Supplemental information

CAR T cell therapy is effective for CD19-dim B-lymphoblastic leukemia but is impacted by prior blinatumomab therapy

Vinodh Pillai[1*], Kavitha Muralidharan[2], Wenzhao Meng[1], Asen Bagashev[1], Derek A. Oldridge[1], Jaclyn Rosenthal[2], John Van Arnam[1], Jos J. Melenhorst[1], Amanda M. DiNofia[4], Minjie Luo[1], Sindhu Cherian[3], Jonathan R. Fromm[3], Gerald Wertheim[1], Andrei Thomas-Tikhonenko[1], Michele Paessler[1], C.H. June[1], Eline T. Luning Prak[1], Vijay G. Bhoj[1], Stephan A. Grupp[4], Shannon L. Maude[4**], and Susan R. Rheingold[4**]

[1]Department of Pathology and Laboratory Medicine, University of Pennsylvania.

[2]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

[3]Department of Laboratory Medicine, University of Washington.

[4]Division of Oncology, Cancer Immunotherapy Program, Children's Hospital of Philadelphia.

[**]Equal contribution

[*]**Corresponding author:** Vinodh Pillai, The Children's Hospital of Philadelphia, 3400 Civic Center Blvd, Philadelphia, PA 19104, Phone: 215-590-1890, Email: pillaiv1@email.chop.edu.

Genetic analyses

Chromosome analysis by Giemsa-trypsin banding was performed on metaphase spreads prepared from unstimulated bone marrow or peripheral blood samples after 24-48 hours of culture. Fluorescence in situ hybridization (FISH) analyses using a series of probe set for *ETV6/RUNX1*, *KMT2A/MLL*, *BCR/ABL1*, *CRLF2*, and centromeres 4 and 10 were performed per modified Children's Oncology Group protocol. Genomic DNA and RNA for next generation sequencing (NGS) and single nucleotide polymorphism (SNP) array analyses were extracted from uncultured samples. Library preparation for DNA-based NGS panel and RNA-based fusion panel was performed using customized SureSelectQXT (Agilent) and ArcherTM FusionPlex (Archer) kits respectively per manufacturer instructions. Sequencing was performed on Illumina MiSeq or HiSeq 2500 instruments. The NGS panels interrogate 118 genes for sequence and copy number variants (CNV) and 110 genes for known and novel fusions. Genome-wide SNP array analysis was performed using Illumina Human Bead Chips. *CD19* gene locus at 16p11.2, chr16:28,943,260-28,950,668 (hg19) was examined for deletions. *CD19* gene Sanger sequencing and analysis was performed as reported.[15] Sanger chromatograms were visualized using the Bioconductor package, sangerseqR (version 1.14.0), through in the R programming language (version 3.4.4). Base calling for coding exons and intronic splice donor/acceptor sites was performed using the makeBaseCalls function of sangerseqR with parameter ratio=0.15. All mutations were manually reviewed and were only called when supported by bi-directional Sanger tracings.

Cell lines

K562 and Nalm6 cells were purchased from ATCC and cultured in RPMI medium with 10% Fetal Bovine Serum (Sigma), 1% Hepes (Gibco) and 1% Penicillin/Streptomycin antibiotic (Gibco). K562 and Nalm6 cells were each transduced with a lentiviral vector encoding click beetle green luciferase for use in luciferase-based cytotoxicity assays.

Supplemental table and figure legends

Supplemental table 1. Flow cytometric panels and antibodies used in clinical flow cytometry at the Children's Hospital of Philadelphia.

Supplemental Figure 1. CAR T cell killing of control Nalm6 and CD19 mRNA transduced K562 cells at various effector: target ratios are shown.

