CAR Detection Reagents – pioneering advances in CAR T analytics

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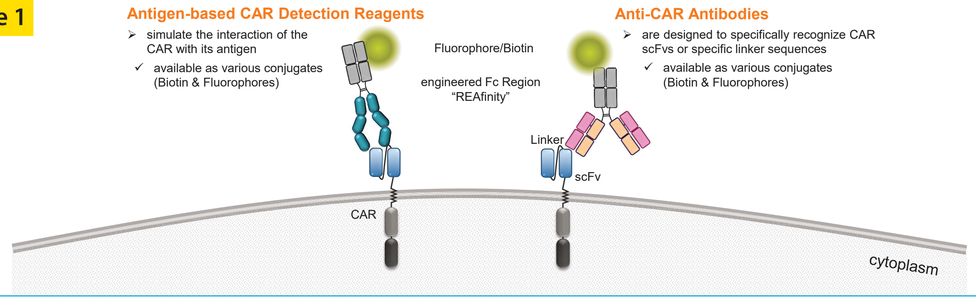
Stephan Wilmes, Desiré Missing, Anja Brauchle, Nadine Borgelt, Johanna Müllers, Monika Winkels, Larissa Möckel, Michaela Niemöller, Dagmar Häußler-Kuballa, Franziska Antonia Bertram, Olga Goldbach, Dominik Poschen, Astrid Imann, Silvia Rüberg, Anijutta Appelshoffer, Fabio El Yassouri, Marsilius Mues, Volker Nölle, Andreas Bosio, Christian Dose and Anne Richter. Miltenyi Biotec B.V. & Co. KG, Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany

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Introduction

Chimeric Antigen Receptor (CAR) cell therapies are pivotal in treating hematological malignancies demonstrating remarkable efficacy. Specific and reliable detection of CAR expression is of utmost importance, not only for research and pre-clinical applications, but also for clinical manufacturing of cellular products and patient immunomonitoring. To fulfill these critical requirements, we developed CAR Detection Reagents (CAR DRs) for flow cytometry, offering outstanding quality and specificity. Our CD19 CAR FMC63 Idiotype Antibody (REA1297) and Antigen-based CD19 and BCMA CAR DRs facilitate fast magnetic isolation or enrichment of CAR T cells from whole blood or PBMCs. Moreover, the CD19 CAR FMC63 Idiotype Antibody (REA1297) enables precise detection and enumeration of CD19 CAR T cells through immunofluorescence microscopy.

Figure 1



We developed different CAR DRs that can be classified into two groups (Fig. 1). Antigen-based CAR DRs consist of the extracellular domain of the target protein that is fused to a Fc part and thereby mimic the target-epitope-binding. We also developed Anti-CAR Antibodies that specifically target the scFv of the CAR (e.g. anti-Idiotype Antibodies) or specific linker sequences within the scFv (e.g Whitlow/218 Linker). These CAR detection strategies take advantage of a recombinantly engineered human IgG Fc part that prevents Fc receptor binding ("REAfinity" technology) and thereby reduces unspecific background staining. These CAR DRs are provided either as Biotin-conjugates or they are directly conjugated to a variety of fluorophores (FITC, Vio® Bright B515, PE, APC).

