

Comparison of single copy gene-based duplex quantitative PCR and digital droplet PCR for monitoring of expansion of CD19-directed CAR T cells in treated patients

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Abstract. Chimeric antigen receptor (CAR) T cell therapy with axicabtagene ciloleucel, tisagenlecleucel and brexucabtagene ciloleucel has been adopted as the standard of care for patients with refractory and/or relapsed CD19-positive lymphoid

malignancies. Monitoring of kinetics of CAR T cells after administration is crucial for patient follow-up and important to guide clinical decisions for patients subjected to CAR T cell therapy. Information of transgene copies within a CAR T cell product prior to administration, i.e. vector copy numbers, is of high importance to warrant patient safety. However, experimental assays for quantitative CAR T cell monitoring in the open domain are currently lacking. Several institutions have established in-house assays to monitor CAR T cell frequencies. In the present study, the quantitative (q)PCR assay established at the Heidelberg University Hospital (Heidelberg, Germany), i.e. single copy gene-based duplex qPCR, was compared with the digital droplet PCR assay established at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). Both methods that were independently developed enable accurate and comparable CAR T cell frequency assessment and are useful in the clinical setting.

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Abbreviations: ALL, acute lymphoblastic leukemia; axi-cel, axicabtagene ciloleucel; brexu-cel, brexucabtagene ciloleucel; CAR, chimeric antigen receptor; CR, complete remission; CRS, cytokine release syndrome; DLBCL, diffuse large B cell lymphoma; dPCR, digital droplet PCR; EMA, European Medicines Agency; FC, flow cytometry; gDNA, genomic DNA; GTMP, gene therapy medicinal products; ICANS, immune effector cell-associated neurotoxicity syndrome; PBMC, peripheral blood mononuclear cell; PD, progressive disease; PR, partial remission; qPCR, quantitative PCR; scFv, single chain variable fragment; SD, stable disease; t/r, refractory and/or relapsed; RPPH1, ribonuclease P RNA component H1; RT, room temperature; SCG-DP-PCR, single copy gene-based duplex qPCR; tisa-cel, tisagenlecleucel; TRM, treatment-related mortality; UKE, University Medical Center Hamburg-Eppendorf; UKHD, Heidelberg University Hospital

Key words: chimeric antigen receptor, CAR T cell monitoring, real-time quantitative PCR, digital droplet PCR, copy number assessment, axicabtagene ciloleucel, brexucabtagene ciloleucel, tisagenlecleucel

Introduction

Cellular immunotherapy with CD19-directed chimeric antigen receptor (CAR) T cells is altering the treatment landscape of lymphoid malignancies. CARs are composed of an extracellular antigen-specific domain derived from an antibody's single chain variable fragment (scFv), a hinge and transmembrane segment and an intracellular domain to activate and co-stimulate the T cell expressing the CAR. Hence, CAR T cells combine the antigen-specific properties of antibodies with the effector functions of T cells. In contrast to physiologic T cell receptors (TCRs), CARs are able to recognize unprocessed extracellular antigens and may therefore act in a human leukocyte antigen-independent manner. The intracellular CAR domain defines the different CAR generations: First-generation CARs contain only the tyrosine-based ζ-signal-transducing subunit from the TCR/CD3 receptor complex (1). Second-generation CARs carry costimulatory domains, e.g. CD28 or 4-1BB (CD137), adjacent to the

TCR/CD3 ζ -domain. Costimulation has been indicated to enhance CAR T cell activity and has resulted in improved clinical efficacy compared to first-generation CAR T cells (2).

In 2018, two second-generation CAR T cell products, i.e. axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel) were approved by the European Medicines Agency (EMA) for the treatment of patients with relapsed and/or refractory (r/r) B-cell lymphoid malignancies (3-6). While axi-cel, carrying CD28 as a costimulatory domain, has been approved for the treatment of patients with r/r diffuse large B cell lymphoma (DLBCL) and primary mediastinal B cell lymphoma (7), tisa-cel, carrying 4-1BB as a costimulatory domain, has been approved for the treatment of patients with r/r DLBCL and patients with r/r acute lymphoblastic leukemia (ALL) below 26 years of age (8). In 2020, the EMA approved the third CD19-directed CAR T cell product in Europe, i.e. brexucabtagene autoleucel (brexu-cel), for the treatment of patients with r/r mantle cell lymphoma (9). The CAR construct of brexu-cel is identical to axi-cel, but the two products differ in their manufacturing process, as brexu-cel includes the selection of T cells to exclude circulating malignant B-cells prior to T cell transduction. All three CAR T cell products are generated from autologous cells and are indicated for the respective underlying disease after two or more lines of therapy.

Quantification of CAR T cells and monitoring of CAR T cell kinetics are crucial diagnostic variables in patients treated with CAR T cells, as clinical response and toxicity have been indicated to depend on CAR T cell engraftment, expansion and persistence (10-16). Hence, the assessment of CAR T cell frequencies by widely available and easily applicable CAR T cell monitoring assays is important.

