THE LANCET

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* 2014; published online Oct 13. http://dx.doi.org/10.1016/S0140-6736(14)61403-3.

Supplementary Appendix:

Methods

Toxicity Monitoring:
The following toxicities were not considered dose limiting toxicities: tumor lysis syndrome, abnormal electrolytes responding to supplementation, hypoalbuminemia, liver dysfunction resolving to ≤grade 2 within 14 days, transient (<72 hours) grade 4 hepatic enzyme abnormality, and grade 3 or 4 fever or neutropenic fever.

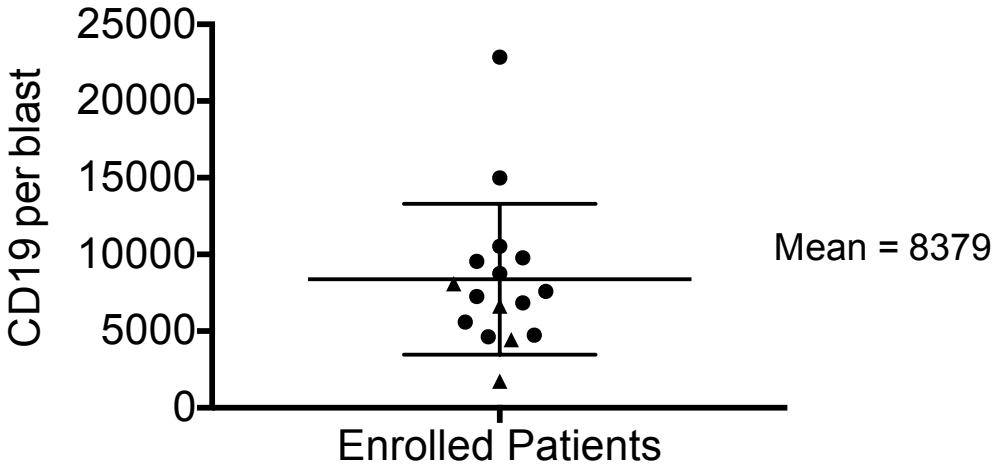
Proliferation Assays and HAMA Monitoring:
For assessment of humoral immune responses to CD19-CAR transduced T cells, thawed serum obtained prior to and four weeks following CD19-CAR T cell infusion was tested in duplicate for human anti-mouse antibodies according to the manufacturer's instructions (ALPCO). For assessment of T cell immune responses to CD19-CAR transduced T cells, thawed elutriated lymphocytes obtained prior to CD19-CAR therapy and 28 days following CD19-CAR therapy were plated in triplicate at 10^5 cells/well in 96-well flat-bottomed plates (Nunc) with 20 IU/ml rhIL-2 (Peprotech). Targets (10^5 cells/well) were autologous non-transduced T cells or CD19-CAR transduced T cells that were PHA activated for 72h (10 ug/ml, Life Technologies), then irradiated (3000 Rad). Cultures were incubated at 37°C with 5% CO_2 for five days, then cell proliferation was measured by incorporation of ^{3}H-thymidine (1 uCi/well, Perkin Elmer) after a 16–18h pulse using a microplate scintillation counter (Top Count, Packard). Two patients who received a second infusion of CD19-CAR T cells were also tested following the second infusion.

