Method validation of a flow cytometry assay for sensitive detection of CD20 CAR+ T cells in peripheral blood

Introduction

Autologous T cells, genetically modified to express a second-generation chimeric antigen receptor (CAR), are under investigation for treatment of several hematopoietic and other malignancies. Since CAR T cell persistence after autologous T cell transfer correlates with effective disease clearance and protection from recurrence, the quantification of such cells in the blood of patients is a valuable monitoring tool. Multicolor flow cytometry offers the opportunity to analyze the presence as well as the phenotype of CAR T cells during follow-up.

Methods

1 Detection and enumeration of CD20 CAR+ T cells

As a test system, peripheral blood (obtained from healthy donors) with or without spiking with CD20 CAR–transduced T cells (generated by the GMACs Protein) were used. Per sample, 600 µL of peripheral EDTA-anticoagulated blood, stored for 24 h, were lysed and resuspended in a final volume of 100 µL. As “Negative control,” lysed blood was used without further additions. For a “Sample,” lysed blood was spiked with CD20 CAR–transduced T cells. The “Positive control” was a test system, peripheral blood (obtained from healthy donors) with or without spiking with CD20 CAR–transduced T cells.

CD3-PE, CD20 CAR detection reagent (PE), 7-AAD, dump markers (CD14-PerCP-Vio® 700, CD15-PerCP-Vio 700, CD45RO-APC, and CD8-APC-Vio 770). Staining was followed by a washing and centrifugation step, aspiration of the supernatant, and resuspension of cell pellets in 100 µL of PBS supplemented with 0.5% BSA and 2 mM EDTA. The flow cytometric analysis of 450 µL cell suspension was performed on a MACSquant® Analyzer 10 by a software tool for automated acquisition and analysis (Stemline Analyze CAR T Cell Persistence, v. 2.0). In total, 1.7 to 3.5×10⁶ events were acquired per sample. Within the automated analysis, samples were gated for CD3+ T cells with the software tool for automated acquisition and analysis (Express Mode CAR T Cell Persistence).

Analytical sensitivity was determined according to the CLSI guideline EP17-A2, which defines the limit of blank (LoB) as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested. Therefore, ten replicates “Blank” from six different donors were analyzed regarding overall mean (0.003% CAR+ cells among CD3+ cells) and standard deviation (0.0023% CAR+ cells among CD3+ cells). Then, the LoD was calculated according to the formula above (A) with an α-failure of 5%, B = number of blank results (≥ 5) and K = number of different samples (≥ 6).

Limit of detection (LoD) was determined by utilizing both the measured LoB and the value measured for “Samples” spiked with low concentration of analyte (i.e., CAR+ T cells).

The obtained coefficient of determination for CD3+ T cells (fig. 3) with a standard error (SE) of 0.0013% and a confidence interval (CI) of 0.0002%. Furthermore, the LoB of the CAR detection was determined to be 0.0139% CAR+ cells among CD3+ cells.

Results

1 Repeatability of CD20 CAR+ T cell detection

Repeatability of CD20 CAR+ T cell detection was determined by using EDTA-anticoagulated whole blood from six different donors for processing and analyzing ten replicates each of “Negative controls” and CD20 CAR+ T cell–spiked “Samples” under the same operating conditions over a short interval of time. The mean and coefficient of variation (CV) of CAR+ cells among CD3+ cells were calculated for each donor. The CV of “Blank” samples ranged from 5.1% to 9.99%, whereas the CV of CAR+ cells (fig. 3) with SE of 0.0013% CAR+ cells among CD3+ cells.

2 Analytical sensitivity of the assay

Analytical sensitivity was determined according to the CLSI guideline EP17-A2, which defines the limit of blank (LoB) as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested. Therefore, ten replicates “Blank” from six different donors were analyzed with regard to overall mean (0.003% CAR+ cells among CD3+ cells) and standard deviation (0.0023% CAR+ cells among CD3+ cells). Then, the LoD was calculated according to the formula above (A) with an α-failure of 5%, B = number of blank results (≥ 5) and K = number of different samples (≥ 6).

Limit of detection (LoD) was determined by utilizing both the measured LoB and the value measured for “Samples” spiked with low concentration of analyte (i.e., CAR+ T cells). Ten low-positive, spiked “Sample” replicates (8–donors) were analyzed with regard to overall mean (0.003% CAR+ cells among CD3+ cells) and standard deviation (0.0023% CAR+ cells among CD3+ cells). The LoD was calculated according to the formula above (B) with a β-failure of 5%, B = number of blank results (≥ 5) and K = number of different samples (≥ 6).

The LoD was determined to be 0.0139% CAR+ cells among CD3+ cells (fig. 3) with a standard error (SE) of 0.0013% and a confidence interval (CI) of 0.0002%. Furthermore, the LoB of the CAR+ cell count was determined to be 0.0139% CAR+ cells among CD3+ cells (fig. 3) with SE of 0.0013% and CI of 0.0002%. Furthermore, the LoD of the CAR+ cell count was determined to be 0.0139% CAR+ cells among CD3+ cells (fig. 3) with SE of 0.0013% and CI of 0.0002%.

3 Linearity of the assay

For evaluation of assay linearity, CD3+ CAR–transduced T cells were spiked into bulk lysed, EDTA-anticoagulated whole blood from three different donors (10 replicates per spike-in) to generate samples with different frequencies of CD3+CAR+ cells over a broad range, starting from 0.32% CAR+ cells among CD3+ cells down to 0.09% CAR+ cells among CD3+ cells. The obtained coefficient of determination for CD3+CAR+ cell frequency averaged at R² = 0.996 (fig. 4A).

Conclusion

The results obtained for reproducibility and linearity of the assay, as well as determination of LoD, LoQ, and LoB, demonstrate that our analytical flow cytometry method is well suited for determination of the persistence of CD20 CAR–transduced T cells in EDTA-anticoagulated whole blood.

References