Sensitive monitoring of CAR+ T cells in peripheral blood by flow cytometry and real-time qPCR

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Introduction

Autologous T cells genetically modified to express a second-generation chimeric antigen receptor (CAR) are under investigation for treatment of several human hematopoietic and other malignancies. Initial clinical data indicate that CAR T cell persistence and expansion after autologous T cell transfer show positive correlation with effective disease clearance and protection from recurrence. Therefore, quantification of such cells in the perpheral blood of patients is a valuable monitoring tool. Multicolor flow cytometry offers the opportunity to analyze the presence as well as the phenotype of circulating CAR T cells with CAR protein expression on the cell surface during patient follow-up. The detection of transgenes in CAR+ T cells by real-time quantitative polymerase chain reaction (qPCR) is the more sensitive detection method with the restriction that qPCR only quantitates the integration of the used CAR construct into the T cell genome and therefore does not measure the surface expression of the CAR molecule. Since it was previously reported that several patients showed CAR T cell expansion by qPCR but no functional CAR T cell expression on the cell surface and therefore no tumor clearance, both monitoring methods should be used in combination to determine the cell surface expression as well as the genome integration of the CAR. Here we present the results obtained during assay verification of CAR qPCR, were we correlated the CAR T cell detection by flow cytometry and by qPCR of spike-in experiments with CAR T cells targeting different antigens, such as CD19, CD20, or both antigens in combination.

Methods

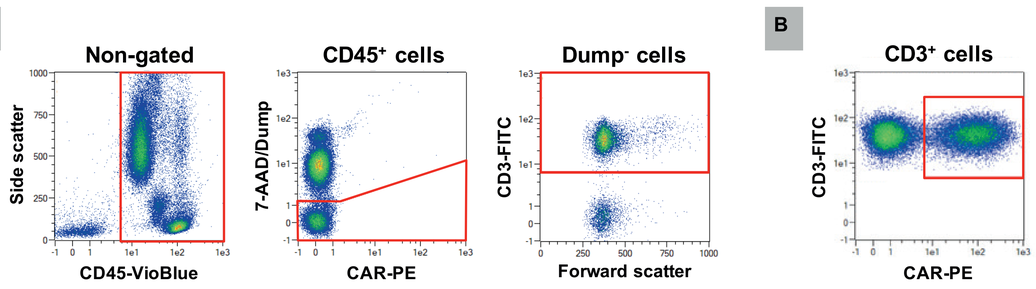
1 Preparation of cellular material

CD19 CAR+ T cells (generated by the CliniMACS Prodigy®) were spiked into EDTA-whole blood samples from three healthy donors. Serial dilution of these samples was prepared and divided into parallel aliquots for flow cytometric determination of CAR T cells and for genomic DNA isolation followed by qPCR. Furthermore, biological negative control samples (EDTA-whole blood of healthy donors; n = 3) and positive control samples (CD19, CD20, and CD2019 *in vitro* cultivated CAR+ T cells) were prepared for both analytical methods.

2 Flow cytometric CAR T cell detection

For flow cytometric analysis 1 mL of peripheral EDTA-anticoagulated blood, with or without spike-in of CD19 CAR–transduced T cells were lysed and resuspended in a final volume of 100 µL. As a positive control 5×10^6 *in vitro* cultivated CAR transduced T cells were stained in a final volume of 100 µL. All samples, except the CD20 CAR-positive T cells, were indirectly stained with the CD19 CAR Detection Reagent (Biotin), as well as with CD45-VioBlue®, CD4-VioGreen™, CD3-FITC, Anti-Biotin-PE, 7-AAD, dump markers (CD14-PerCP-Vio® 700, CD15-PerCP-Vio 700), and CD8-APC-Vio 770. Staining was followed by a washing and centrifugation step, aspiration of the supernatant, and resuspension of cell pellets in 900 µL of PBS supplemented with 0.5% BSA and 2 mM EDTA. CD20 CAR+ T cells were stained directly with the CD20 CAR Detection Reagent (PE) and the other prior listed fluorochrome-conjugated antibodies. The flow cytometric analysis of 450 µL cell suspension was performed on a MACSQuant® Analyzer 10. The gate for CD19 CAR+ T cells was set on the positive control (fig. 1B).