Flow Cytometry for T Cell Subsets:
For T cell subset analyses, analysis of the apheresis product and CD19-CAR product was undertaken using thawed cryopreserved samples. For analysis of peripheral blood subsets, specimens were processed within 12h of collection. Red blood cells were pre-lysed by incubating with hypertonic ammonium chloride solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 10 minutes at room temperature (maintained at 21-23^{o}C) at a ratio of 1:9 (volume of sample: volume of lysing solution). Cells were incubated for 15 minutes in the dark at room temperature with a 1/100 dilution of Aqua Live Dead (InVitrogen/Molecular Probes, Grand Island, NY) Cells were then washed with PBS and re-suspend in sterile azide free RPMI with 4% FBS before incubation for 20 min in the dark at 37 C with CCR7-FITC (Clone: 150503, BD Biosciences, San Jose, CA). Cells were washed with cold PBA before incubation for 30 minutes in the dark at room temperature with a panel of antibodies including the following: CD4 Brilliant Violet 421(clone OKT4), CD8a Brilliant Violet 421 (clone RPA-T8),and CD45RO PE (clone UCHL1) (Biolegend, San Diego, CA); CD3 AH7 (Clone: SK7), CD27PC7 (clone M-T271) and CD95PerCP (clone DX2) (BD Biosciences, San Jose, CA); and the anti-idiotype mAb 136.20.1 CD19scFv Alexa 647 (provided by Dr. Lawrence Cooper, M.D.Anderson, Tx). Cells were then washed once with cold PBA and resuspended in 0.5% formalin and stored at 4°C for up to 12 h before acquisition. Approximately 2- 3 million cells were acquired for each cocktail using an 8-color multiparametric approach on a 3-laser FACS Canto II (BD Biosciences, San Jose, CA) with DiVa 6.1.1 software. The data was analyzed by FCS Express 4 software (DeNovo Software, Los Angeles, CA). An analysis gate of Aqua Live Dead negative and CD3 positive was used to analyze live T-cells, with CD4 and CD8 to separate these subsets for further evaluation. Naive T-cells were defined as positive for CCR7 and CD27 but negative for CD95 and CD45 RO. Terminal effector T-cells were defined as positive for CD95 but negative for CCR7, CD27 and CD45RO. Effector memory T-cells were defined as positive for CD45RO and CD95 but negative for CCR7 and CD27. Central memory T-cells were defined as positive for CCR7, CD27, CD45RO and CD95. T memory stem cells were defined as positive for CCR7, CD27 and CD95 but negative for CD45RO.

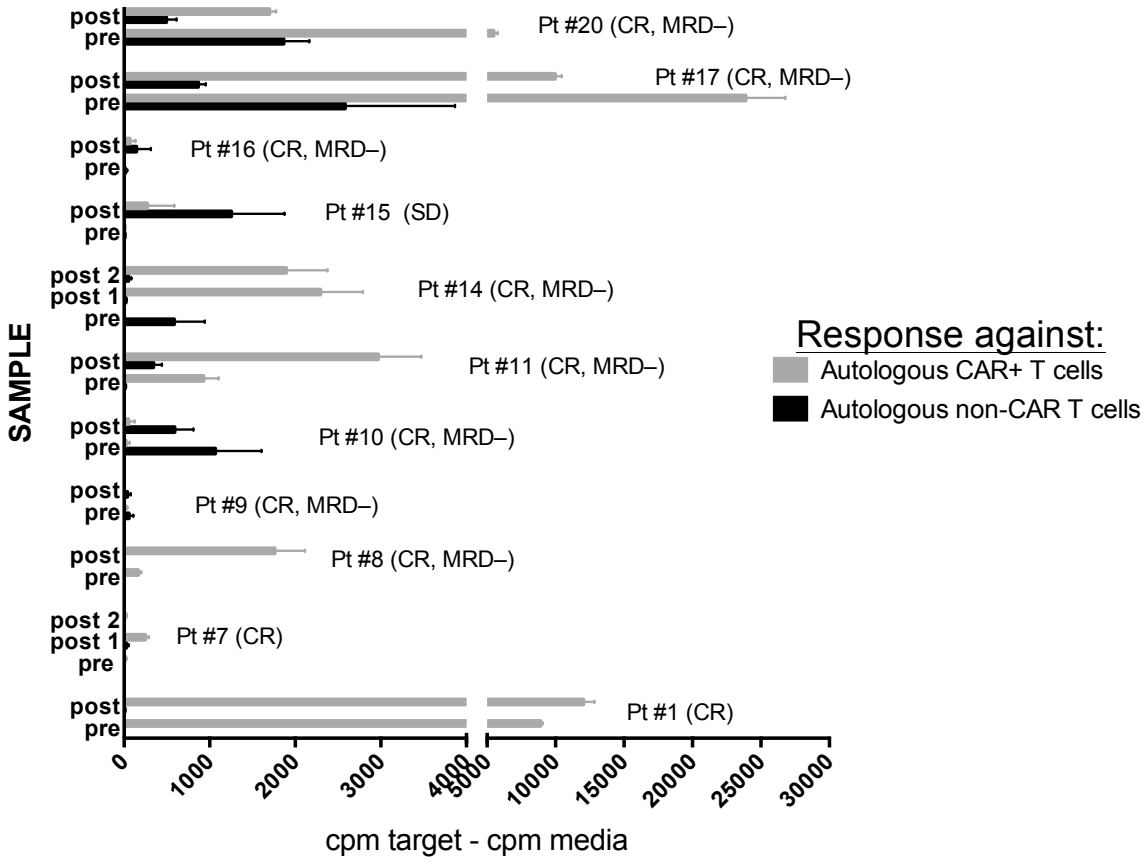
Statistical Analyses:
Relationships between peak IL-6 and CRP and circulating CAR T cells and copies of the CAR gene/100ng genomic DNA were analyzed using a Spearman correlation, with Fisher's z transformation used to estimate the confidence interval. Log_{10} values were used to transform data as appropriate, with 0·01 substituting for values of 0. Correlations of $|r| > 0{\cdot}70$ were interpreted as strong; $0{\cdot}5 < |r| < 0{\cdot}7$, as moderately strong; $0{\cdot}3 < |r| < 0{\cdot}5$, as weak to moderately strong, and $|r| < 0{\cdot}30$ as weak. Differences between two categories with respect to continuous parameters were determined using an exact Wilcoxon rank sum test. For analysis of T cell