Results

1 Antigen-based CAR Detection Reagents for Flow Cytometry

Figure 2

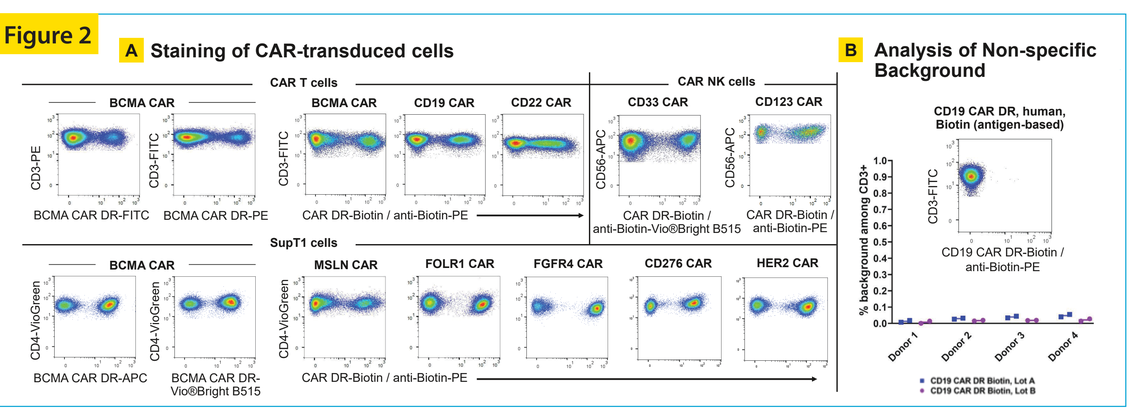


Figure 2A: Representative density plots of CAR T cells, CAR NK cells, and CAR-transduced SupT1 cells stained with antigen-based CAR Detection Reagents containing ectodomains of BCMA, CD19, CD22, CD33, CD123, MSLN, FOLR1, FGFR4 FGFR4 (CD334), CD276 (B7-H3) or HER2. CAR T and NK cells were gated on CD45 (REA747)-positive, 7-AAD-negative, and CD3 (REA613)-positive populations; SupT1 cells on 7-AAD-negative and CD4 (REA623)-positive cells.

Figure 2B: Non-specific background staining of two different lots of CD19 CAR Detection Reagent, human, Biotin, tested on whole blood from four donors. Inset shows a representative density plot from one donor. Biotinylated reagents were detected using anti-Biotin Antibody (REA746) conjugated to PE or Vio®Bright B515. Flow cytometric data was acquired using the MACSQuant® Analyzer 10.

2 Anti-CAR Antibodies for Flow Cytometry

Figure 3 Staining of Primary CAR T cells

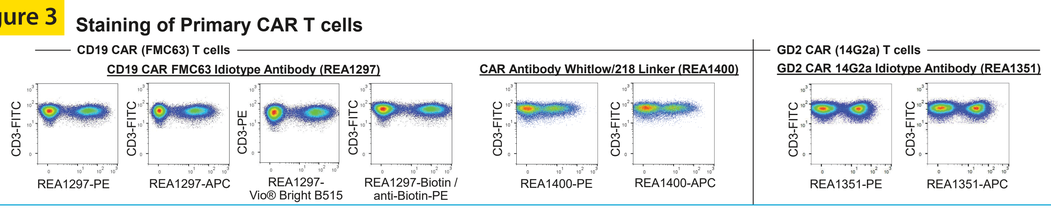


Figure 3: Representative density plots of CD19 CAR (FMC63) T cells stained with either CD19 CAR (FMC63) Idiotype Antibody (REA1297) conjugated to PE, APC, VioBright B515, or Biotin, or with the CAR Antibody Whitlow/218 Linker (REA1400) conjugated to PE or APC. Additionally, GD2 CAR (14G2a) T cells were stained using GD2 CAR (14G2a) Idiotype Antibody (REA1351) conjugated to PE or APC. Cells were gated on CD45 (REA747)-positive, 7-AAD-negative, and CD3 (REA613)-positive populations. Flow cytometric data was acquired using the MACSQuant® Analyzer 10.