Supplemental figure 2. MRD level CD19-negative populations before CAR T-cell therapy. a. Sequential gating of CD10+ CD58bright CD20variable blasts (red) in HP-10 (no recurrence) revealed 0.07% CD19-negative blasts before CAR infusion. Negative control gate was based on internal CD3+ T cells (teal) b. Sequential gating of CD10+ CD34dim CD38dim blasts in HP-102 revealed 0.76% CD19-negative blasts before CAR infusion. Patient recurred with CD19-negative leukemia 3 months after infusion. c. Sequential gating of CD10+ CD38dim CD58bright blasts in HP-6 revealed 0.07% CD19-negative blasts before CAR infusion. Patient recurred with CD19-positive leukemia 12 months after infusion. d. Proportion of CD19-negative blasts among total blasts in the remission, CD19-posMRD/Relapse and CD19-negMRD/Relapse groups

Supplemental figure 3. Morphologic and immunophenotypic comparison of representative KMT2A rearranged leukemia with ALL to AML switch (HP-78). Pre-CAR CD45dim lymphoblasts (a) with multiple B cell antigens (CD19, CD22) showed switch to myeloid lineage by morphology (increased size and cytoplasm) and immunophenotype (CD13, CD33, loss of CD19 and CD22) post-CAR T-cell therapy.

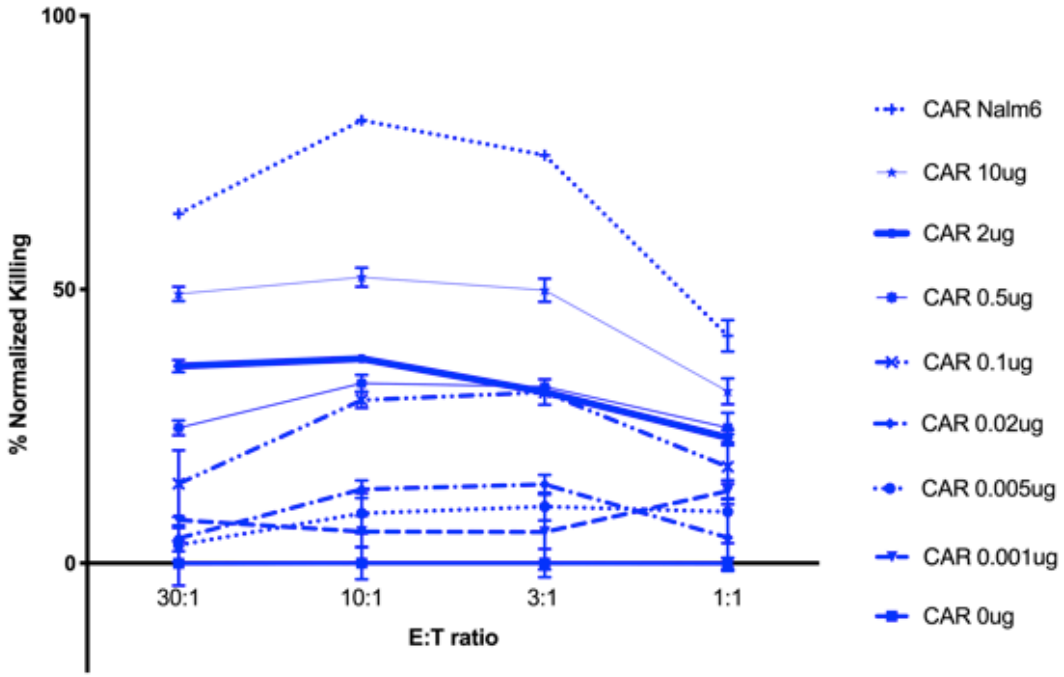
Supplementary data file. Sequences of Dominant Antibody Heavy Chain Rearrangements. Data from individual (biological) replicates are shown on the highest copy number gene rearrangement from each sequencing library. The dominant clone ID refers to its copy number rank in the library (1 = highest copy number rearrangement; 2 = second highest copy number rearrangement). Total reads indicate the number of valid reads in the sample. Freq. refers to the frequency of the dominant rearrangement in the sample, expressed as a fraction. V, D and J provide the most similar germline variable, diversity and joining gene segments, respectively. CDR3aa provides the amino acid sequence of the third complementarity determining region. CDR3nt provides the nucleotide sequence of the CDR3. Functional indicates whether the rearrangement is productive or non-productive (in the latter case, rearrangements are non-productive either by virtue of being out of frame or having a premature termination codon or both). The raw nucleotide sequence of the rearrangement is also provided.