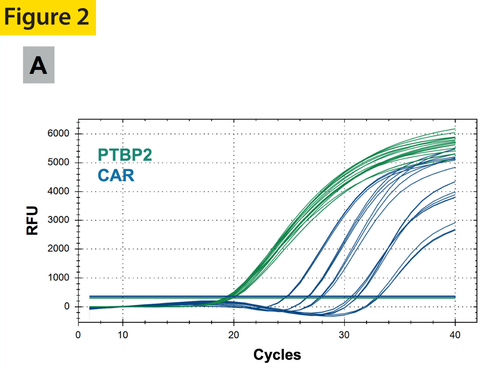
Figure 1



3 DNA isolation and real-time qPCR

Isolation of gDNA from 1 mL of peripheral EDTA-anticoagulated blood, with or without spike-in of CD19 CAR–transduced T cells was done with the QIAamp DNA Blood Midi Kit according to the manufacturers protocol. DNA was then concentrated by a vacuum concentrator to a volume of 100 µL. Real-time qPCR was performed using the single-copy reference gene PTBP2 (Polypyrimidine tract binding protein 2) as a control for cell numbers. The real-time qPCR reaction comprised 12.5 µL of DNA and 12.5 µL of TaqMan™ Fast Advanced Master Mix including primers/probes targeting the single-copy reference gene as well as a gene coding for a CAR-specific gene. Real-time PCR was performed using a BIO-RAD CFX96 Touch™ Real-Time PCR Detection System with an appropriate 40-cycles thermal protocol. A circular plasmid carrying the information of PTBP2 and a CAR-specific gene was used as a standard for absolute quantification (fig. 2A – amplification curves; fig. 2B standard curves). qPCR products of negative and positive controls were analyzed according to their fragment length with the Agilent® 2100 Bioanalyzer® system using a DNA 7500 chip.

Figure 2



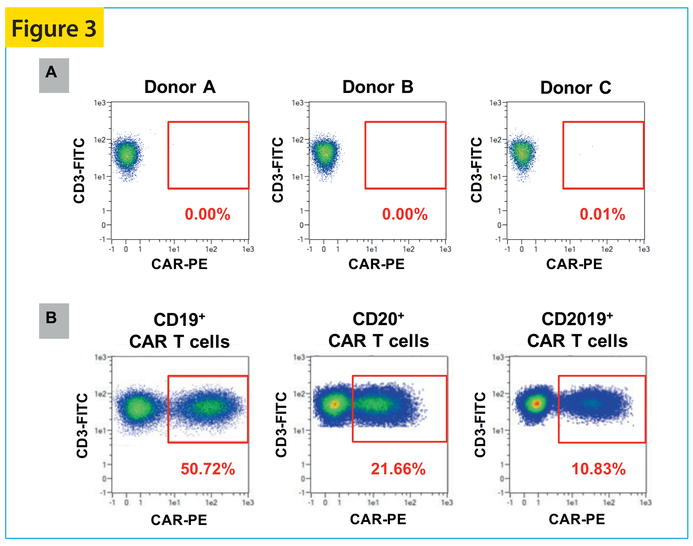
	Slope	PCR efficiency	R^2	Amplification factor
PTBP2	-3.336	99.42%	0.9955	1.99
CAR	-3.405	96.65%	0.9945	1.97