However, to date, precise and fast assays that enable the quantification of commercially available CAR T cells have been largely unavailable. Recently, our groups developed PCR-based in-house assays to detect and quantify CD19-directed CAR T cells (17-20). However, systematic comparison of different approaches for CAR T cell quantification has remained an open task, and it requires to be elucidated whether different PCR approaches with diverse technical elements and methodical parameters, i.e. quantitative PCR (qPCR) (19,20) and digital PCR (17,18), are able to provide comparable data when assessing CAR T cell frequencies. To address this, the qPCR assay established at the Heidelberg University Hospital (UKHD), i.e. single copy gene-based duplex qPCR (SCG-DP-PCR) (20), from here onwards referred to as qPCR, was compared to the digital droplet PCR (dPCR) assays established at the University Medical Center Hamburg-Eppendorf (UKE) (17,18). Both methods target the FMC63-based scFv that is incorporated within the CD19-directed CAR T cells commercially available in Europe, i.e. axi-cel, tisa-cel and brexu-cel.

Materials and methods

Patient samples. Samples for CAR T cell quantification were obtained from a total of 20 patients, i.e. 10 patients with axi-cel and tisa-cel, respectively, at different time-points until up to six months after CAR T cell treatment. Each 10 patients had been treated at the UKHD or the UKE in accordance with the institutional guidelines (see Table I). Informed consent had

been obtained from all patients prior to CAR T cell treatment and the study was approved by the local ethics committees at the UKHD (Heidelberg, Germany; no. S-254/2016) and UKE (Hamburg, Germany; no. PV7091).

Response to treatment was assessed according to revised response criteria for malignant lymphoma (21) or according to standard criteria for ALL (22). Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were graded according to the consensus guidelines defined by the American Society for Transplantation and Cellular Therapy (23).

Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells (PBMCs) as described in the following and samples of gDNA were exchanged between the two institutions in a blinded manner. Quantification was performed using qPCR (20) at the UKHD and dPCR (17,18) at the UKE. Overall, 113 genomic DNA samples, 56 from patients treated with axi-cel and 57 from patients treated with tisa-cel, were analyzed.

qPCR at the UKHD. SCG-DP-PCR relies on the simultaneous amplification of the FMC63 sequence of the CAR (24) and the human SCG ribonuclease (RNase) P RNA component H1 (RPPH1; in the following referred to as RNaseP) as the internal standard. The following primer and probe sets were used: i) Primer/probe reaction mix (cat. no. 4331348; Applied Biosystems; Thermo Fisher Scientific, Inc.) targeting the FMC63 sequence of the CAR (24): Forward primer, TGAAACTGCAGGAGTCAGGA; reverse primer, CTGAGACAGTGCATGTGACG; probe, FAM-CTGGCCTGGTGGCGCCCTCA-minor groove binder/non-fluorescent quencher; ii) RPPH1 (RNaseP) primer/probe reaction mix (cat. no. 4403326; TaqMan; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used as described previously (19,20).

SCG-DP-PCR was performed on gDNA isolated from PBMCs. PBMC isolation was performed by Ficoll density gradient (Linaris GmbH) following the manufacturer's protocol, washed and suspended in PBS.

gDNA was extracted using a commercially available DNA extraction kit and following the manufacturer's instructions (cat. no. 51104; QIAamp DNA Blood Mini; Qiagen GmbH). The concentration of extracted gDNA was measured using UV spectroscopy (NanoDrop OneC; Applied Biosystems; Thermo Fisher Scientific, Inc.), and samples were diluted to a final concentration of 20 ng/ μ l gDNA in nuclease-free H₂O. The PCR mixture had a final volume of 25 μ l and contained 1.25 μ l mix containing primers (900 nM) and probes (250 nM) targeting the CAR-transgene and RNaseP, 12.5 μ l of PCR master mix (cat. no. 4304437; Applied Biosystems; Thermo Fisher Scientific, Inc.), 5 μ l of gDNA sample and 5 μ l of nuclease-free H₂O. Non-template control and a biological negative control (non-transduced cells) were included within all experiments. All reactions were performed in triplicates.

The following amplification conditions were used (19,20): 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Thermal cycling was performed using a StepOnePlus real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Table I. Characteristics of patients treated with CAR T cells.

