Supplementary Data

Supplementary Tables

Table S1. Summary of patient key eligibility criteria for trials

	ELIANA (NCT02435849)	ENSIGN (NCT02228096)	JULIET (NCT02445248)
Age	≥3 years at screening and ≤21 years at diagnosis	≥3 years at screening and ≤21 years at diagnosis	≥18 years
Disease	Relapsed or refractory pediatric B-ALL	Relapsed or refractory pediatric B-ALL	Relapsed or refractory adult DLBCL
Prior lines of therapy	≥2 bone marrow relapses, or Primary refractory after 2 cycles of chemotherapy	≥2 bone marrow relapses, or Primary refractory after 2 cycles of chemotherapy	≥2 lines of therapy
Bone marrow blasts	Bone marrow with ≥5% lymphoblasts by morphologic assessment at screening	Bone marrow with ≥ 5% lymphoblasts by morphologic assessment at screening	Adequate bone marrow reserve
Prior alloSCT	Allowed	Allowed	Not allowed
Active CNS involvement	No	CNS-3 allowed after reduced to CNS-1/2	No
Prior gene therapy	No	No	No
Prior anti-CD19 therapy	No	No	No

Abbreviations: alloSCT, allogeneic stem cell transplant; B-ALL, B-cell acute lymphoblastic leukemia; CNS, central nervous system; DLBCL, diffuse large B-cell lymphoma.

Table S2. Summary of individual patients with T-cell responses ≥1%

Patient	Trial	BOR	DOR, days	Maximum CRS grade	Peptide pool	Net CD3+ CD4+ IFN-γ response, %	Net CD3+ CD8+ IFN-γ response, %
1	JULIET	CR	443[a]	—	1	11.9	—
2	JULIET	PD	—	—	1, 2	4.32	3.91, 1.68
3	JULIET	PD	—	—	1	1.145	—
4	JULIET	PD	—	—	2	1.82	—
5	JULIET	SD	—	3	2	1.6	2.63
6	JULIET	PR	55[b]	—	2	1.47	1.69
7	JULIET	PD	—	4	2	1.075	—
8	JULIET	SD	520[c]	2	2	—	1
9	ELIANA	CR	69[d]	3	1	1.17	1.346

Abbreviations: BOR, best overall response; CR, complete response/remission; CRi, CR with incomplete blood count recovery; CRS, cytokine release syndrome; DOR, duration of response; IFN-γ, interferon gamma; PD, progressive disease; PR, partial response; SD, stable disease.
[a]Death due to reasons other than underlying cancer.
[b]Disease progression.
[c]Ongoing without an event.
[d]Underwent hematopoietic stem cell transplant.

Table S3. Response categories and CRS grade among patients with B-ALL and the 90th percentile for pretreatment anti-mCAR19 antibody levels[a]

	Patients in the 90th percentile anti-mCAR19 levels (N=14), n (%)	Overall patient population (N=137), n (%)
Response category		
CR	5 (36)	33 (24)
CRi	6 (43)	61(45)
NR	1 (7)	11 (8)
Maximum CRS grade		
No CRS	3 (21)	24 (18)
Grade 1/2	4 (29)	47 (34)
Grade 3	5 (36)	22 (16)
Grade 4	1 (7)	29 (21)

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; CAR, chimeric antigen receptor; CR, complete remission; CRi, CR with incomplete blood count recovery; CRS, cytokine release syndrome; MFI, median fluorescence intensity; NR, no response.
[a]Pretreatment anti-mCAR19 antibody levels as determined by flow cytometry assay. Patients are from the ELIANA and ENSIGN trials.

Table S4. Summary of baseline anti-mCAR19 antibody levels by rituximab therapy prior to tisagenlecleucel infusion

	Time of rituximab therapy before tisagenlecleucel infusion, months					
	0-3 (n=18)	**3-6 (n=63)**	**6-9 (n=16)**	**9-12 (n=9)**	**≥12 (n=7)**	**Missing (n=2)**
Baseline anti-mCAR19 antibody, MFI						
Median	1820	2750	2100	5000	3150	2220

Abbreviations: mCAR19, murine CAR19; MFI, median fluorescence intensity.