Supplementary table 1. Flow cytometric panels and antibodies used.

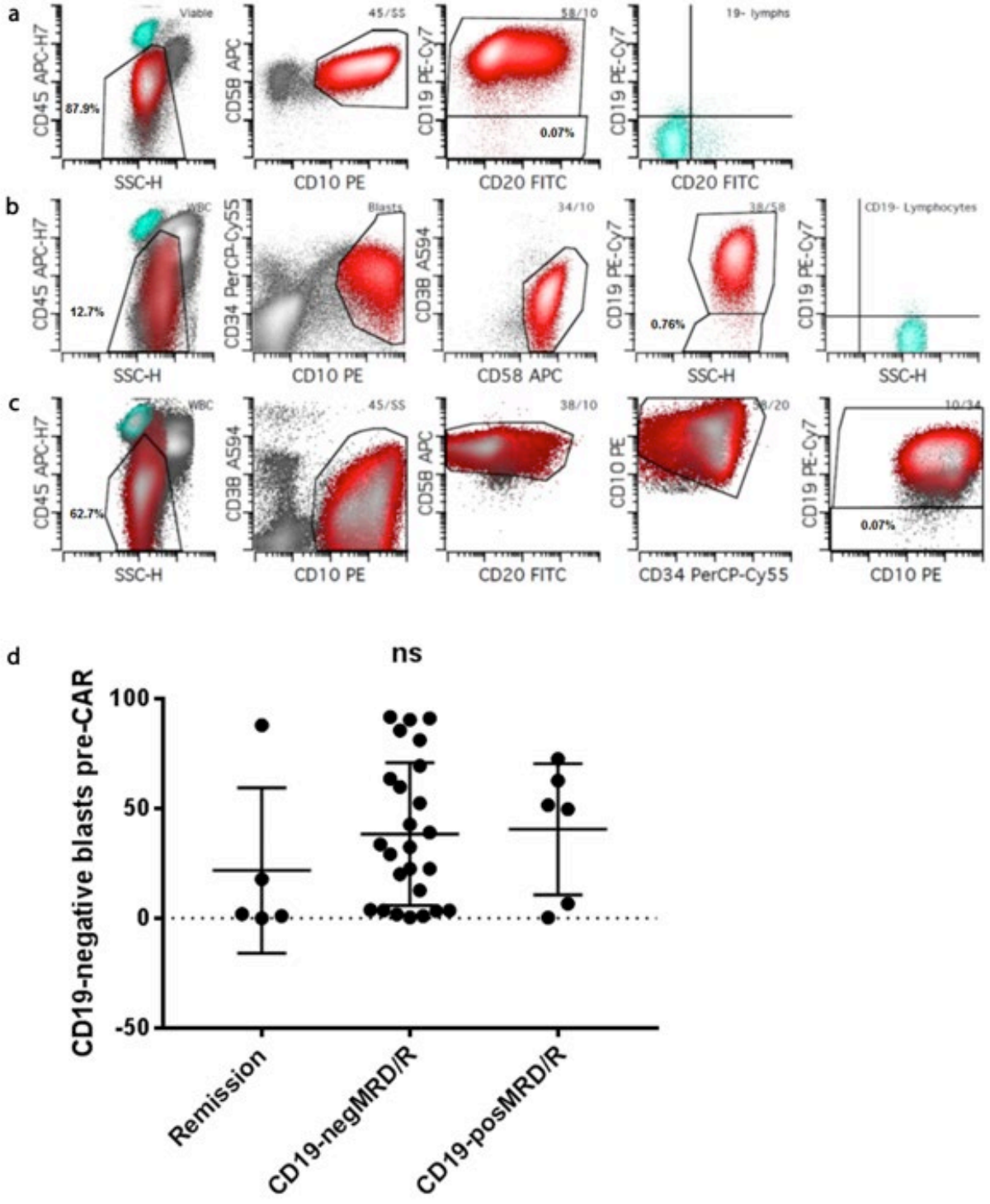
Tube #	FITC	PE	ECD	PC5.5 / PerCP-Cy5.5	PC7	APC	APC-AF700	APC-AF750	Pacific Blue	Krome Orange
Isotype	MsIgG1 BC IM0639U	MsIgG1 BC IM0670U	MsIgG1 BC IM2714U	MsIgG1 BC A62834	MsIgG2a BC A12692	MsIgG1 BC IM2475U	MsIgG1 BC A71118	MsIgG1 BC A71120	MsIgG1 BC A74766	CD45 BC A96416
1	Kappa D F0434	Lambda D R0437	CD20 BC IM3607	CD38 (PerCP-Cy5.5) BD 656050	CD5 BC A51075	CD22 BC A60791	CD19 BC A78837	CD10 BC A89310	IgM BC B30656	CD45 BC A96416
2	CD2 BC B36533	CD30 INV 12-0309-42	CD7 BC A70202	CD3 BC A86327	CD5 BC A51075	CD56 BD 341026	CD34 BC A86354	CD4 BC A94685	CD8 BC A82791	CD45 BC A96416
3	CD9 BD 341636	CD10 BD 340920	CD7 BC A70202		CD5 BC A51075	CD1a BC IM3645	CD34 BC A86354	CD4 BC A94685	CD8 BC A82791	CD45 BC A96416
4	CD13 BC IM0778U	CD33 BD 340679	HLA-Dr BC IM3636		CD5 BC A51075	CD56 BC IM3701	CD34 BC A86354			CD45 BC A96416
5	CD13 BC IM0778U	CD33 BD 340679	HLA-Dr BC IM3636	CD38 (PerCP-Cy5.5) BD 656050	CD19 BC IM3628U	CD117 BC IM3538	CD34 BC A86354	CD71 BC A89313	CD15 BC A74775	CD45 BC A96416