3 Comparison of Antigen-based CD19 CAR DR and CD19 CAR FMC63 Idiotype Antibody (REA1297)

Figure 4

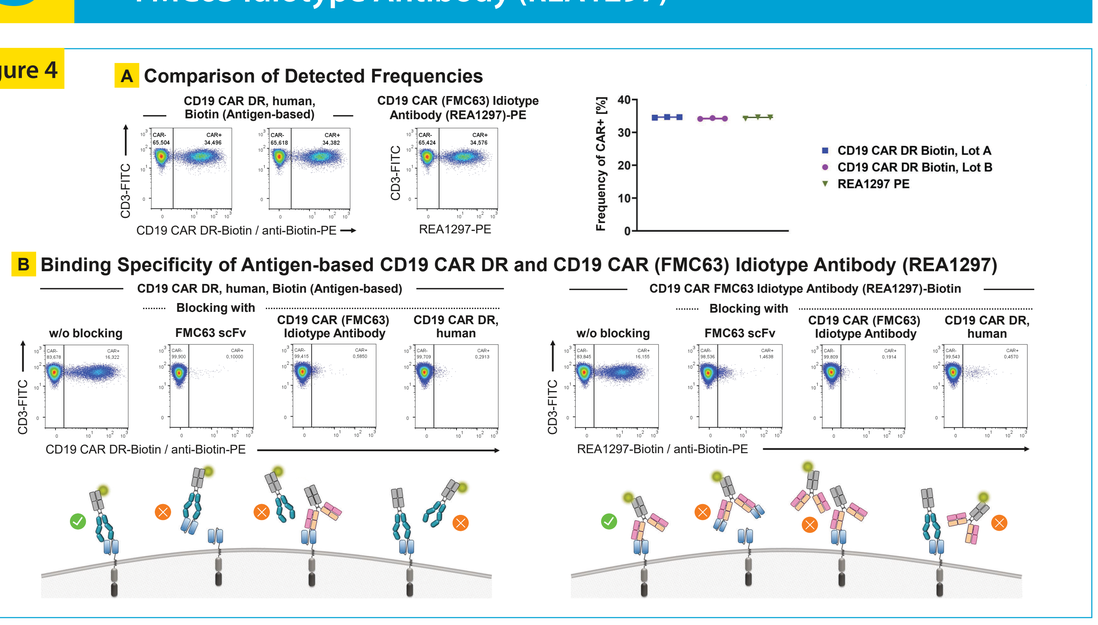


Figure 4A: Representative density plots of CD19 CAR (FMC63) T cells stained with two different lots of CD19 CAR Detection Reagent, human, Biotin, and one lot of CD19 CAR (FMC63) Idiotype Antibody (REA1297)-PE. CAR-positive cell frequencies were reproducibly detected with all tested reagents. The experiment was performed in technical triplicates. Figure 4B: Representative density plots of CD19 CAR (FMC63) T cells stained with either CD19 CAR Detection Reagent, human, Biotin or CD19 CAR (FMC63) Idiotype Antibody (REA1297)-Biotin. Staining specificity was assessed by pre-incubation with blocking agents: FMC63 scFv, pure CD19-Fc protein, or pure REA1297 Antibody. Cells were gated on CD45 (REA747)-positive, 7-AAD-negative, and CD3 (REA613)-positive populations. Secondary detection of biotinylated reagents was performed using anti-Biotin Antibody (REA746). Flow cytometric data was acquired using the MACSQuant® Analyzer 10