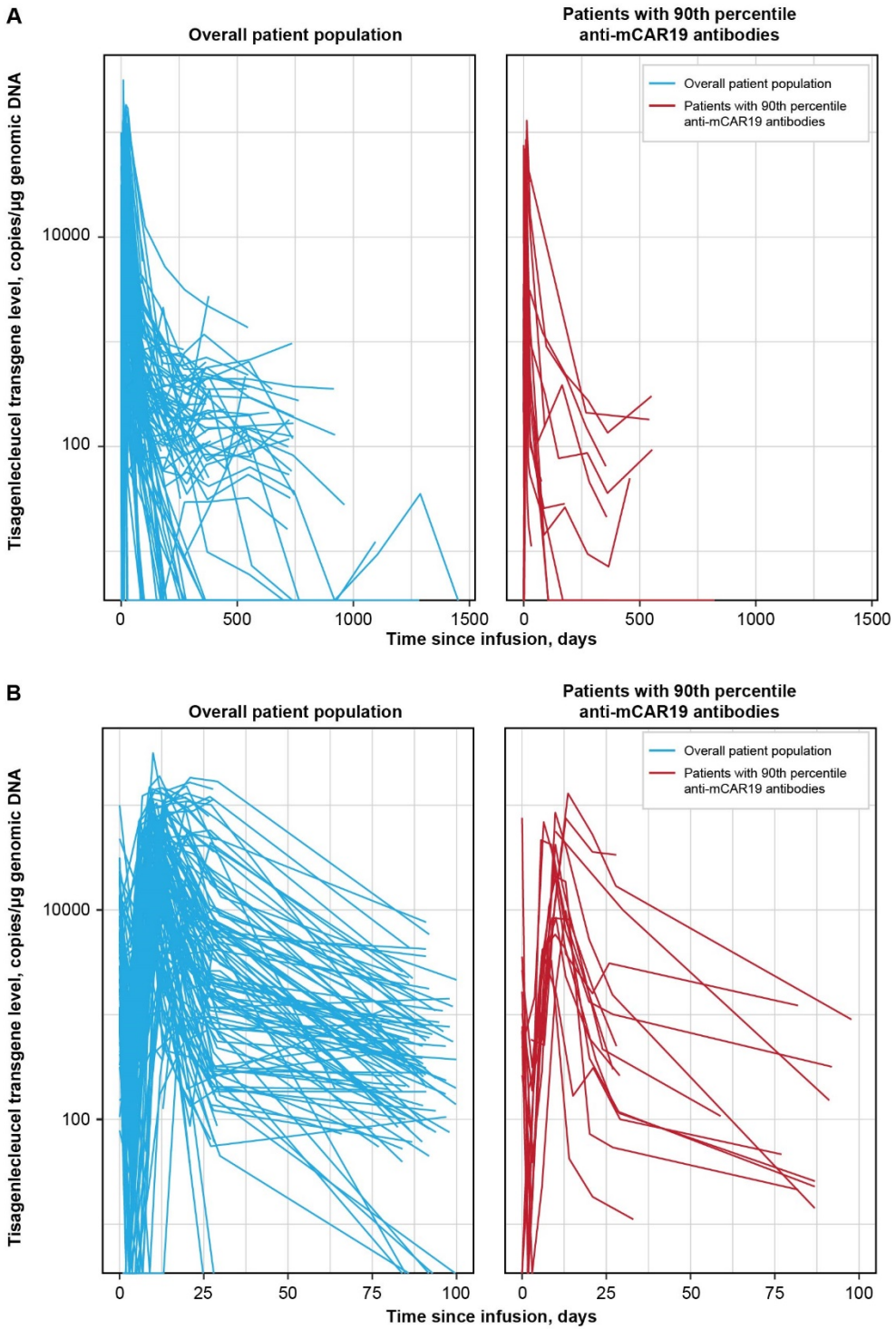
Table S5. Summary of cellular kinetics parameters in peripheral blood by B-cell aplasia status in patients with B-ALL

Parameter	**B-cell recovery <6 months (n=23)**	**B-cell recovery >6 months (n=15)**	**Sustained B-cell aplasia (n=53)**
$AUC_{0\text{-}28d}$, copies/µg × d			
geo-mean	138000	225000	380000
geo CV%	176	170	158
C_{max}, copies/µg			
geo-mean	16200	28600	42600
geo CV%	194	158	145
T_{max}, d			
median	9.91	9.89	8.95
(min, max)	(5.84, 17.8)	(6.92, 54.8)	(5.7, 27.8)
T_{last}, d			
median	78.8	86	546
(min, max)	(17.8, 554)	(20.9, 380)	(100, 921)
C_{last}, copies/µg			
geo-mean	152	158	179
geo CV%	209	170	121

Abbreviations: $AUC_{0\text{-}28d}$, area under the curve from baseline to 28 days after infusion; B-ALL, B-cell acute lymphoblastic leukemia; C_{last}, last detectable transgene level; C_{max}, maximum transgene level; CV, coefficient of variation; T_{last}, time of last detectable transgene; T_{max}, time to maximal transgene level.

