasmic reticulum membrane	11.10
MOZ/MORF histone acetyltransferase complex	11.03

GO Molecular Function

Term Name	-log10 Binomial P-Value
volume-sensitive anion channel activity	44.50
CD8 receptor binding	30.67
adenosine kinase activity	27.54
protein kinase B binding	27.36
tumor necrosis factor receptor binding	26.26
T cell receptor binding	25.07
BH domain binding	25.04
BH3 domain binding	24.28
thyroid hormone receptor binding	22.62
cysteine-type endopeptidase activity involved in apoptotic process	20.46
calcium-dependent protein serine/threonine kinase activity	20.34
chromo shadow domain binding	20.24
histone acetyltransferase activity (H4-K5 specific)	19.82
histone acetyltransferase activity (H4-K8 specific)	19.82
histone acetyltransferase activity (H4-K16 specific)	19.82
calcium-dependent protein kinase activity	19.36
dynactin binding	19.19
type 5 metabotropic glutamate receptor binding	18.27
lysine-acetylated histone binding	18.26
phosphatidylinositol phosphate 4-phosphatase activity	18.01

PB/BM

GO Biological Process

Term Name	-log10 Binomial P-Value
T cell receptor V(D)J recombination	14.05
negative regulation of eosinophil extravasation	9.82
myo-inositol transport	9.59
negative regulation of eosinophil migration	9.59
D-ribose catabolic process	9.54
activated T cell proliferation	9.49
astral microtubule organization	9.37
T-helper 17 cell differentiation	9.35
negative regulation of integrin activation	9.20
negative regulation of interleukin-4 production	9.08
negative regulation of cAMP-mediated signaling	9.05
lymphoid lineage cell migration into thymus	8.93
olfactory bulb axon guidance	8.84
regulation of eosinophil migration	8.12
neutrophil differentiation	7.64
positive regulation of chemokine-mediated signaling pathway	7.00
plasma membrane to endosome transport	6.96
cortical microtubule organization	6.91
pentose catabolic process	6.80
regulation of gluconeogenesis by regulation of transcription from RNA polymerase II promoter	6.62

GO Cellular Component

Term Name	-log10 Binomial P-Value
calcineurin complex	6.30
proteasome core complex, alpha-subunit complex	4.68
integrin alphav-beta3 complex	4.60
CD95 death-inducing signaling complex	4.52
ATF1-ATF4 transcription factor complex	4.24
lateral loop	4.20
activin receptor complex	3.77
IPAF inflammasome complex	3.74
postsynaptic recycling endosome	3.53
SMAD3-SMAD4 protein complex	3.27
chromosome passenger complex	3.18
tricellular tight junction	3.09
macrophage migration inhibitory factor receptor complex	3.09
NLRP3 inflammasome complex	3.00
cyclin D2-CDK6 complex	2.91
Toll-like receptor 1-Toll-like receptor 2 protein complex	2.81
Rix1 complex	2.80
Rb-E2F complex	2.74
alphav-beta3 integrin-vitronectin complex	2.73
cuticular plate	2.73