proliferative responses to CD19-CAR transduced cells, comparisons were made between the mean net proliferation of all 22 samples analyzed (11 obtained pre-therapy and 11 obtained post-therapy) to autologous CD19-CAR transduced T cells vs mean net proliferation to autologous non-transduced T cells using a Wilcoxon matched pairs signed rank test. A Hodges-Lehmann estimator was used to report the difference and the confidence interval on the difference between two groups as reported. All p-values are two-tailed and presented without any formal adjustment for the limited number of statistical comparisons performed.

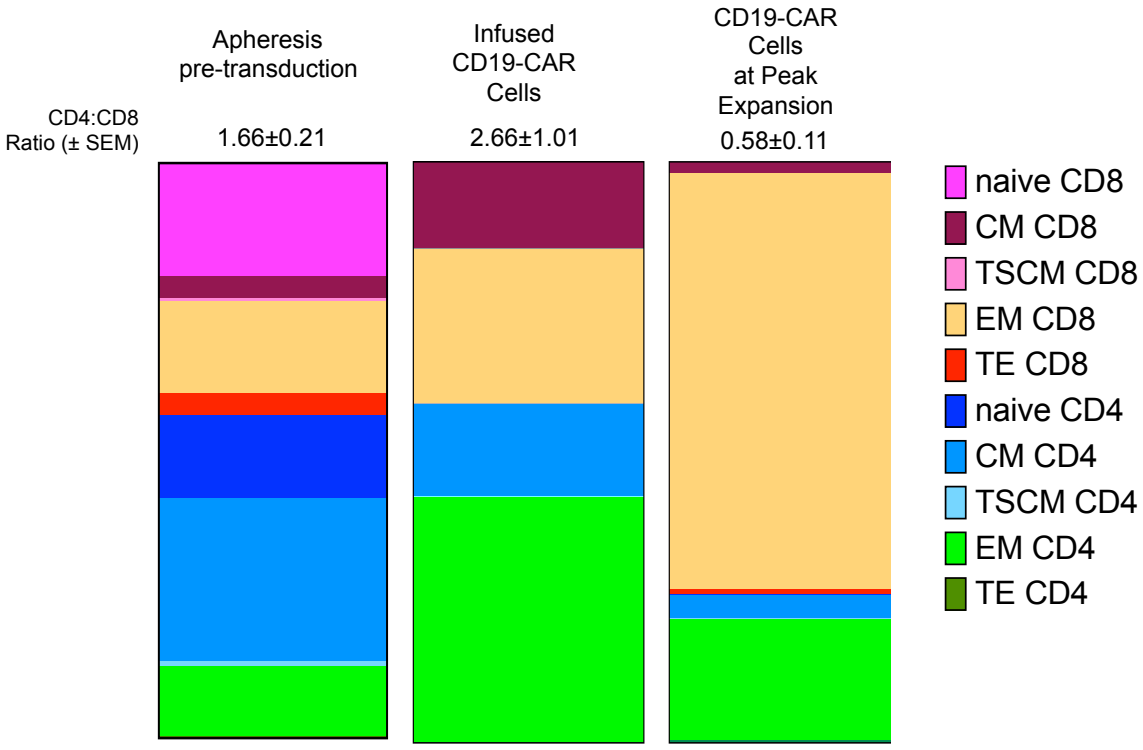
Supplementary Figures



Supplementary Figure 1. CD19 is highly expressed on patient ALL blasts.
CD19 site density was evaluated for 16 B-ALL patients enrolled from whom samples were available. The mean site density was 8,379 CD19 molecules per blast (SD 4,921). Circles represent responding patients. There was no statistical difference in CD19 site density in responders vs non-responders (p=0.073, data not shown).