Supplementary Figures

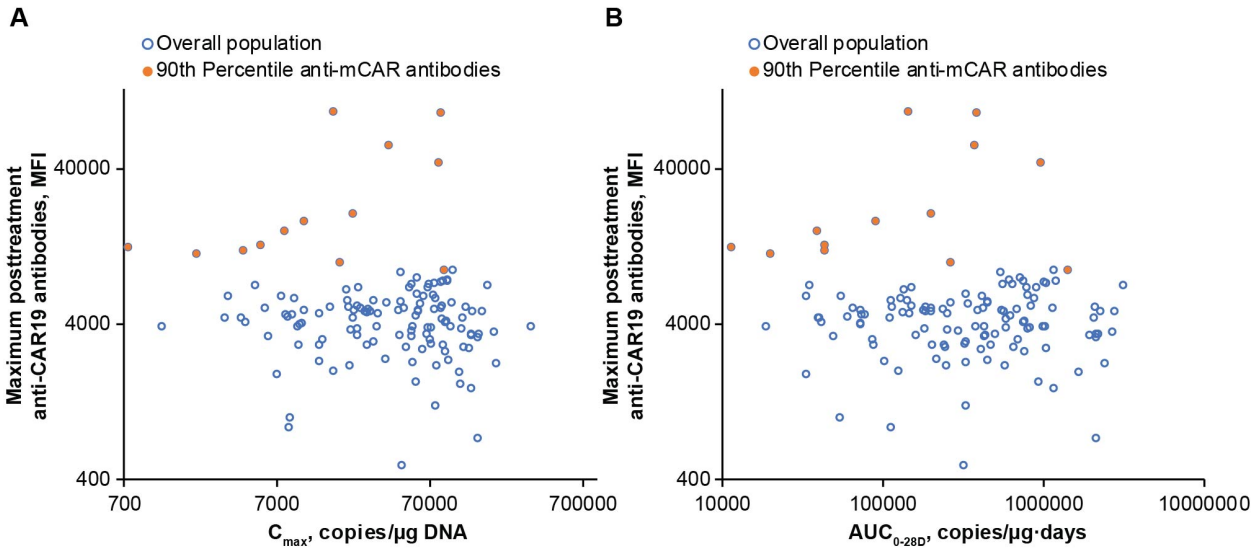
S1



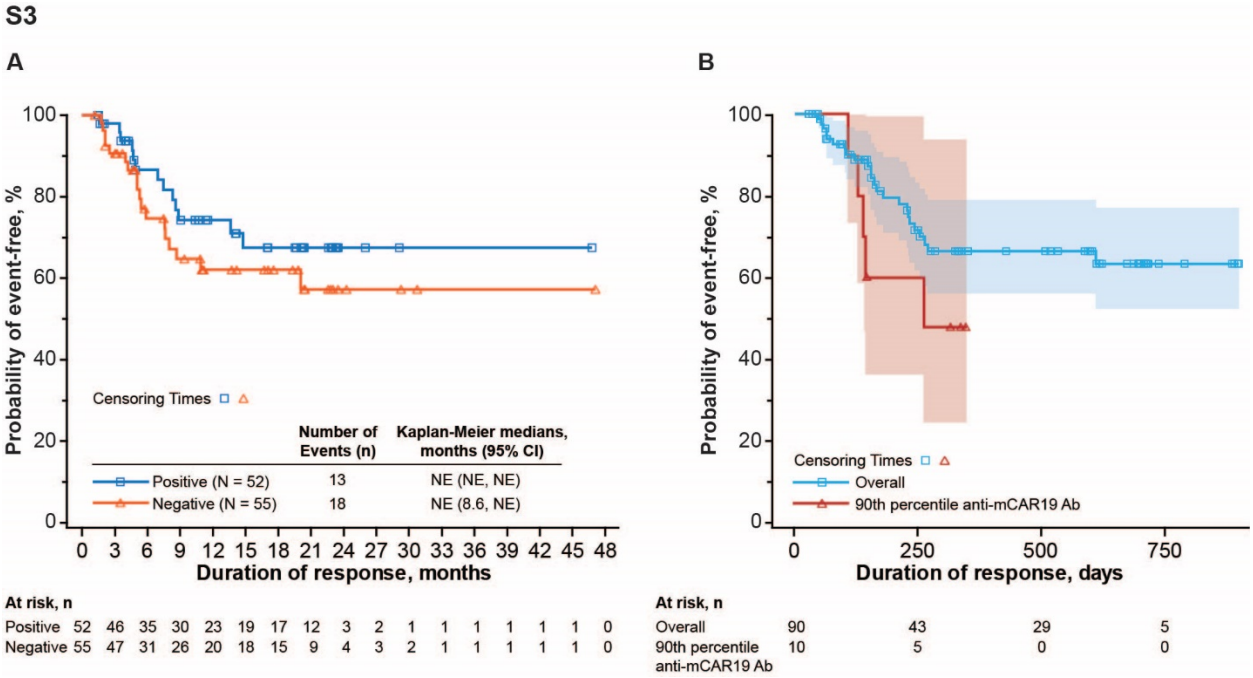
Supplementary Figure S1. Effects of anti-mCAR19 antibodies on transgene expression in