UPN #	Age, years/sex	Institution administering CAR Ts	CAR T product/entity	Prior therapy lines	Bridging therapy	Status prior to CAR T therapy	CRS grade	ICANS grade	Best response
001	41/M	UKHD	Axi-cel/DLBCL	3	Yes	PD	I	III	PR
002	56/M	UKHD	Axi-cel/DLBCL	3	Yes	PD	I	-	PR
003	40/F	UKHD	Axi-cel/DLBCL	4	Yes	PD	I	III	CR
004	58/M	UKHD	Axi-cel/DLBCL	3	Yes	SD	-	-	SD
005	55/M	UKHD	Axi-cel/DLBCL	3	Yes	SD	I	-	PR
006	53/M	UKE	Axi-cel/DLBCL	4	Yes	PD	I	I	CR
007	44/M	UKE	Axi-cel/DLBCL	4	Yes	PD	III	II	CR
008	58/M	UKE	Axi-cel/DLBCL	8	Yes	SD	-	-	SD
009	52/F	UKE	Axi-cel/DLBCL	3	No	PD	II	I	CR
010	69/M	UKE	Axi-cel/DLBCL	4	Yes	CR	III	-	CR
011	66/M	UKHD	Tisa-cel/DLBCL	5	Yes	SD	I	-	SD
012	49/M	UKHD	Tisa-cel/DLBCL	3	No	PR	-	-	SD
013	71/M	UKHD	Tisa-cel/DLBCL	4	Yes	PD	II	-	PR
014	57/M	UKHD	Tisa-cel/DLBCL	4	Yes	PD	I	II	PR
015	67/M	UKHD	Tisa-cel/DLBCL	4	Yes	CR	I	-	CR
016	10/M	UKE	Tisa-cel/ALL	3	No	PD	I	-	CR
017	59/F	UKE	Tisa-cel/DLBCL	4	Yes	PD	IV	-	n.e./TRM
018	51/M	UKE	Tisa-cel/DLBCL	3	Yes	SD	-	-	PR
019	67/F	UKE	Tisa-cel/DLBCL	7	Yes	PR	I	-	SD
020	59/M	UKE	Tisa-cel/DLBCL	2	Yes	PR	II	-	CR

ALL, acute lymphoblastic leukemia; axi-cel, axicabtagene ciloleucel; CAR T, chimeric antigen receptor T cells; CR, complete remission; CRS, cytokine release syndrome; DLBCL, diffuse large B cell lymphoma; F, female; M, male; ICANS, immune effector cell-associated neurotoxicity syndrome; n.e., not evaluable; PR, partial remission; PD, progressive disease; SD, stable disease; tisa-cel, tisagenlecleucel; TRM, treatment-related mortality; UKE, University Medical Center Hamburg-Eppendorf; UKHD, Heidelberg University Hospital; UPN, unique patient number.

The calculation of copy numbers via qPCR was performed applying a $2^{-\Delta Ct}$ calculation method as previously described using the following formula (19,20): Copy number/ μ m PBMC DNA = $2^{-\Delta Ct} \text{FMC63-Ct RNaseP}$ x 2x140,370.

dPCR at the UKE. gDNA from patient samples was prepared as previously described (17,18). In brief, PBMCs were isolated by Ficoll gradient centrifugation using SepMate (Stemcell Technologies, Inc.) following the manufacturer's protocol, washed and suspended in PBS. If available, $\sim 1 \times 10^6$ PBMCs (in 200 μ l) were loaded on columns of the QIA-Amp Blood Kit (Qiagen GmbH) and genomic DNA was isolated following the manufacturer's protocol. DNA was eluted in a final volume of 200 μ l. If $< 1 \times 10^6$ mononuclear cells were available, all cells were used for DNA preparation and the elution volume was adjusted accordingly.

Typically, 100 ng gDNA corresponding to $\sim 15,000$ diploid genomes (cells) were subjected to dPCR analysis. For axi-cel samples, dPCR was performed as previously described (17), and for tisa-cel samples, the approach outlined in (18) was used. Primers and probes for both dPCRs are available as Expert Design assays from Bio-Rad Laboratories, Inc. (axi-cel: Cat. no. dEXD45718942; tisa-cel/universal: Cat. no. dEXD88164642). All dPCRs were performed in

duplex reactions using the diploid hematopoietic cell kinase gene as a reference gene (17,18). Final concentrations of primers (900 nM) and probes (250 nM) were according to standards suggested for dPCR by Bio-Rad Laboratories, Inc. To reduce sample viscosity and improve target accessibility, 5 units *EcoRI* (Thermo Fischer Scientific, Inc.) were added to the reaction mix, which was incubated at RT for 5 min prior to starting the PCR. Droplets were analyzed with the QX100 droplet reader (Bio-Rad Laboratories, Inc.) and data were processed with QuantaSoft_v1.7 software (Bio-Rad Laboratories, Inc.) that included automatic Poisson correction (17,18).

Flow cytometric assessment of CAR expression. Frozen PBMCs of selected patients were thawed and flow cytometry (FC) was performed as recently described (17). In brief, CD19 CAR-expressing T cells were determined using the CD19 CAR detection reagent Biotin (cat. no. 130115965; Miltenyi Biotec GmbH) following the manufacturer's protocol. In brief, PBMCs were washed with FC buffer [PBS (Gibco, Thermo Fisher Scientific) containing 2% fetal bovine serum (MilliporeSigma) and resuspended in 100 μ l FC buffer. Cells were stained with CD19 CAR Detection Reagent Biotin for 10-15 min at RT, washed twice with FC buffer and stained with anti-CD45 Vioblue, anti-CD3 FITC and anti-biotin