4 Improving the Analytical Sensitivity of CAR T Cell Detection by Magnetic Enrichment

Figure 5

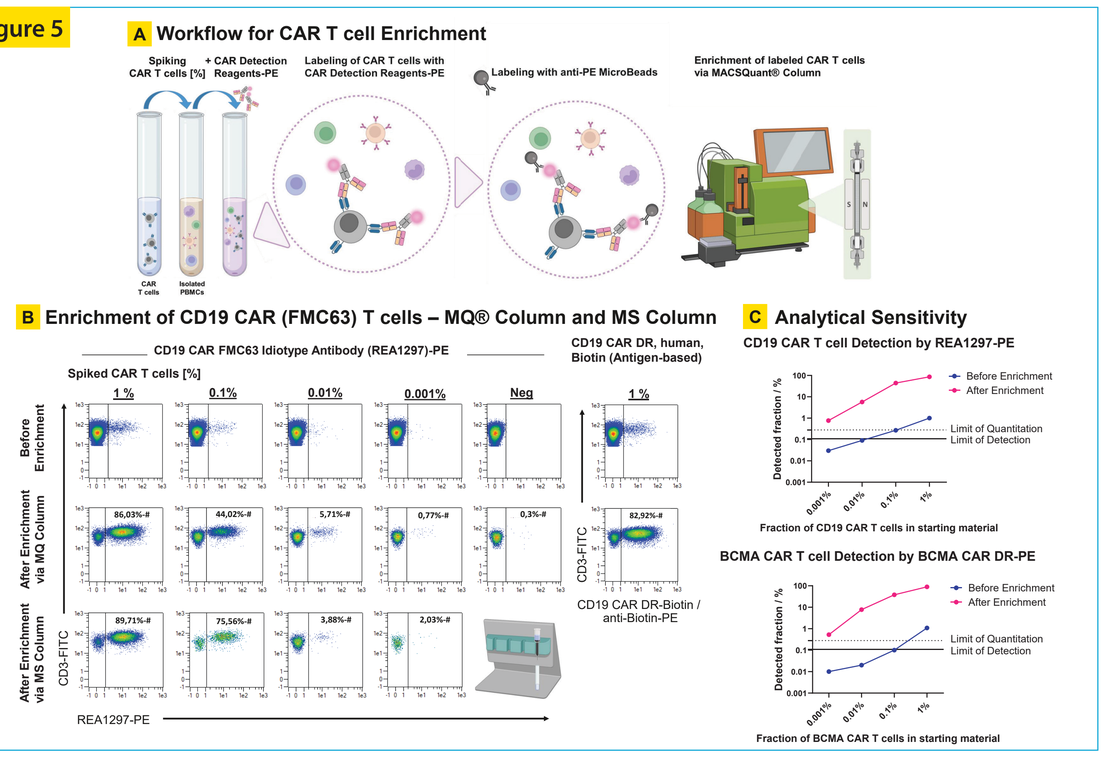


Figure 5A: Workflow illustrating the magnetic enrichment of CAR T cells from freshly isolated PBMCs using the integrated MACSQuant® Column. Figure 5B: CD19 CAR (FMC63) T cells were spiked into 1×10^7 PBMCs at defined frequencies (0–1%) and labeled using CAR Detection Reagents. Representative density plots of CD19 CAR (FMC63) T cells stained with CD19 CAR FMC63 Idiotype Antibody (REA1297)-PE or CD19 CAR DR, human, Biotin before and after enrichment are shown. Samples were gated on CD45 (REA747) positive, 7-AAD negative and CD3 (REA613) positive cells. Secondary staining of the biotinylated CAR DR was done with anti-Biotin Antibody (REA746)-PE. Samples were labeled with Anti-PE Microbeads, enriched by using the MQ® Column and analyzed by MACSQuant®10 in a fully automated workflow. A direct comparison of manual CAR T cell enrichment via the MS-column is shown in the bottom panel. Figure 5C: Comparison of CAR T cell frequencies before (blue) and after (red) magnetic enrichment by MQ® Column with RE1297-PE and BCMA CAR DR, human, PE. The Magnetic enrichment strongly increased the detectable frequency of CAR T cells, especially at low spike-in levels (≤0.1%), where detection without enrichment was observed to be challenging and inconsistent. This demonstrates a substantial gain in analytical sensitivity, allowing the reliable identification and enumeration of rare CAR T cell populations. As a result, both the limit of detection (LOD) and the limit of quantitation (LOQ) were substantially improved after enrichment.