pediatric and young adult patients with r/r B-ALL. Individual concentration/time profiles for the overall patient population and patients in the 90th percentile for anti-mCAR19 antibody levels among pediatric and young adult patients with r/r B-ALL shown for time since infusion (**A**) 0 to 1500 days to show persistence of tisagenlecleucel transgene and (**B**) 0 to 100 days to show expansion of tisagenlecleucel transgene. Patients in the 90th percentile for anti-mCAR19 antibody levels were determined by mean fluorescence intensity. B-ALL, B-cell acute lymphoblastic leukemia; CAR, chimeric antigen receptor; mCAR19, murine CAR19.

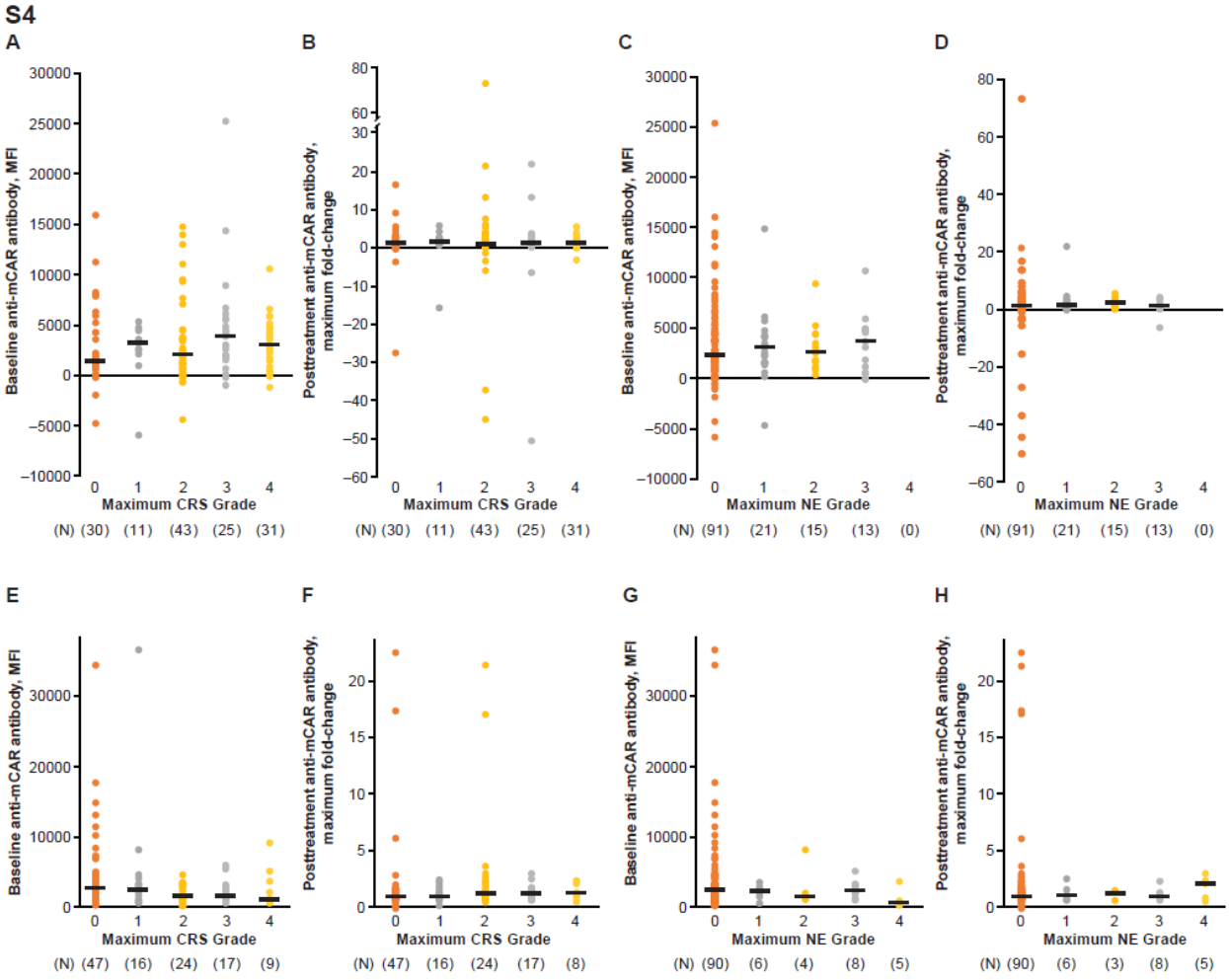
S2



Supplementary Figure S2. Effects of posttreatment anti-mCAR19 antibodies on tisagenlecleucel cellular kinetics in pediatric and young adult patients with r/r B-ALL. (**A**) Maximum tisagenlecleucel expansion (C_{max}) and (**B**) exposure ($AUC_{0\text{-}28d}$). Open circles represent the overall patient population in the ELIANA and ENSIGN trials. Closed circles represent patients in the 90th percentile for posttreatment anti-mCAR19 antibodies. $AUC_{0\text{-}28d}$, area under