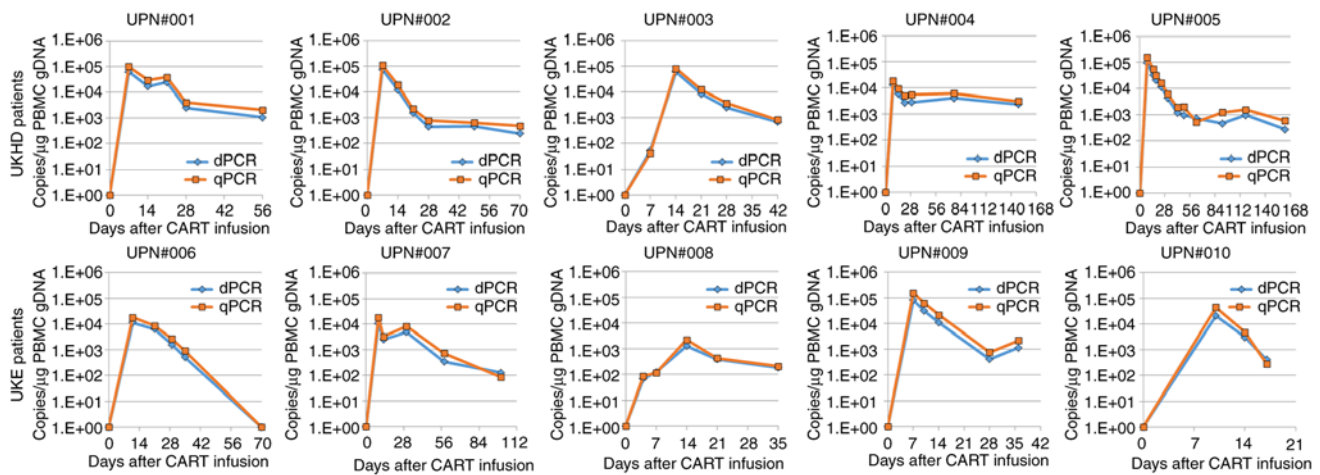


Figure 1. Expansion curves of patients after axi-cel administration determined using qPCR and dPCR. Assessment and monitoring of the CAR T cell frequencies in patients treated with axi-cel ($n=56$). At each of the two institutions, five patients were treated and samples were interchanged between the institutions in a blinded manner. CAR T cell frequencies were measured using qPCR at the UKHD and dPCR at the UKE. Semi-logarithmic representation of CAR transgene copies/ μg PBMC gDNA over time provided almost overlapping curves. Each curve displays the result for a single patient. UPN, unique patient number; axi-cel, axicabtagene ciloleucel; CAR, chimeric antigen receptor; qPCR, quantitative PCR; dPCR, digital droplet PCR; PBMC, peripheral blood mononuclear cell; UKE, University Medical Center Hamburg-Eppendorf; UKHD, Heidelberg University Hospital.

phycoerythrin (cat. no. 130110951; Miltenyi Biotec GmbH) for 10 min at RT. After washing with FC buffer, cells were resuspended in 500 μl FC buffer supplemented with Cytotfix reagent (BD Biosciences) and subsequently analyzed on a FACS Canto Analyzer (BD Biosciences). At least 125,000 cells were analyzed in the lymphocyte gate to ensure high accuracy. Dead cells were excluded using a fixable viability dye (eFluor 506; Ebioscience; Thermo Fischer Scientific, Inc.). Results were analyzed using FlowJo software, version 10.6.2 (BD Biosciences).

Statistical analysis. Copy numbers of individual samples measured using qPCR and dPCR were compared: qPCR results were set as 100% and the relative difference of the corresponding dPCR result to qPCR was calculated. Two-tailed Pearson statistics with a confidence interval of 95% were applied to determine correlation coefficients (R^2) between data-points obtained with qPCR and dPCR. A correlation was considered statistically significant if $P < 0.05$. For statistical analyses, GraphPad Prism, version 8.4.3 (GraphPad Software, Inc.) was used.

Results

Response and toxicity in patients treated with CAR T cells. The copy numbers in 113 gDNA samples of 20 patients treated with axi-cel [$n=10$, five patients per institution (Fig. 1)] and tisa-cel [$n=10$, five patients per institution (Fig. 2)] were assessed by qPCR and dPCR. The patient data are summarized in Table I. Of the patients treated with tisa-cel, nine patients were treated for DLBCL and one for ALL [unique patient no. (UPN) #016]. Most patients ($n=16$) were male, the median age of the treated patients was 56.5 years (range, 10–71 years), and patients had received 2–7 prior treatment lines. The majority of patients ($n=17$) received bridging therapy between lymphodepletion and CAR T cell administration due to a high burden of the hematologic disease or progressive disease (PD). Of those

patients, four patients achieved complete remission (CR, $n=2$) or partial remission (PR, $n=2$), five patients displayed stable disease (SD) and eight patients had PD despite treatment. Of the three patients that did not receive any bridging therapy, one patient had PR and two patients PD prior CAR T cell treatment. Following CAR T cell administration, 16 patients developed CRS with 3 cases of high-grade CRS ($>$ grade III CRS). ICANS was observed in 6 patients, with high-grade ICANS ($>$ grade III ICANS) evident in 2 patients. Peak levels of CAR T cell copies ranged between 43 and 159,304 copies/ μg PBMC DNA. High-grade ICANS was observed in patients with high peak CAR T cell expansion (UPN#001 and #003).