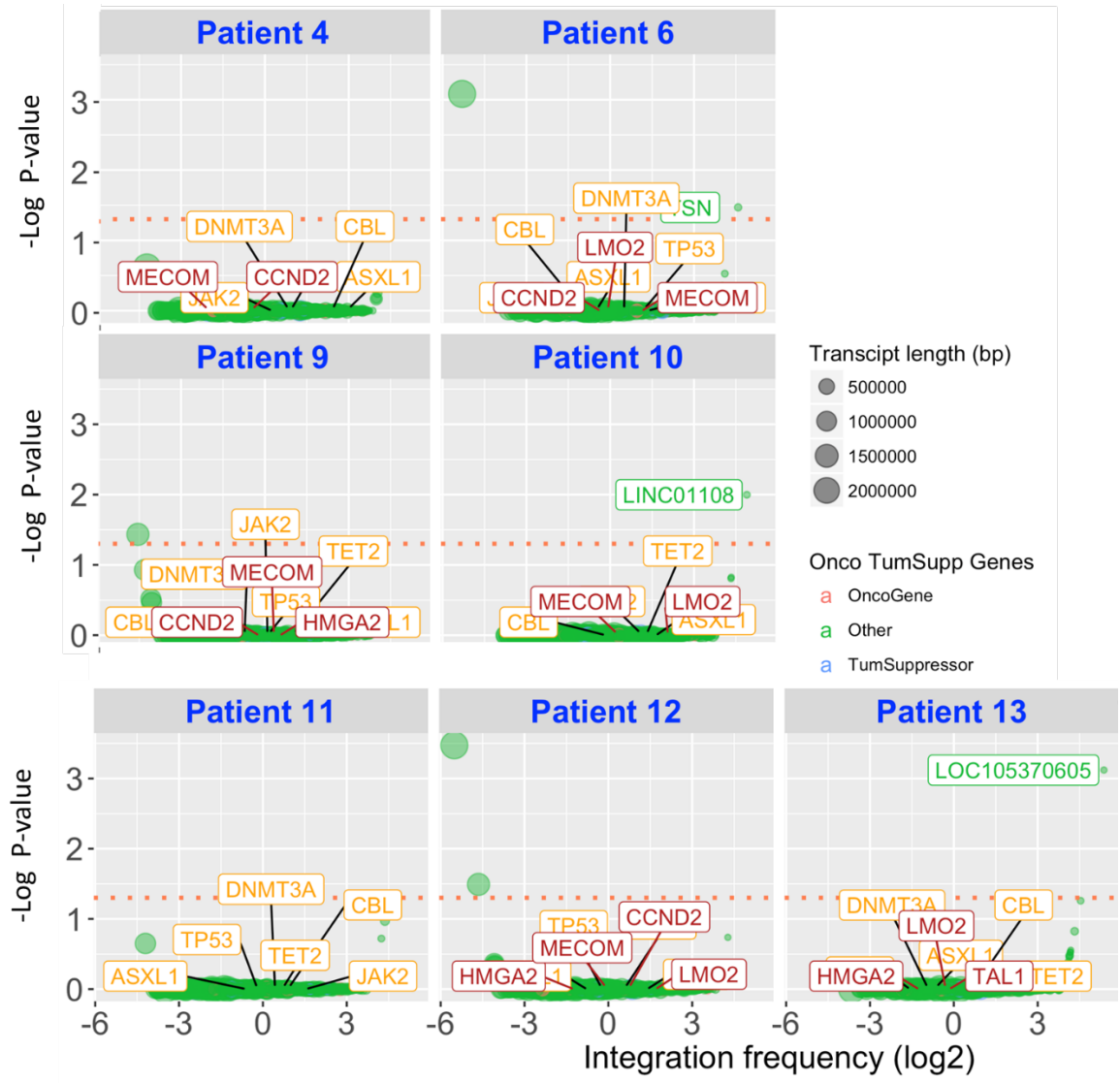
GO Molecular Function

Term Name	-log10 Binomial P-Value
ribokinase activity	11.31
cysteine-type endopeptidase activity involved in apoptotic process	9.83
phosphatidylinositol-3,4-bisphosphate 4-phosphatase activity	8.72
inositol-1,3,4-trisphosphate 4-phosphatase activity	8.72
inositol-3,4-bisphosphate 4-phosphatase activity	8.72
estrogen receptor activity	8.67
phosphatidylinositol-4,5-bisphosphate 4-phosphatase activity	8.24
inositol bisphosphate phosphatase activity	8.12
cysteine-type endopeptidase activity involved in execution phase of apoptosis	7.70
volume-sensitive anion channel activity	7.10
protein kinase B binding	6.23
C-rich single-stranded DNA binding	5.84
cysteine-type endopeptidase activity involved in apoptotic signaling pathway	5.65
adenosine kinase activity	5.64
neuregulin binding	4.78
cysteamine dioxygenase activity	4.75
small ribosomal subunit rRNA binding	4.72
mannose transmembrane transporter activity	4.67
interleukin-7 receptor activity	4.60
interleukin-1, Type II receptor binding	4.50

Supplemental Figure 7.