the curve from baseline to 28 days after infusion; B-ALL, B-cell acute lymphoblastic leukemia; CAR, chimeric antigen receptor; C_{max}, maximum concentration; mCAR19, murine CAR19; MFI, median fluorescence intensity; r/r, relapsed or refractory.

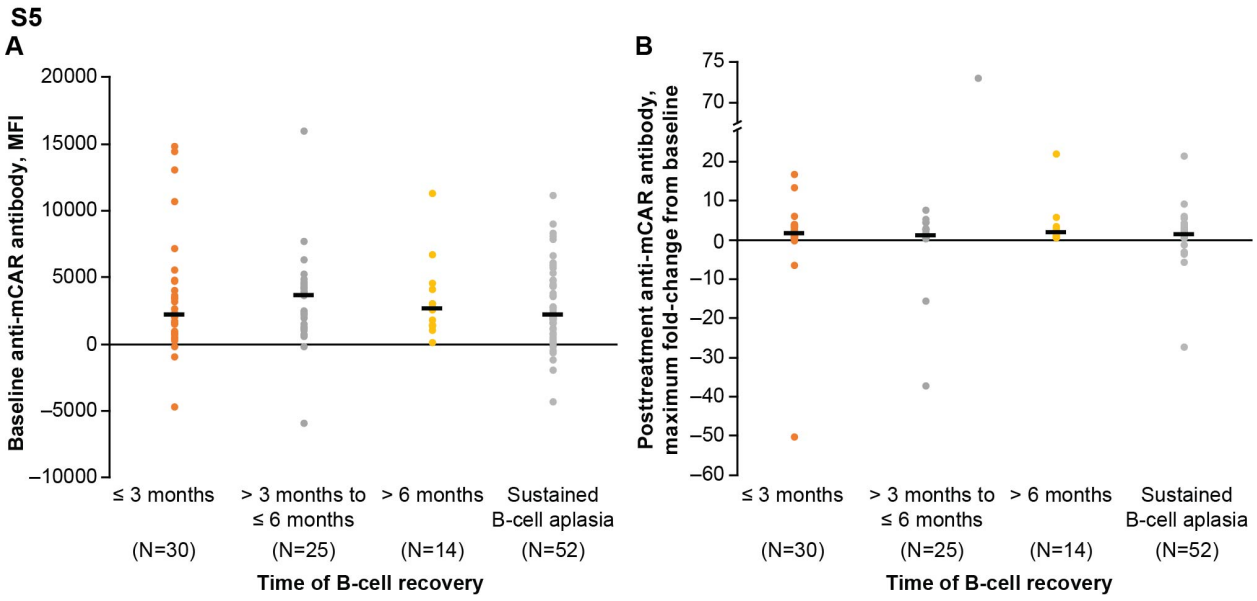


Supplementary Figure S3. Effects of posttreatment anti-mCAR19 antibodies on duration of response. Kaplan-Meier analysis of duration of response for patients (**A**) who were positive and negative for posttreatment anti-mCAR19 antibodies, and (**B**) patients in the 90th percentile for anti-mCAR19 antibody MFI levels post infusion. The red curve represents 10 patients (nonresponding patients were not included in the analysis). Among these 10 patients, 5 patients relapsed on days 107, 261, 140, 127, and 143. The other 5 patients were censored: 1 patient for adequate assessment no longer available on day 348, 2 patients for new therapy other than HSCT on days 337 and 347, and 2 patients for HSCT on days 146 and 316. Ab, antibody; CAR, chimeric antigen receptor; HSCT, hematopoietic stem cell transplant; mCAR19, murine CAR19; MFI, median fluorescence intensity.