Results

1 Analysis of negative and positive controls

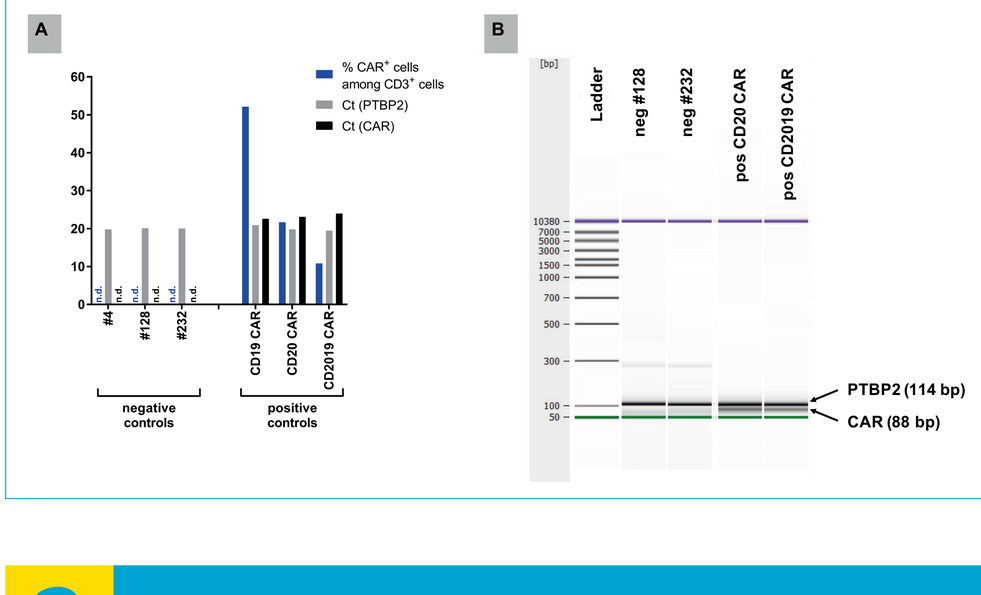
Biological negative control samples (EDTA-whole blood of healthy donors; n = 3) and positive control samples (CD19, CD20, and CD2019 *in vitro* cultivated CAR+ transduced T cells) were analyzed by either flow cytometry (fig. 3) or real-time qPCR (fig. 4A). In flow cytometry, negative controls showed no or only minor CAR-background using the staining with CD19 CAR Detection Reagent (Biotin) and Anti-Biotin-PE (fig. 3A), while CAR detection was not possible by real-time qPCR (fig. 4A). *In vitro* CAR transduced T cells showed a clearly distinguishable CAR-positive population by flow cytometry (fig. 3B) and an amplification of the CAR-gene by real-time qPCR (fig. 4A).

Figure 3



Real-time qPCR products of two negative and two positive controls were additionally evaluated by an automated microfluidic chip-based electrophoresis to confirm qPCR results. Negative controls showed only bands for PTBP2, while positive controls showed also a second band for the CAR qPCR product (fig. 4B). qPCR product sizes correlated to theoretical sizes of DNA fragments, which should be amplified (CAR – 88 bp; PTBP2 – 114 bp).

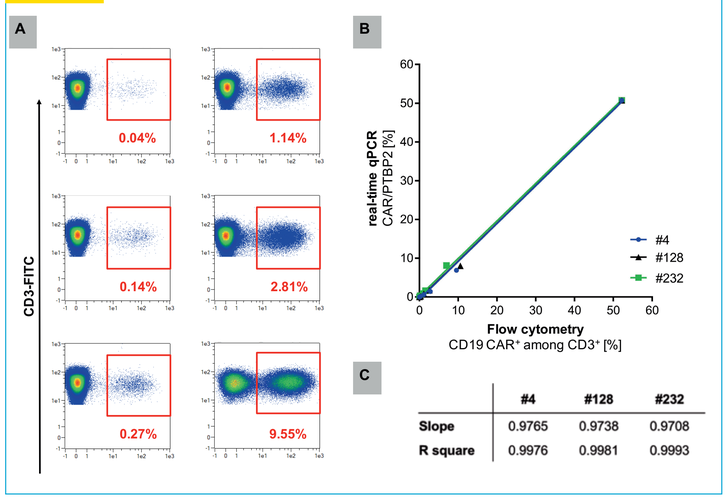
Figure 4



2 Spike-in experiment

To correlate flow cytometry and qPCR results, CD19 CAR+ transduced T cells were spiked to EDTA-anticoagulated whole blood of healthy donors. Serial dilutions of those samples were performed and samples for both detection methods were prepared simultaneously. Analysis of serial dilution samples by flow cytometry (fig. 5A; donor #232) as well as real-time qPCR (data nor shown) resulted in decreasing CAR frequencies. Data generated by flow cytometry and real-time qPCR were correlated by a linear regression. Spike-in samples of all donors (n = 3) showed a good correlation including the corresponding positive and negative controls with an average R square of 0.9983 ± 0.0001 and an average slope of 0.9737 ± 0.0023 (fig. 5B).

Figure 5



	#4	#128	#232
Slope	0.9765	0.9738	0.9708
R square	0.9976	0.9981	0.9993

Conclusion

The results obtained for determination of CAR-transduced T cells by flow cytometry and real-time qPCR determination demonstrated good correlation between both assays. Therefore, we were able to set-up and verify two different CAR T cell detection methods, which should be standardized and validated for CAR T cell persistence determination in multicenter trials.