One patient (UPN#017) died within 1 week after CAR T cell treatment due to hemophagocytic lymphohistiocytosis/macrophage activation syndrome. The remaining 19 patients were evaluable for assessment of clinical response: 14 patients (74%) responded to treatment, with 8 patients (42%) achieving CR and 6 patients PR (32%) as their best response. SD was observed in 5 patients (26%). Those patients with the lowest CAR T cell expansion [UPN#008 (axi-cel) and UPN#012 (tisa-cel)] did not respond to treatment.

Comparison of qPCR and dPCR for CAR T cell quantification.

For all analyzed patient samples, qPCR and dPCR provided highly similar, overlapping logarithmic curves of CAR vector copies/ μg PBMC gDNA over time (Figs. 1 and 2). Data sets obtained for each patient with qPCR and dPCR displayed a high degree of correlation with statistical significance for all measurements (Table II). For patient samples with low CAR T cell expansion levels (i.e. UPN #008, #012 and #018; maximum CAR T cell levels $< 5,000$ copies/ μg PBMC gDNA), a statistically significant correlation persisted ($R^2 > 0.78$; $P < 0.05$), reaffirming the comparability of qPCR and dPCR even at low CAR T cell levels. When copy numbers of individual samples were compared by relating dPCR to qPCR results (qPCR set as 100%), the mean quantification results of dPCR were $70 \pm 34\%$, i.e. a mean relative difference of -30% from

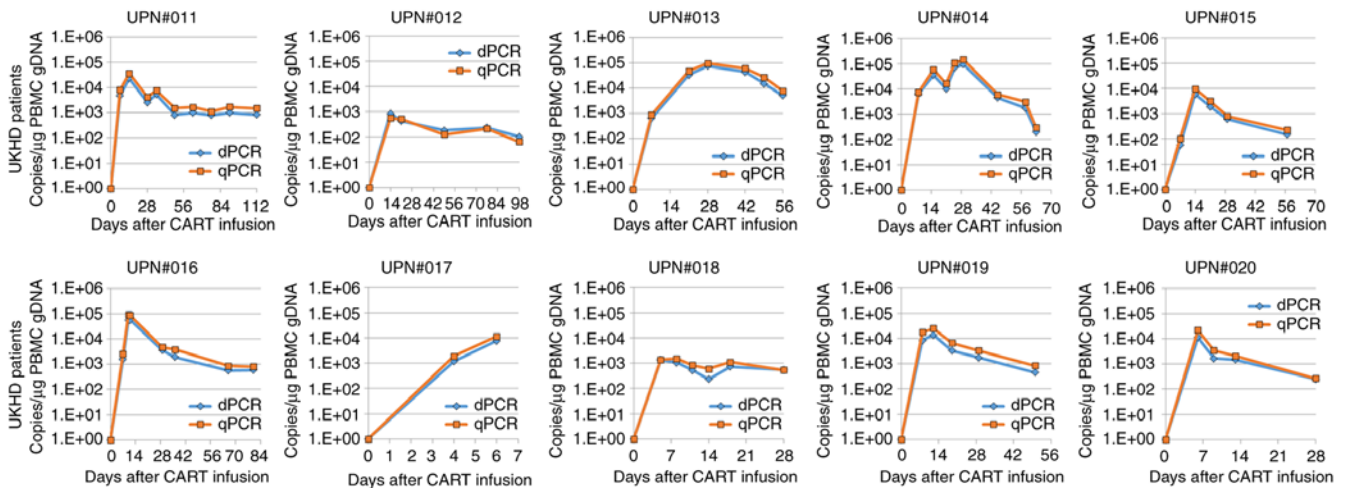


Figure 2. Expansion curves of patients after tisa-cel administration determined using qPCR and dPCR. Assessment and monitoring of the CAR T cell frequencies in patients treated with tisa-cel (n=57). At each of the two institutions, five patients were treated and samples were interchanged between the institutions in a blinded manner. CAR T cell frequencies were measured using qPCR at the UKHD and dPCR at the UKE. Semi-logarithmic representation of CAR transgene copies/ μ g PBMC gDNA over time provided almost overlapping curves. Each curve displays the result of a single patient. UPN, unique patient number; tisa-cel, tisagenlecleucel; CAR, chimeric antigen receptor; qPCR, quantitative PCR; dPCR, digital droplet PCR; PBMC, peripheral blood mononuclear cell; UKE, University Medical Center Hamburg-Eppendorf; UKHD, Heidelberg University Hospital.

qPCR was observed for dPCR (Fig. 3). Indeed, copy numbers determined using dPCR were lower for almost all measured samples (Figs. 1 and 2). This observation was independent of the dPCR (axi-cel or tisa-cel) assay used. Finally, the numbers of CAR-expressing T cells were assessed by FC for patients treated with axi-cel and tisa-cel. FC was retrospectively performed for UPN#009 (axi-cel) and UPN#020 (tisa-cel) on PBMCs frozen at 5 different time-points after CAR T cell administration (Figs. 4 and S1). CAR T cell numbers were determined per μ l blood and set in relation to the data obtained by digital PCR for the same patients. As evident from Fig. 4, a high convergence of CAR T cell numbers determined with either method was observed. Of note, a resurgence of CAR T cell numbers as seen in UPN#009 at day 35 was detected by all three methods-FC, dPCR and qPCR (Figs. 1 and 4). These data are in line with previous observations by our group on the high concordance of PCR- and FC-based quantification (18).