GO enrichment for Biological Processes and Molecular Function for the transposon IS.

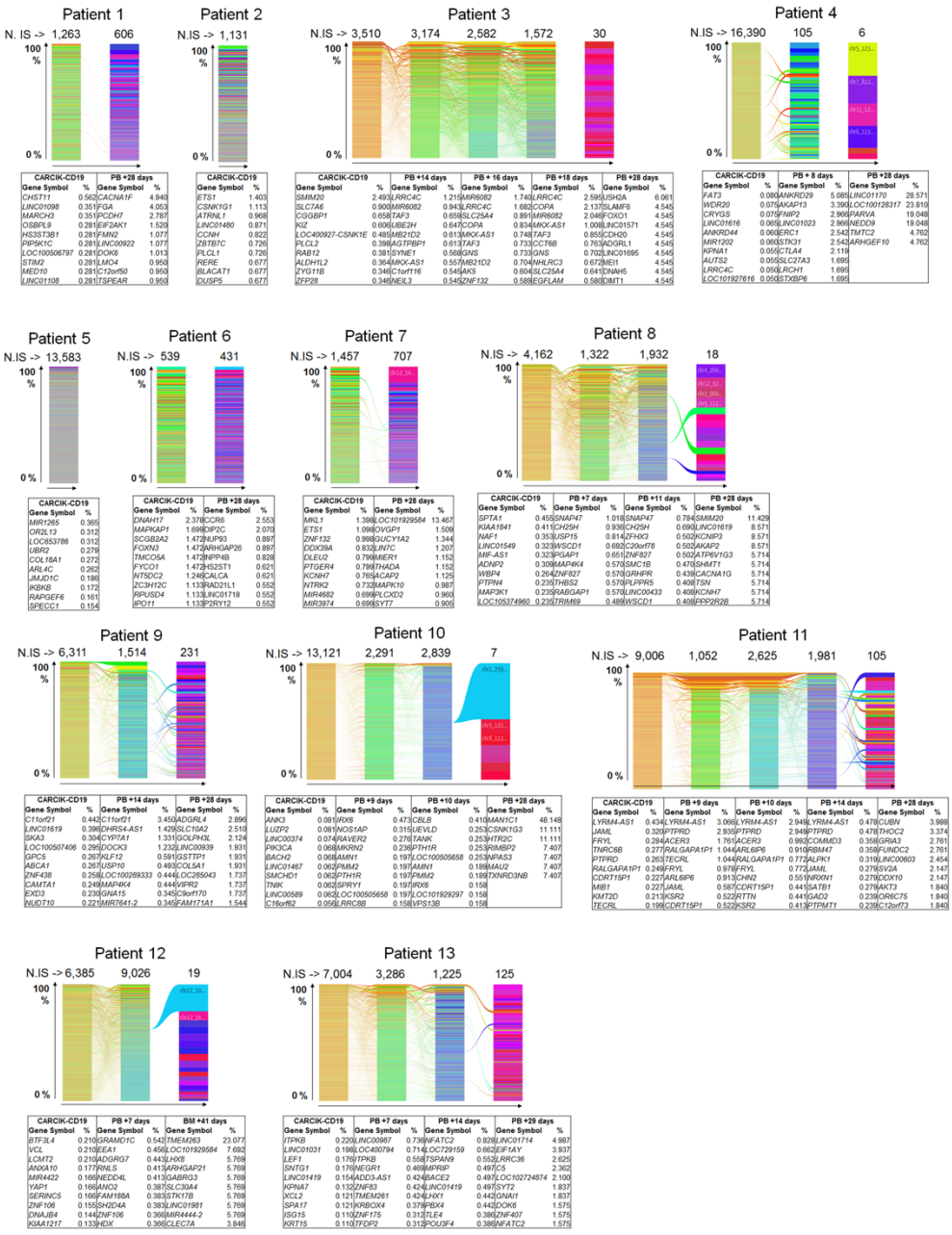
Coordinates of transposon IS retrieved from CARCIK-CD19 medicinal products and patients' whole PB and BM were analyzed by the GREAT enrichment discovery software (http://bejerano.stanford.edu/great/public/html/), interrogating Gene Ontology (GO) Biological Process, Molecular Function and Cellular Component databases. Significantly overrepresented gene classes are shown. For this analysis, the IS obtained from the pre- (CAR-CIK) and post- (whole PB and BM) transplant IS were merged (n=40). In the CAR-CIK IS group we observed enriched ontology terms mostly related to T cell biology.