Supplementary Figure 2. Anti-CD19 CAR T cell immune reactivity.
T cells collected before (pre) or after (post) therapy with anti-CD19 CAR T cells were tested for anti-CAR T cell responses (grey bars) when sufficient cell numbers were available (n=11) using a proliferation assay. Comparison is made to proliferation directed against autologous, non-CAR transduced cells (black bars). All samples were run in triplicate, and error bars represent the standard deviation. Each set of pre- and post-therapy proliferation is annotated with patient number and clinical response.



Supplementary Figure 3. Phenotypic composition of starting apheresis, CD19-CAR product and expanded CD19-CAR cells *in vivo.*
CD4+ and CD8+ CAR T cells were analyzed for the T cell subsets listed in the apheresis used to manufacture CAR T cells, in the CAR T cell product that was infused, and *in vivo* at the time of peak expansion. Apheresis products contained sizable fractions of naïve and central memory cells. Infused products were predominantly effector memory cells with more CD4 > CD8 cells, whereas composition at the time of peak expansion was predominantly effector memory with CD8 > CD4 cells. CM = central memory, TSCM = T stem cell memory, EM = effector memory, TE = terminal effector

Supplementary Table S1. Cytokine release syndrome grading system.

Grade 1	Mild reaction; infusion interruption not indicated; intervention not indicated.
Grade 2	Therapy or infusion interruption indicated but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics, IV fluids); prophylactic medications indicated for <= 24 hours.
Grade 3	Severe symptoms including any one or more of the following: • A drop in blood pressure of 20% or more from the patient's baseline (systolic, diastolic or MAP) not responsive to fluid therapy within 24 hours or not responsive to IV fluid bolus of at least 20 ml/kg • Grade 3 Respiratory dysfunction • Grade 3 Creatinine indicative of renal dysfunction • Grade 3 Neurologic dysfunction
Grade 4	Life-threatening consequences; vasopressor or ventilator support indicated.

Supplementary Table S2. Definition of M status for B-ALL.

	Percent blasts (at least 200 cells counted)
M1	<5%
M2	5 – 25%
M3	>25%

*Modified from: Cheson BD, et al. Revised recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. J Clin Oncol 2003;21:4642-4649

Supplementary Table S3. Definition of CNS status for B-ALL.

	CSF cell count and cytology
CNS1	No blasts on cytology
CNS2	<5/uL WBCs and cytology positive for blasts or Traumatic spinal tap with ≥ 10/µL RBCs, WBC ≥ 5/µL, cytospin positive for blasts but negative by Steinherz/Bleyer algorithm*
CNS3	≥ 5/µl WBCs, cytospin positive for blasts or Traumatic spinal tap with ≥ 10/µL RBCs, cytospin positive for blasts, and positive Steinherz/Bleyer algorithm*

*Steinherz/Bleyer algorithm method of evaluating traumatic lumbar punctures:
If the patient has leukemic cells in the peripheral blood and the lumbar puncture is traumatic and contains ≥ 5 WBC/µL and blasts, the following algorithm should be used to distinguish between CNS2 and CNS3 disease:

CSF WBC/RBC > 2X Blood WBC/RBC

Supplementary Table S4. Prior therapies for all enrolled patients.