6	CD13 BC IM0778U		HLA-Dr BC IM3636	CD38 (PerCP-Cy5.5) BD 656050	CD64 BC B06925	CD123 BC B06376	CD34 BC A86354	CD4 BC A94685	CD14 BC B00846	CD45 BC A96416
7		CD33 BD 340679		CD38 (PerCP-Cy5.5) BD 656050	CD5 BC A51075	CD56 BD 341026	CD34 BC A86354		CD7 BC B06499	CD45 BC A96416
8	CD235a BC IM2212U	CD33 BD 340679	HLA-Dr BC IM3636	CD56 BC A79388	CD64 BC B06925	CD123 BC B06376	CD34 BC A86354	CD71 BC A89313	CD65 BC B08164	CD45 BC A96416
9	CD42b BC IM0648U	CD24 BC IM1428U		CD33 BC A70198	CD61 BC IM3716	CD41 BC B16894		CD10 BC A89310	CD15 BC A74775	CD45 BC A96416
Cytoplasmic	TdT BC IM3524	MPO BC IM3455U	CD3 BC IM2705U		CD22 BC A74788	CD79a BC A60793			IgM BC B30656	CD45 BC A96416
Limited CART panel	CD66b BC IM0531U	CD24 BC IM1428U	CD20 BC IM3607	CD3 BC A86327	CD19 BC IM3628U	CD22 BC A60791	CD34 BC A86354	CD10 BC A89310		CD45 BC A96416

Vendor abbreviations followed by catalog numbers; BC = Beckman Coulter, BD = Becton Dickinson, D = Dako-Agilent, Inv = Invitrogen;

Supplemental figure 1.



Supplemental figure 2.



Supplemental figure 3.