Supplemental Figure 8.

Common Integration site analysis.

Common Insertion Site (CIS) analysis was performed on the IS identified for each patient using the Grubbs test for outliers and volcano plot representation. In each volcano plot, all genes targeted by IS are tested and plotted with dots of size proportional to the gene length; gene integration frequency normalized by gene length is placed on the x-axis, while the y-axis shows the P-value of CIS Grubbs test for outliers (-log base 10 of P-value). According to the annotation of genes in the main classes (tumor suppressor in blue, proto-oncogene in red, and a generic "other" in green for the remaining genes. Dots with significant P-values (alfa threshold of 0.05 are above the dashed horizontal line and labelled with the closest gene name (RefSeq). Gene ID labels of genes in involved in clonal expansion of genetically modified cells were annotated using red labels, tumor suppressor genes using orange labels or genes with other functions in blue. Seven out of 11 patients are represented.



Supplemental Figure 9.

Clonal abundance from whole peripheral blood samples over time.

For each patient, the clonal abundance is represented over time (x axis, days after therapy) with a stacked bar plot in which each clone (each represented by a different color) and the height shows the percentage of genomes with a specific integration site over the total genomes; ribbons connect tracked clones between two consecutive time points. Below each plot, the ten most abundant clones annotated with the closest gene are reported. N. IS, number of integration sites above each bar. 13 patients are represented.

Supplemental Table 1.
Batch's release specifications and compliance with quality requirements

Parameter	Method	Specification	n of compliant batches	Significance
% of CD3+ cells	Flow Cytometry	≥90%	19/19	purity/identity
% of CD3+/CD56+ cells (CIK)	Flow Cytometry	≥30%	19/19	purity/identity
% of CD3+/CAR+ cells	Flow Cytometry	≥20%	18/19	purity/identity
Cytotoxicity	Flow cytometry	≥25%	19/19	potency
Vector copy number	Q-PCR	<5 copies/cell	19/19	safety
Transposase expression	Q-RT-PCR	<20 copies/10000 GUS	17/19	safety
Cell viability	Trypan blue dye exclusion	≥80%	19/19	purity
Sterility	Eu Ph 2.6.1	sterile	18/19	safety
Mycoplasma	Eu Ph 2.6.7 – 2.6.21	absent	19/19	safety
Endotoxin	Eu Ph 2.6.14	<0.5 Eu/mL	19/19	safety

Supplemental Table 2.
Patient blood values pre-lymphodepletion

Patient no.	LDH pre-LT CT[A] (U/L)	PLT count pre-LT CT (mmc)	WBC pre-LT CT (mmc)	neutrophils pre-LT CT (mmc)
1	271	12000	4570	110
2	306	145000	770	650
3	595	143000	120	60
4	454	33000	1330	950
5	503	41000	570	180
6	148	16000	360	60
7	284	237000	5200	4150
8	252	131000	3380	1530
9	435	24000	270	200
10	415	68000	1270	700
11	162	181000	1390	1230
12	357	18000	180	150
13	187	177000	1080	1000