Discussion

CAR T cells as cellular products display variable pharmacokinetic and pharmacodynamic profiles that depend not only on patient-specific characteristics but also on the administered CAR T cell dose, lymphodepletion therapy and targeted disease (25). Engraftment, expansion and persistence of CAR T cells have important clinical and therapeutic implications (10,11,14,26-28). Hence, assessing CAR T cell kinetics after CAR T cell treatment is of crucial importance for patient follow-up. Also, given that CAR genes are stably integrated into the T cell genome via viral vectors, CAR T cells are classified as gene therapy medicinal products (GTMPs). Hence, precise tools to assess vector copy numbers in CAR T cell products are important to ensure GTMP product quality and patient safety.

The present study compared qPCR (19,20) and dPCR (17,18), two broadly applicable quantification assays

for monitoring CD19-targeting CAR T cells established and validated at independent laboratories and institutions.

Both methods target the FMC63-based scFv incorporated within CD19-directed CAR T cells that are commercially available in Europe, i.e. axi-cel, tisa-cel and brexu-cel. Although the present study did not formally include patients treated with brexu-cel, in our experience, quantification by qPCR and dPCR is also suitable for brexu-cel monitoring (data not shown), given that both products are composed of the same CAR construct and differ only with regards to manufacturing.

When compared to traditional qPCR approaches such as the absolute copy number method, qPCR, i.e. SCG-DP-PCR and dPCR, offer methodological advantages and simplify CAR T cell quantification, e.g. by operating independently from calibrator samples or standards (17-20). While SCG-DP-PCR was specifically developed to exclude the requirement for calibrator and standard samples, dPCR intrinsically does not rely on calibrators or standards. Independence from these samples economizes material and time resources, minimizes procedure parameters and simplifies mathematical analysis. Consequently, technical complexity of CAR T cell quantification is reduced. qPCR and dPCR independently fulfill the requirements for Good Clinical Laboratory Practice for CAR quantification (29) and are highly suitable for being established in other clinical and diagnostic laboratories due to easy technical transfer and implementation. Transfer of qPCR and dPCR to other laboratories is supported by a transparent procedural description: For SCG-DP-PCR, all PCR oligo sequences are published [(19,20), see also materials and methods section], and for dPCR, all required supplements are available as a commercialized, ready-to-use assay kit [(17,18), see also materials and methods section].

qPCR and dPCR provided very similar quantification results when measuring axi-cel and tisa-cel levels; the resulting high levels of concordance of CAR T cell kinetics assessed with both methods underlines their equal precision.

Table II. Correlation and statistical significance of data points obtained with qPCR and dPCR.

UPN #	Time-points, n	R ²	P-value
001	5	0.999	<0.0001
002	6	1	<0.0001
003	5	1	<0.0001
004	6	0.985	<0.0001
005	11	0.999	<0.0001
006	5	0.993	0.0003
007	5	0.996	<0.0001
008	5	0.990	0.0004
009	5	1	<0.0001
010	3	0.999	0.0162
011	9	0.999	<0.0001
012	5	0.802	0.0398
013	6	0.994	<0.0001
014	8	0.999	<0.0001
015	5	1	<0.0001
016	7	0.998	<0.0001
017	2	n. a.	n. a.
018	6	0.779	0.0199
019	5	0.994	0.0002
020	4	0.998	0.0008

Chimeric antigen receptor T cell frequencies at distinct time-points obtained with qPCR and dPCR (axicabtagene ciloleucel, n=56; tisagenlecleucel, n=57) correlated significantly ($R^2=0.9846$). All correlations were statistically significant. In UPN#017, correlation analysis was not performed as only 2 time-points were measured. $P<0.05$ was considered to indicate a statistically significant correlation. UPN, unique patient number; R^2 , correlation coefficient; n.a., not available; qPCR, quantitative PCR; dPCR, digital droplet PCR.

This is to be expected, since qPCR and dPCR are based on the same amplification and signal-generation principle, i.e. the hydrolysis probe technique. However, lower dPCR values when compared to qPCR were obtained for almost all tested samples, by a mean magnitude of 30%. Compared to the qPCR approach that applies real-time measurement of the signal generated from PCR products in a reaction cycle when a detection threshold is reached, dPCR fractionates samples in smallest portions with an endpoint detection of every sample fraction. This diverse detection principle, as well as differing assay preparation steps, primer/probe sets and analytical procedures, may have resulted in the observed variations. However, patterns of CAR T cell *in vivo* kinetics assessed by the two methods were identical, making the differences clinically irrelevant.