5 Detecting CD19 CAR (FMC63) T Cell Tissue Infiltration by Fluorescence Microscopy

Figure 6

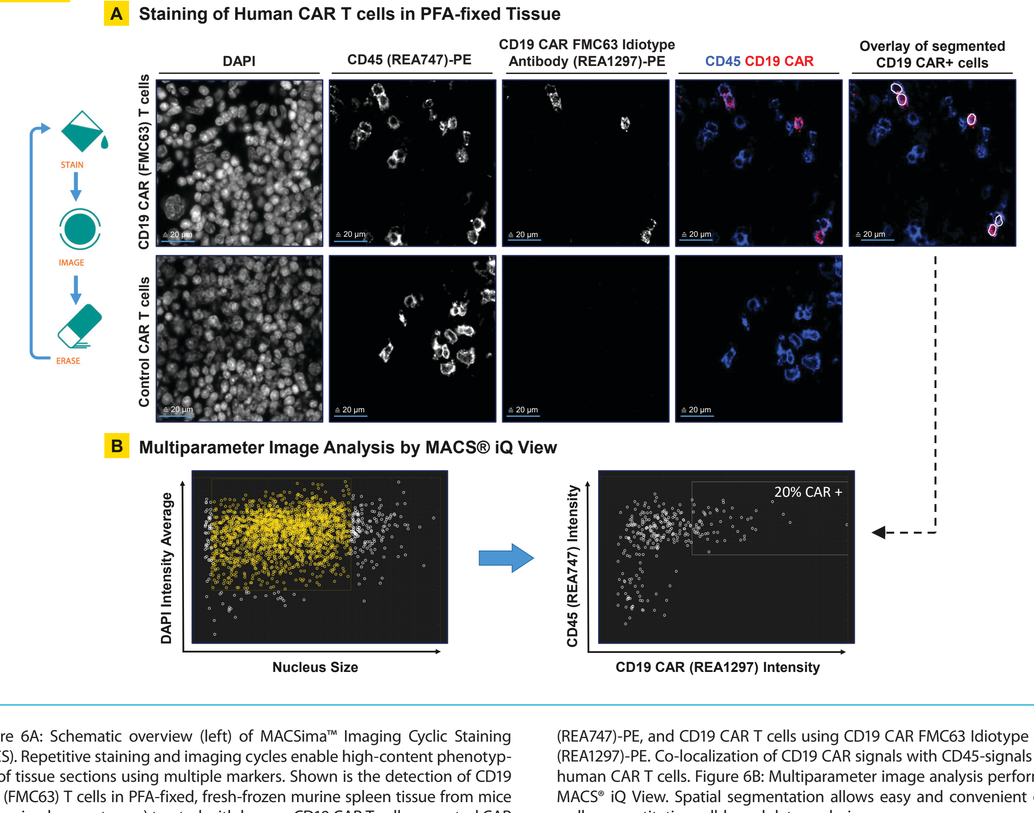


Figure 6A: Schematic overview (left) of MACSima™ Imaging Cyclic Staining (MICS). Repetitive staining and imaging cycles enable high-content phenotyping of tissue sections using multiple markers. Shown is the detection of CD19 CAR (FMC63) T cells in PFA-fixed, fresh-frozen murine spleen tissue from mice (harboring human tumor) treated with human CD19 CAR T cells or control CAR T cells. Nuclei were stained with DAPI. Human T cells were identified via CD45 (REA747)-PE, and CD19 CAR T cells using CD19 CAR FMC63 Idiotype Antibody (REA1297)-PE. Co-localization of CD19 CAR signals with CD45-signals indicates human CAR T cells. Figure 6B: Multiparameter image analysis performed with MACS® iQ View. Spatial segmentation allows easy and convenient gating as well as quantitative cell-based data analysis.

Conclusions

We developed CAR Detection Reagents to enable robust flow cytometric analysis of CAR-transduced cells. These reagents are characterized by high brightness, excellent specificity, minimal unspecific background staining, and consistent performance across multiple production lots. These are fundamental prerequisites for quality control of CAR T cell manufacturing, immunomonitoring purposes, and research applications. In addition to standard flow cytometric detection, our CAR DRs support the enrichment of CAR-positive cells, strongly enhancing analytical sensitivity. Automated enrichment for analytical purposes can be conveniently performed using the built-in MQ® Column of the MACSQuant® Analyzer. This workflow allows the detection of sparse amounts of CAR T cells e.g. for persistence monitoring. The performance is comparable to established preparative enrichment workflows using MACS® MS Columns. Here, isolated CAR T cells with high purity are suitable for downstream NGS-based approaches such as gene expression profiling or TCR sequencing, as well as for further cell culture and *ex vivo* functional assays. Finally, we demonstrate that our Anti-CAR Antibodies can be used in immunofluorescence microscopy. This enables detection of tissue infiltration by CAR T cells and detailed phenotyping. CAR Detection Reagents represent a versatile toolbox for various applications and we are aiming to further expand our CAR DR portfolio, for more specificities and considering fluorochrome-conjugated antigen-based CAR DRs.

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