[A]CT= count

Supplemental Table 3.
Pharmacokinetic analysis

Patient no.	AUC[A]-28d[B]	AUC-last[C]	Cmax[D]-28d	Cmax-last	Tmax[E]-28d	Tmax-last	Tlast[F]
1	1,970,613	2,004,593	343403.8	343,403.8	14	14	31
2	35,341.5	40,861.5	4645	4,645	14	14	300
3	602,761	827,256.5	127376	127,376	14	14	42
4	53,049.5	181,578	6476	6,476	8	8	84
5	3,915.5	224,424.5	287	5,930	22	60	130
6	190,078	608,980	15487	15,487	21	21	150
7	1,262,209	3,306,133	240263	240,263	9	9	210
8	487,126.5	516,150.5	68668	68,668	11	11	60
9	4,804,764	13,218,179	977992	977,992	14	14	150
10	1,077,839	1,378,135	183119	183,119	9	9	94
11	1,762,137	2,255,599	137726	137,726	14	14	94
12	3,461,141	15,331,986	850090	850,090	7	7	50
13	2,708,937	2,825,280	384750	384,750	7	7	22
range	3,915.5-4,804,764	40,861.5-15,331,986	287-977,992	4,645-977,992	7-22	7-60	22-300
Median	1,077,839	1,378,135	137,726	137,726	14	14	94
Geometric mean (CV%)	487,225 (910.98)	1,133,644 (395.73)	68,831.1 (1654.87)	86,886.5 (583.26)	11.81 (39.19)	12.75 (61.90)	84.82 (88.98)

[A]AUC= area under the curve (transgene copies/μg DNA); [B]28d= measurement from time 0 to 28; [C]last= measurement from time 0 to last; [D]Cmax= maximum concentration; [E]Tmax= time to reach peak expansion; [F]Tlast= days to last measurement

Supplemental Table 4.
Summary of toxicities and intervention

Patient no.	Fever (days)	Low-flow oxygen (days)	Seizure (days)	ICU care (days)	Tocilizumab (days)	Methylprednisolone (days)
1	13	4	1	0	0	0
3	5	0	0	0	0	0
5	4	36	0	10	1	15
6	0	0	1	0	0	0
10	2	0	0	0	0	0
12	7	6	0	0	2	0
13	9	0	0	0	0	0

Supplemental Table 5.
Summary of SB IS retrieved from in vitro and in vivo samples

Patient no.	Cell Type	Time point	N IS[A]	ng DNA[B]	genome equivalents[C]	N IS / genome equivalents[D]	N IS norm by seq[E]
Patient 1	CIK	0	3,234	903	125,762	0.026	0.002
	PB	28	1,565	5,400	752,089	0.002	0.000
Patient 2	CIK	0	1,966	899	125,223	0.016	0.001
Patient 3	CIK	0	3,942	1,000	139,276	0.028	0.003
	PB	14	3,725	900	125,298	0.030	0.011
		16	3,040	1,320	183,861	0.017	0.013
		18	1,777	1,106	154,028	0.012	0.007
		28	2,043	592	82,451	0.025	0.071
Patient 4	CIK	0	19,400	1,000	139,276	0.139	0.017
	PB	8	2,901	429	59,766	0.049	0.002
		28	285	399	55,599	0.005	0.003
Patient 5	CIK	0	15,344	1,000	139,276	0.110	0.011
Patient 6	CIK	0	768	903	125,762	0.006	0.001
	PB	28	13,384	450	62,674	0.214	0.006
Patient 7	CIK	0	2,242	900	125,348	0.018	0.002
	PB	28	7,408	901	125,448	0.059	0.001
Patient 8	CIK	0	4,652	1,000	139,276	0.033	0.005
	PB	7	1,488	1,341	186,819	0.008	0.004
		11	2,190	976	135,978	0.016	0.009
		28	2,036	1,000	139,276	0.015	0.022
Patient 9	CIK	0	6,993	1,000	139,276	0.050	0.006
	PB	14	1,684	230	32,089	0.052	0.004
		28	2,807	638	88,858	0.032	0.002
Patient 10	CIK	0	15,882	1,000	139,276	0.114	0.013
	PB	9	2,743	907	126,351	0.022	0.013
		10	3,374	900	125,298	0.027	0.016
		28	2,028	664	92,479	0.022	0.030
Patient 11	CIK	0	10,246	1,000	139,276	0.074	0.010
	PB	9	1,304	1,005	140,039	0.009	0.001
		10	3,107	901	125,479	0.025	0.014
		14	2,313	904	125,965	0.018	0.009
		28	475	732	101,950	0.005	0.001
Patient 12	CIK	0	7,244	1,000	139,276	0.052	0.007
	PB	7	12,141	698	97,170	0.125	0.040
	BM	41	2,852	840	116,992	0.024	0.031
Patient 13	CIK	0	9,107	899	125,245	0.073	0.035
	PB	7	3,859	752	104,791	0.037	0.011
		14	4,135	1,225	170,574	0.024	0.002
		29	4,935	1,349	187,947	0.026	0.004