Supplementary Figure S4. Effects of pretreatment and posttreatment anti-mCAR19 antibodies on CRS and neurological event grade in pediatric and young adult patients with r/r B-ALL and adult patients with r/r DLBCL. Horizontal bars represent medians. **B-ALL:** (**A**) Preexisting humoral immunogenicity and (**B**) maximum fold-change in posttreatment anti-mCAR19 antibodies versus CRS grade. (**C**) Preexisting humoral immunogenicity and (**D**) maximum fold-change in posttreatment anti-mCAR19 antibodies versus NE. **DLBCL:** (**E**) Preexisting humoral immunogenicity and (**F**) maximum fold-change in posttreatment anti-mCAR19 antibodies versus

CRS grade. (**G**) Preexisting humoral immunogenicity and (**H**) maximum fold-change in posttreatment anti-mCAR19 antibodies versus NE. B-ALL, B-cell acute lymphoblastic leukemia; CAR, chimeric antigen receptor; CRS, cytokine release syndrome; DLBCL, diffuse large B-cell lymphoma; mCAR19, murine CAR19; MFI, median fluorescence intensity; NE, neurological events; r/r, relapsed or refractory.



Supplementary Figure S5. Effects of pretreatment and posttreatment anti-mCAR19 antibodies on B-cell recovery in pediatric and young adult patients with r/r B-ALL. Horizontal bars represent medians. (**A**) Baseline and (**B**) maximum fold-change of anti-mCAR19 antibodies and B-cell recovery. B-ALL, B-cell acute lymphoblastic leukemia; CAR, chimeric antigen receptor; mCAR19, murine CAR19; MFI, median fluorescence intensity; r/r, relapsed or refractory.

Supplementary Appendix

Patient Case Summary

Patient 104 is a white 6-year-old male with B-cell acute lymphoblastic leukemia diagnosed in August 2010. The patient first relapsed in March 2012 after prior allogeneic stem cell transplant (cord blood, unrelated) without any residual donor engraftment and was refractory to subsequent salvage chemotherapy, with most recent relapse in September 2012. He was enrolled in the CHP959 study on December 18, 2012. The patient received lymphodepleting chemotherapy consisting of cytarabine and etoposide, and then was infused with a single dose consisting of 4.80×10^7 CTL019 transduced cells on January 8, 2013 (day 1). At infusion, there was no evidence of blasts in the bone marrow or minimal residual disease by flow cytometry.

On day 10 (January 17, 2013), 9 days after the CTL019 infusion, the CTL019 transgene showed rapid expansion in the peripheral blood by quantitative polymerase chain reaction, and had peak expansion by day 11 (53,026 copies/µg genomic DNA) with early rapid complete loss of chimeric antigen receptor (CAR) transgene by day 28 (**Supplementary Table S6**). At the same time, the patient was diagnosed with grade 2 cytokine release syndrome (CRS) on day 10 and was treated with supportive care; CRS resolved on day 12 (January 19, 2013). No concomitant infections were observed during this period; however, the patient experienced several episodes of pyrexia without concomitant neutropenia throughout the study follow-up period, was treated with broad-spectrum antibiotics, and on day 369 (January 11, 2014) the patient was diagnosed with grade 2 otitis media (verbatim term: infection with normal absolute neutrophil count otitis media) and treated with amoxicillin-clavulanic acid.

Patient was in complete remission on day 28 but also had a ~6-fold increase from baseline in anti-mCAR19 antibodies that were among the highest anti-mCAR19 antibodies detected in the study. On day 185 post infusion (July 11, 2013), the patient had an isolated skin relapse, received localized radiotherapy, and achieved remission of the skin lesion.

The patient developed a CD19-positive bone marrow relapse on day 374 and was discontinued from the study on day 386 (January 28, 2014). The patient then went on to receive reinduction chemotherapy followed by stem cell transplant, and ultimately died of disease progression 1152 days after the first infusion of CTL019 transduced cells.

Table S6. Patient case: Anti-mCAR19 antibody, CTL019 transgene levels, and response category over time

Time, days	Anti-mCAR19 antibody level, MFI	CTL019 level, copies/μg genomic DNA[a]			Response category
		Blood	CSF	Bone marrow	
-1	4323	0	—	0	—
1	—	27	—	—	—
4	—	34	—	—	—
8	—	110	—	—	—
10	—	5878	—	—	—
11	—	53,026	—	—	—
14	2896	—	—	—	—
15	—	1168	—	—	—
18	—	70	—	—	—
28	24,548	0	137	0	CR
87	—	0	—	0	CR
148	—	0	—	—	—
185	—	0	—	0	PD[b]
280	—	0	0	0	CR
374	—	0	—	0	PD[c]

Abbreviations: CAR, chimeric antigen receptor; CR, complete remission; CSF, cerebral spinal fluid; mCAR19, murine CAR19; MFI, median fluorescence intensity; PD, progressive disease.