Patient	Previous therapies administered
1	-As per POB protocol 95-C-2001 with craniospinal radiation (1800 cGy) -As per COG AALL0132 -Matched related BMT with TBI/cyclophosphamide (Cy) prep -BFM-REZ -Second matched related BMT -Rituximab & ICE x 4 cycles -POB protocol 08-C-0123 (Phase I: moxetumomab pasudotox) -4 drug re-induction -POB protocol 08-C-0051 (Pilot: WT-1 vaccine trial with donor lymphocyte infusion) -4 drug re-induction followed by weekly asparaginase -POB protocol 10-C-0220 (Phase I: liposomal vincristine) -Re-induction with single dose vincristine, PEG-asparaginase 2 doses of IV methotrexate & dexamethasone -XRT to bilateral testes (2400 cGy) -Vincristine, 6-mercaptopurine, dexamethasone, & PEG-asparaginase -Triple intrathecal (IT) therapy and IT methotrexate
2	-COG-AALL0331, SR-low arm -4 drug re-induction per AALL01P2 -Matched sibling BMT with TBI/Cy/ATG prep -Ifosfamide & etoposide per AALL0331 Consolidation Block 1 -Clofarabine & cytarabine per AALL01P2 Treatment Group 1 Arm A Blocks 1 & 2
3	-Prednisone & weekly methotrexate -Mycophenolate mofetil -Rituximab, vincristine, cyclosphosphamide & doxorubicin (R-CHO without prednisone) -Rituximab, ifosfamide, carboplatin & etoposide -MUD BMT with TBI/Cy prep -vinorelbine/gemcitabine -local radiation to multiple sites -Donor lymphocyte infusion -Brentuximab vedotin (anti-CD20 Ab) -Rituximab -Dexamethasone, 6-mercaptopurine and oral methotrexate
4	-AALL1131 Induction without dexamethasone -As per AALL0031 consolidation block 1 & 2
5	-AALL0932 Induction -St. Jude TOTXV -As per AALL07P1 Re-Induction Block 1 without bortezomib -As per AALL07P1 Re-Induction Block 2 Days 1-5 without bortezomib
6	-Standard 4-drug induction with vincristine, prednisone, L-asparaginase, daunorubicin and triple intrathecals -Re-induction and another salvage attempt (international records not available) -As per AALL1131 Induction -As per AALL07P1 Re-Induction Block 2
7	-Hyper-CVAD x8 cycles -POMP maintenance therapy -POB protocol 08-C-0123 (Phase I: moxetumomab pasudotox)
8	-COG AALL1131 Induction and consolidation -MUD BMT
9	-As per AALL0232 Induction + 14 day extended induction -As per COG AALL07P1 Reinduction Block 2 with bortezomib -Myeloablative haploidentical BMT with Cy/TBI prep

	-POETIC 10-03 (etoposide, cytarabine, plerixafor) -POB protocol 08-C-0123 (Phase I: moxetumomab pasudotox)
10	-COG AALL1131 Induction, consolidation, and high-dose methotrexate as per interim maintenance course 1 (3 courses) -Vincristine, 6-mercaptopurine, methotrexate
11	-As per AALL0232 through maintenance cycle 6 -AALL02P2 for isolated CNS relapse
12	-As per protocol AHOPCA 2008 -As per a modified ALL protocol 90 (4 doses cytarabine & 3 doses etoposide) -6-mercaptopurine, methotrexate, prednisone -As per AALL0433 re-induction and induction #2 -POB protocol 10-C-0220 (Phase I: liposomal vincristine)
13	-Larson protocol for induction & re-induction then 2 years of therapy -Re-induction with hyper-CVAD alternating with high dose methotrexate & cytarabine for 3 cycles -Maintenance with 6-mercaptopurine, prednisone, methotrexate and vincristine
14	-CCG protocol 1991 -As per AALL0433 regimen A -As per AALL01P1 -Clofarabine-based regimen -UCBT as per BMT protocol CTN0501 with fludarabine, cyclophosphamide and TBI. -8 months of low-dose chemotherapy with IV methotrexate, IT methotrexate & 6-mercaptopurine
15	-As per AALL0232 for Induction -As per AALL0031 Consolidation Block 1 & 2 with imatinib -As per AALL0031 Re-induction Block 1, Intensification Block 1, Re-induction Block 2 with imatinib -Matched sibling BMT with Cy/TBI prep -As per AALL0031 matintenance with vincristine, 6-mercaptopurine, methotrexate, and dasatinib
16	-Cycle 1A and 1B of hyper-CVAD + IT methotrexate -Re-induction cycle 1 as per COG AALL07P1
17	-As per AALL1131 Induction & consolidation (standard arm) -As per AALL0031 (1 cycle) including ifosfamide/etoposide
18	-Standard risk ALL group stratification on protocol 9900/9905 -AALL0433 Induction, Induction II, Induction III, Intensification I -Testicular radiation therapy -As per AALL07P1 with bortezomib -Matched sibling BMT with TBI/Cy/Thiotepa prep with post transplant cyclophosphamide
19	-CCG 1961 -As per AALL0433 -Re-induction with decitabine/vorinostat plus UK R3 backbone therapy -As per AALL0433 Induction 2 -As per AALL1131 Consolidation therapy (2 cycles) -As per AALL1131 maintenance regimen (Non-DS patients)
20	-AALL1131 Induction with PEG-asparaginase -As per AALL1131 VHR Control Arm Consolidation (1 dose PEG-asparaginase then Erwinia) -As per AALL1131 VHR Control Arm Interim Maintenance 1 -Cyclophosphamide & etoposide
21	-AALL0932 Induction -As per AALL1131 VHR ALL Consolidation -As per AALL1131 VHR control arm Interim Maintenance 1 (through Day 15 high dose methotrexate)