[A]N IS= number of unique IS retrieved; [B]ng DNA= amount of DNA used for the PCR reactions; [C]genome equivalents= calculated by dividing the total amount by the weight of a diploid genome (7,18 pg); [D]IN IS / genome equivalents= frequency of integrations for each genome; [E]Theoretical IS= the number of total IS present in the input DNA, calculated as the product by the number of genome equivalents and the VCN.

Supplemental Table 6.
Dose transition rules

Number of DLTs	Cumulative number of patients treated at each dose					
	3	6	9	12	15	18
0	E	E	E	E	E	E
1	S	E	E	E	E	E
2	DU	S	E	E	E	E
3	DU	D	S	S	E	E
4		DU	D	S	S	S
5		DU	DU	D	S	S
6		DU	DU	DU	D	S
7			DU	DU	DU	D
8			DU	DU	DU	DU
9			DU	DU	DU	DU
10				DU	DU	DU
11				DU	DU	DU
12				DU	DU	DU
13					DU	DU
14					DU	DU
15					DU	DU
16						DU
17						DU
18						DU

E= Escalate to the next higher dose; S= Stay at the same dose; D= De-escalate to the previous lower dose; DU= De-escalate to the previous lower dose and the current dose will never be used again in the trial (safety rule).

Supplemental Table 7.
CRS grading system

CRS Parameter	Grade 1	Grade 2	Grade 3	Grade 4
Fever	Temperature >=38°C	Temperature >=38°C	Temperature >=38°C	Temperature >=38°C
Hypotension	None	Not requiring vasopressors	Requiring a vasopressor with or without vasopressin	Requiring multiple vasopressors (excluding vasopressin)
Hypoxia	None	Requiring low-flow nasal cannula or blow-by	Requiring high-flow nasal cannula, facemask, non rebreather mask or Venturi mask	Requiring positive pressure (C-PAP, B-PAP, intubation and mechanical ventilation)

Supplemental Table 8.
Assessment of CAR T-cell persistence by flow cytometry

Panel: CAR persistence							
FITC	PE	PerCP Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
CD45RO	CD62L	CD3	CD56	CD19 HIS + Anti HIS	CD8	CD4	CD45
UCHL1	REG-56	SK7	NCAM 16.2		SK1	RPA-T4	HI30
BD	BD	Biolegend	BD	Thermo Fisher + Miltenyi	BD	Biolegend	Invitrogen
555492	555544	344814	335826	A42605 130-119-820	641400	300521	MHCD453