[a]Lower limit of quantitation is 25 copies/μg genomic DNA.

[b]Isolated skin relapse.

[c]Bone marrow relapse.

Supplementary Methods

Floating immunogenicity cutpoint

The floating immunogenicity cutpoint (CP) was calculated for each analysis based on the assay signal of a negative control (immunoglobulin-depleted individual sera from 62 healthy volunteers) multiplied by a cutpoint factor (CPF) established during validation.[1] As previously described, the method to calculate the CPF as the 99.9% percentile from individual immunoglobulin-depleted healthy sera (0.1% false-positive rate [ie, less than the 1% currently required by health authorities]) differs from the evaluation of a CPF for a protein-based biotherapeutic.[1] For these, sera from the desired disease population are used and a 5% false-positive antidrug antibody rate is aimed for, in combination with a confirmatory assay with a 1% false-positive rate. The CPF gives a measure of inherent assay signal variability. Further, a patient-specific CP tier was applied to identify treatment-induced or -boosted responses. The patient-specific CP was calculated as the predose mCAR19-specific signal multiplied by the mCAR19 cell CPF as factor of the assay variability. Depending on the pre- and postdose sample result, immunogenicity (IG) observed at later timepoints was considered to be treatment induced (predose IG negative, postdose IG positive), treatment unaffected (pre- and postdose negative or pre- and postdose positive but below patient-specific CP), or treatment boosted (predose IG positive, postdose IG positive above patient-specific CP).

Humoral immunogenicity assay

Tisagenlecleucel is made up of autologous T cells transduced with mCAR19, and the humoral immunogenicity assay is specific for mCAR19 expressed on the cell surface. Antibodies binding to mCAR19 (for simplicity referred to here as anti-CTL019 antibodies) in human serum in the ELIANA (NCT02435849) and ENSIGN (NCT02228096) trials were measured using a flow cytometry method. Anti-CTL019 antibodies in human serum samples were captured by Jurkat

cells transfected to express mCAR19. Mock transfected cells were used as reference. The method measures bound immunoglobulin G/M on viable cells.

The humoral immunogenicity assay was developed and validated according to the US Food and Drug Administration 2016 guidance (https://www.fda.gov/media/77796/download), and validation included the determination of the screening assay CPF, sensitivity, assay precision/reproducibility, the establishment of a procedure to titrate immunogenicity-positive samples and assessment of potential hook effect. The validation experiments were conducted with a surrogate positive control (human anti-mCAR19 antibody), with the determined assay parameters being specific to this antibody. The method had a sensitivity of 100 ng/mL of positive control antibody in 100% human serum. Gating of signals was done to view live Jurkat cells. In addition, healthy volunteer samples were screened during assay development and found to be positive for anti-mCAR19 antibodies in approximately 90% of samples. Hence, this method was utilized for qualitative/semiquantitative determination of anti-mCAR19 antibodies in clinical studies. The method was found to be precise, accurate, and sensitive. No hook effect was observed with high levels of positive control antibody.

The development of this assay also included an assessment of the impact of the coadministration of intravenous immunoglobulin (IVIG), which is frequently administered for the treatment of hypogammaglobulinemia in patients receiving tisagenlecleucel. Due to the high prevalence of anti-mCAR19 antibodies in naive human serum as determined during assay development, it was assessed whether IVIG preparations can contain antibodies specifically binding to mCAR19. By testing IVIG preparation in the fluorescence-activated cell sorting-(FACS)–based CTL019 humoral immunogenicity assay, anti-mCAR19 antibodies were detected.

To conclude whether a humoral immune response against mCAR19 was induced or boosted after a patient received the autologous CTL019 cells, a patient-specific CP was additionally calculated using the following formula: 2.1578 × anti-mCAR19 antibody median

fluorescence intensity at preinfusion. The 2.1578 corresponds to the CP factor on CTL019 Jurkat cells.