PCR-based approaches for quantification amplify small vector fragments integrated within genomic DNA and do not necessarily provide information on the functional expression of the detected CAR. CAR T cell numbers obtained by dPCR and FC were compared for two representative patients, one treated with axi-cel and one with tisa-cel, at five different time-points post-infusion. Convergence of the data obtained

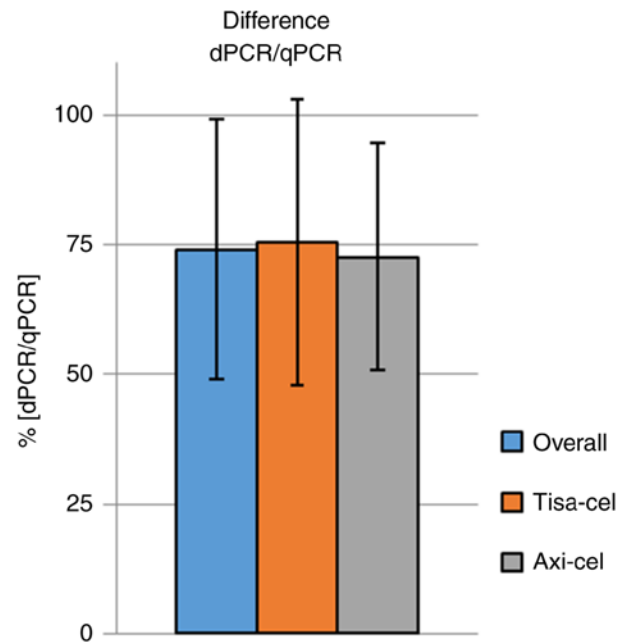


Figure 3. Relative difference of CAR T cell frequency results of dPCR compared to qPCR. Results of CAR transgene numbers of individual samples obtained with both quantification methods were compared relating the dPCR to the qPCR results. The qPCR results were set as 100% and the relative difference from the corresponding dPCR result was calculated. The mean quantification result of dPCR was $70\pm34\%$, i.e. a mean relative difference of -30% of dPCR to qPCR was observed. Data-points: axi-cel, n=56; tisa-cel, n=57; total, n=113. CAR, chimeric antigen receptor; qPCR, quantitative PCR; dPCR, digital droplet PCR; tisa-cel, tisagenlecleucel; axi-cel, axicabtagene ciloleucel.

with the different detection methods was observed. (Minor differences were to be expected, since the FC analyses were performed on previously frozen samples. It has been established that cryopreservation may not only lead to reduced viability, but also has an impact on the expression of different proteins/markers in T cells (30). PCR-based approaches, in turn, are less prone to variations in sample quality, i.e. fresh and intact cells, when compared to FC. In addition, FC-based approaches depend on the target population size, as well as total event counts, and are considered to be less sensitive when compared to PCR-based methods (31). In any case, CAR T cell expansion and persistence strongly depend on the growth signal provided by the CAR. Therefore, the survival of transgenic cells not expressing the CAR is unlikely. Accordingly, high correlation levels of CAR quantification data obtained by PCR-based approaches determining the transgene at the genomic level and FC detecting the CAR protein on the CAR T cell surface have been reported by our group (18,32) and others (10).

In the clinical setting, both methods are highly useful: Response to treatment was not observed in patients displaying the lowest CAR T cell expansion. This confirms previous findings by our group that low CAR T cell expansion is associated with limited clinical efficacy (16,17). With regard to toxicity, the present study observed that high-grade ICANS developed more frequently in patients with high CAR T cell expansion, again in line with a previous study by our group (20). However, and as previously reported (11), no association of CRS with CAR T cell expansion was determined.

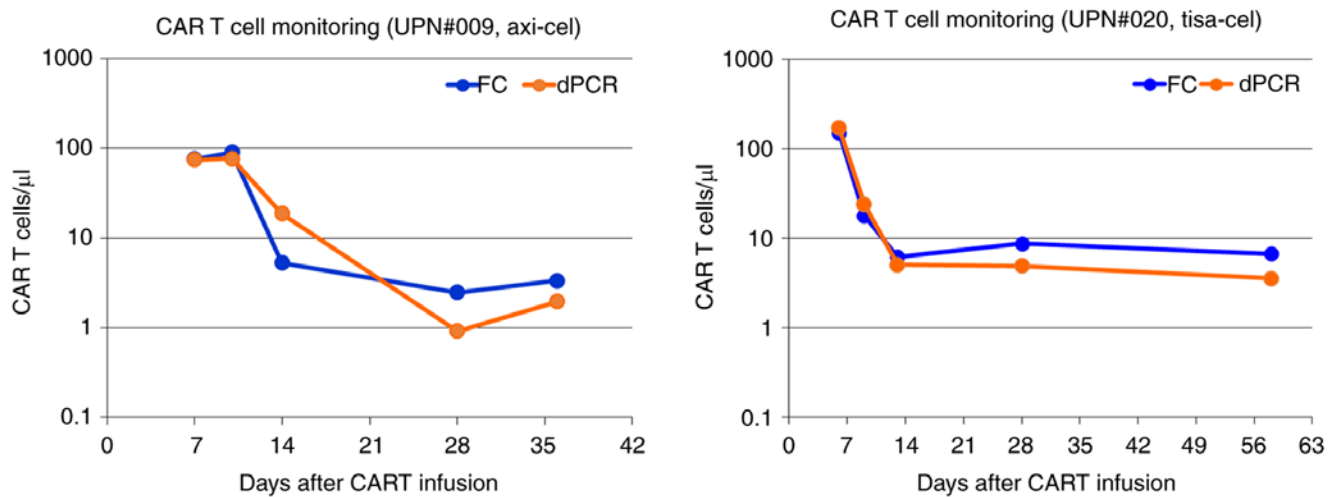


Figure 4. Comparison of CAR T cell numbers assessed by dPCR and FC. CAR T cell numbers of one patient treated with axi-cel (left) and one treated with tisa-cel (right), assessed by dPCR (orange graph) were compared to results obtained using FC (blue graph). FC was performed on frozen/thawed peripheral blood mononuclear cell samples at different time-points after CAR T cell administration. UPN, unique patient number; CAR, chimeric antigen receptor; dPCR, digital droplet PCR; tisa-cel, tisagenlecleucel; axi-cel, axicabtagene ciloleucel; FC, flow cytometry.