References

1. Magnani CF et al. Preclinical Efficacy and Safety of CD19CAR Cytokine-Induced Killer Cells Transfected with Sleeping Beauty Transposon for the Treatment of Acute Lymphoblastic Leukemia. *Hum Gene Ther*. 2018;29(5):602–613.
2. Magnani CF et al. Immunotherapy of acute leukemia by chimeric antigen receptormodified lymphocytes using an improved Sleeping Beauty transposon platform. *Oncotarget*. 2016;7(32):51581–51597.
3. Kolacsek O et al. Excision efficiency is not strongly coupled to transgenic rate: Cell type-dependent transposition efficiency of sleeping beauty and piggy bac DNA transposons. *Hum Gene Ther Methods*. 2014;25(4):241–252.
4. van der Velden VHJ et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: Guidelines for interpretation of real-time quantitative PCR data. *Leukemia*. 2007;21(4):604–611.
5. Lee DW et al. ASTCT Consensus Grading for Cytokine Release Syndrome and Neurologic Toxicity Associated with Immune Effector Cells. *Biol Blood Marrow Transplant*. 2019;25(4):625–638.
6. Lee DW et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014;124(2):188–195.

CONSORT 2010 checklist of information to include when reporting a pilot or feasibility trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a pilot or feasibility randomised trial in the title	NA not randomized
	1b	Structured summary of pilot trial design, methods, results, and conclusions (for specific guidance see CONSORT abstract extension for pilot trials)	Supplementary, page 4
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale for future definitive trial, and reasons for randomised pilot trial	NA not randomized
	2b	Specific objectives or research questions for pilot trial	Supplementary, page 6
Methods			
Trial design	3a	Description of pilot trial design (such as parallel, factorial) including allocation ratio	NA
	3b	Important changes to methods after pilot trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	Supplementary, page 4
	4b	Settings and locations where the data were collected	Page 18
	4c	How participants were identified and consented	Page 22
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Supplementary, page 7
Outcomes	6a	Completely defined prespecified assessments or measurements to address each pilot trial objective specified in 2b, including how and when they were assessed	Page 19; Supplementary, page 8
	6b	Any changes to pilot trial assessments or measurements after the pilot trial commenced, with reasons	NA
	6c	If applicable, prespecified criteria used to judge whether, or how, to proceed with future definitive trial	NA
Sample size	7a	Rationale for numbers in the pilot trial	Page 18
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence	8a	Method used to generate the random allocation sequence	NA

generation	8b	Type of randomisation(s); details of any restriction (such as blocking and block size)	NA
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	NA
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	NA
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	NA
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12	Methods used to address each pilot trial objective whether qualitative or quantitative	NA
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were approached and/or assessed for eligibility, randomly assigned, received intended treatment, and were assessed for each objective	Figure 2
	13b	For each group, losses and exclusions after randomisation, together with reasons	Figure 2
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Page 7
	14b	Why the pilot trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each objective, number of participants (denominator) included in each analysis. If relevant, these numbers should be by randomised group	Figure 2
Outcomes and estimation	17	For each objective, results including expressions of uncertainty (such as 95% confidence interval) for any estimates. If relevant, these results should be by randomised group	NA
Ancillary analyses	18	Results of any other analyses performed that could be used to inform the future definitive trial	NA
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	NA
	19a	If relevant, other important unintended consequences	NA
Discussion			
Limitations	20	Pilot trial limitations, addressing sources of potential bias and remaining uncertainty about feasibility	NA
Generalisability	21	Generalisability (applicability) of pilot trial methods and findings to future definitive trial and other studies	NA
Interpretation	22	Interpretation consistent with pilot trial objectives and findings, balancing potential benefits and harms, and considering other relevant evidence	NA
	22a	Implications for progression from pilot to future definitive trial, including any proposed amendments	NA
Other information			
Registration	23	Registration number for pilot trial and name of trial registry	Page 3, 22

Protocol	24	Where the pilot trial protocol can be accessed, if available	Page 3
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Pages 2-3
	26	Ethical approval or approval by research review committee, confirmed with reference number	Page 22

Citation: Eldridge SM, Chan CL, Campbell MJ, Bond CM, Hopewell S, Thabane L, et al. CONSORT 2010 statement: extension to randomised pilot and feasibility trials. BMJ. 2016;355.
*We strongly recommend reading this statement in conjunction with the CONSORT 2010, extension to randomised pilot and feasibility trials, Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.