Cellular immunogenicity assay

The presence of mCAR19-specific CD4+ and CD8+ T cells has been assessed to investigate cellular immunogenicity against tisagenlecleucel. The response against infused CAR-positive cells was assessed by flow cytometric detection of activated CD4 and CD8 T cells after ex vivo exposure of peripheral blood mononuclear cells (PBMCs) to peptides (pool 1 and 2) derived from the amino acid sequence of mCAR19. Induction of intracellular interferon gamma (IFN-γ) expression was used as a marker of T-cell activation. Intracellular staining was used to identify IFN-γ–positive T cells in samples obtained from patients at different timepoints. Peripheral blood mononuclear cells from each collection were distributed to separate wells and mixed at the desired concentration with SEB (staphylococcal enterotoxin B) strong-positive control, CEF peptides (cytomegalovirus, Epstein-Barr virus, influenza virus peptides; a group of 27 peptides) weak-positive control, or pools of 15-mer overlapping peptides obtained from mCAR19 (pool 1 and 2). Incubation of PBMCs with dimethyl sulfoxide (DMSO) alone (negative control) was used to estimate the nonspecific activation. After incubation of samples, cells were fixed, permeabilized, and stained with fluorescence-labeled antibodies against CD3, CD4, CD8, CD16, and CD56 markers to identify CD4+ and CD8+ T cells and antibodies against intracellular IFN-γ. The stained cells were analyzed by flow cytometry, and the activated T cells were identified and quantitated using FlowJo software. Fluorescence minus one control (the sample stained with all antibodies except anti–IFN-γ) was used to set the background fluorescence level caused by autofluorescence of cells in a channel used for INF-γ detection.

Interpretation of cellular immunogenicity results

The method uses several controls that serve different functions. The SEB superantigen-strong positive control is a universal positive control to confirm the ability of T cells to be activated.[2] It serves as a general control for the INF-γ staining. It has been established during the assay validation that CD4+ and CD8+ T-cell responses can be universally detected following SEB stimulation. Therefore, if all stimulation results from a sample, including the SEB result, were less than the lower limit of quantitation, then the sample was repeated. From the literature, the expected level of activation by SEB should be in the range between 1% and 15% of CD4+ or CD8+ T cells, as was observed during this study. A weak positive response to peptides (0.5% to 5% activated T cells in peripheral blood mononuclear cell samples) was expected to be lower than the strong positive response elicited by the positive control (staphylococcal enterotoxin B; up to 40% activated T cells). To date, no mCAR19 peptides have been identified that activate T cells; therefore, no mCAR19 peptides could be used as positive control for T-cell activation. Net responses were calculated from exposure to 2 nonidentical peptide pools, each composed of ~60 overlapping 15-mer peptides derived from the full-length amino acid sequence of the tisagenlecleucel mCAR19 protein (analytically validated method, unpublished). Net responses (percentage of T cells expressing intracellular IFN-γ) were calculated as the difference between responses to mCAR19 peptide pools and responses to a negative control (dimethyl sulfoxide alone).[2] Positivity was based on percentage of T cells with positive cytokine staining compared with the negative control well (0.1%-1.0% IFN-γ–expressing T cells). A sample was positive if >1.0% of IFN-γ–expressing T cells were observed after mCAR19 peptide stimulation, and the frequency of IFN-γ–expressing T cells after stimulation was at least 2× higher than the negative control for the same sample. Postinfusion IFN-γ levels (maximum net change) were determined by comparing percentage of IFN-γ–positive levels relative to baseline levels.[2]

The CEF positive control was performed to prove the ability of T cells to be activated with peptides. The expected level of activation should vary between 1% and 3%, although some

individuals demonstrated poor response, as expected due to HLA diversity within the tested populations. In most cases, the percentage of CEF-activated cells will reflect the CD8 memory response to CEF.

The DMSO negative control shows the level of nonspecific activation (not specific to the stimulators tested or pre-activation) of T cells. The level observed can vary between timepoints and individuals due to a number of factors, including ongoing infection, high cytokine levels, or disease-related factors. DMSO values were subtracted from the results obtained with stimulators for the calculation of the specific T-cell activation. The fluorescence minus one identifies a level of spread of the fluorochromes into the channel of interest.

Based on SEB and CEF controls, the level of a significant cellular (T cell) immune response against the drug would be expected to be in the range of 1% or more percent activated CD4+ or CD8+ T cells. Our experimental observations led us to investigate potential positive signals above 1% and closer to the CEF control results rather than the high SEB results. Using a threshold of signals equivalent to SEB results would have been unrealistic; therefore, we chose the observed CEF signals as a possible positive result.

References

1. Potthoff B, McBlane F, Spindeldreher S, Sickert D. A cell-based immunogenicity assay to detect antibodies against chimeric antigen receptor expressed by tisagenlecleucel. *J Immunol Methods*. 2020;476:112692.
2. Horton H, Thomas EP, Stucky JA, et al. Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. *J Immunol Methods*. 2007;323(1):39-54.