Treatment with axi-cel carrying CD28 as a costimulatory domain within the CAR construct resulted in higher peak expansion of CAR T cells and side effects were more common in patients treated with axi-cel. Even though based on small patient numbers, this observation is in accordance with previous studies indicating that CD28 is associated with the promotion of the differentiation of CAR T cells into effector CAR T cells with short-lived glycolysis-based metabolism, resulting in rapid and robust CAR T cell expansion (33-35). By contrast, 4-1BB contained as a costimulatory domain within tisa-cel mediates a central memory CAR T cell phenotype with slower expansion, diminished exhaustion and longer CAR T cell persistence (10,33,36-38).

In conclusion, the present study confirmed the validity of qPCR and dPCR for precise CAR T cell quantification and demonstrated that both approaches are comparable and suitable to monitor CD19-directed CAR T cell kinetics. qPCR, i.e. SCG-DP-PCR, and dPCR contribute to the elucidation of the association of CAR T cell kinetics with treatment response and toxicity and are important diagnostic tools to ensure patient safety, enable comprehensive patient follow-up and guide therapeutic decisions in treated patients. In addition, providing dependable real-world data based on the analysis of numerous patients by precise, fast and easily applicable CAR T cell monitoring assays is indispensable for improving the understanding of CAR T cell therapy.

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Availability of data and materials

The datasets used and/or analyzed within this study are available from the corresponding author on reasonable request.

Authors' contributions

AB established and validated the dPCR assays and analyzed and discussed data. AHK was responsible for quality control (QC) of CAR T cell products and discussed data. AK designed the qPCR protocol, performed experiments and acquired, analyzed and discussed data. AS performed leukapheresis of the patients and discussed data. BF designed the study and the dPCR, discussed and interpreted experiments and data and wrote and edited parts of the manuscript. BN performed QC of CAR T cell products and discussed data. CB participated in dPCR development, processed samples, acquired data and designed and organized the study. CMT, FAA, IM and NK treated patients and interpreted and discussed data. KR identified target sequences for dPCRs, discussed the study design and interpreted and analyzed data. LW performed QC of CAR T cell products and discussed data. MLS treated patients, discussed experiments, data and the study design and wrote major parts of the manuscript. MS designed the study and treated patients, and discussed and interpreted experiments and data. PD treated patients and interpreted and discussed experiments and data. SZ performed dPCRs analyses and acquired and analyzed data. All authors revised the manuscript. All authors edited the manuscript and approved the final version of the manuscript for publication. All authors agreed to be accountable for all aspects included in this work. MLS, AK and BF checked and approved the authenticity of the raw data.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committees of the two institutions (no. S-254/2016 at UKHD Heidelberg and no. PV7091 at UKE Hamburg, Germany).

Patient consent for publication

Informed consent including consent for publication was obtained from all patients involved in the study prior to CAR T cell treatment.

Competing interests

AS is a co-founder and part-time employee of TolerogenixX Ltd. BF has performed consultancy work for Celgene/BMS. CMT received research support from Bayer AG and is an Advisory Board member of Pfizer and Janssen-Cilag GmbH. CMT has also received grants and/or investigational medicinal products from Pfizer, Daiichi Sankyo and BiolineRx. FAA has performed consultancy work for Celgene/BMS and is an Advisory board member of Kite/Gilead, Celgene/BMS, Novartis and Janssen. NK has received honoraria from Celgene/BMS, Kite/Gilead, Novartis and Janssen. MLS has performed consultancy work for Kite/Gilead and Takeda, and is an Advisory Board member of Kite/Gilead. MS has received research grants from Apogenix, Hexal and Novartis, and is an Advisory Board member of MSD. MS is also a (co-)principal investigator of clinical trials for MSD, GSK, Kite and BMS, and is a co-founder and a shareholder of TolerogenixX, Ltd. PD has performed consultancy work for AbbVie, AstraZeneca, Gilead, Janssen, Novartis, Riemsler and Roche. The dPCR assay used in the present study was made available as 'Expert Design Assays' by Bio-Rad Laboratories, Inc. based on an agreement between UMC Hamburg-Eppendorf and Bio-Rad Laboratories, Inc.; in accordance with the German law on employee inventions, BF, AB, CB and KR received compensation payments. Note that Bio-Rad Laboratories, Inc. was not involved in the study design, collection, analysis or interpretation of the data, or in the writing of this paper. None of the mentioned sources supported the work described within this manuscript. The remaining authors have no competing interests to declare.

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