Impact of serum-free media on the expansion and functionality of CD19.CAR T-cells

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Abstract. Fetal bovine serum (FBS) or human serum is widely used in the production of chimeric antigen receptor (CAR) T-cells. In order to overcome a lot-to-lot inconsistency, the use of chemically defined medium that is free of animal-components would be highly desirable. The present study compared three serum-free media [Prime-XV™ T Cell CDM, Fujifilm™ (FF), LymphoONE™ T-Cell Expansion Xeno-Free Medium, Takara Bio™ (TB) and TCM GMP-Prototype, CellGenix™ (CG)] to the standard CAR T-cell medium containing FBS (RCF). After 12 days of CD19.CAR T-cell culture, the expansion, viability, transduction efficiency and phenotype were assessed using flow cytometry. The functionality of CAR T-cells was evaluated using intracellular staining, a chromium release assay and a long-term co-culture assay. Expansion and viability did not differ between the CAR T-cells generated in serum-free media compared to the standard FBS-containing medium. The CG CAR T-cells had a statistically significant higher frequency of IFNγ^+ and IFNγ^+TNF-α^+ CAR T-cells than the CAR T-cells cultured with FBS (22.5 vs. 7.6%, P=0.0194; 15.3 vs. 6.2%, P=0.0399, respectively) as detected by intracellular cytokine staining. The CAR T-cells generated with serum-free media exhibited a higher cytotoxicity than the CAR T-cells cultured with FBS in the evaluation by chromium release assay [CG vs. RCF (P=0.0182), FF vs. RCF (P=0.0482) and TB vs. RCF (P=0.0482)]. Phenotyping on day 12 of CAR T-cell production did not reveal a significant difference in the expression of the exhaustion markers, programmed cell death protein 1, lymphocyte-activation gene 3 and T-cell immunoglobulin and mucin-domain containing-3. The CAR T-cells cultured in FF had a higher percentage of central memory CAR T-cells (40.0 vs. 14.3%, P=0.0470) than the CAR T-cells cultured with FBS, whereas the CAR T-cells in FF (6.2 vs. 24.2%, P=0.0029) and CG (11.0% vs. 24.2%, P=0.0468) had a lower frequency of naïve CAR T-cells. On the whole, the present study demonstrates that in general, the functionality and expansion of CAR T cells are maintained in serum-free media. Given the advantages of freedom from bovine material and consistent quality, serum-free media hold promise for the future development of the field of GMP manufacturing of CAR T-cells.

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Introduction

Chimeric antigen receptor (CAR) T-cell therapy is considered to be a promising immunotherapeutic treatment strategy for the treatment of patients with relapsed or refractory B-cell malignancies. Clinical trials have shown promising remission rates for patients with B-lymphoblastic acute leukemia (B-ALL) and B-cell non-Hodgkin lymphoma who were treated with CD19-specific CAR T-cells (1-5). Based on these results, Kymriah™ (Tisagenlecleucel, Novartis) and Yescarta™ (Axicabtagene Ciloleucel, Kite Pharma/Gilead) were the first two CD19 CAR T-cell products to be approved by the Food and Drug Administration (FDA) in the United States in 2017 for the treatment of relapsed or refractory B-ALL (r/r B-ALL) (Kymriah™) and relapsed or refractory diffuse large B-cell lymphoma (r/r DLBCL) (Yescarta™) (6,7). Indications were extended in the following years, and the CD19 CAR T-cell products Tecartus™ (Brexucabtagene Autoleucel, Kite Pharma/Gilead) and Breyanzi™ (Lisocabtagene Maraleucel, Juno Therapeutics/BMS) have recently obtained market access (8). Additionally, academic institutions began their own good manufacturing production (GMP) of CD19 CAR T-cells (9). Whereas the commercially available products contain a second-generation CAR, a third-generation CD19 CAR is currently being evaluated for safety and efficacy within the HD-CAR-1 trial (9). In contrast to second-generation CARs, third-generation CARs contain two intracellular costimulatory domains (e.g., CD28 and 4-1BB) instead of only one (10,11).

For the GMP-compliant manufacturing of CAR T-cell products, different types of media are currently being used. Media containing fetal bovine serum (FBS) is still most commonly used in GMP core facilities worldwide (12). FBS is nutrient-rich containing growth factors, hormones, vitamins, buffering proteins and ions, but does not mirror well the condition within the human body following the application of the CAR T-cells, as the composition of nutrients between fetal bovine and human serum (HS) differs significantly (13). In addition, ethical and ecological aspects have to be taken into consideration whenever FBS is used. Consequently, an increasing number of GMP laboratories have begun to optimize HS containing media formulations for CAR T-cell production (14-16). It is considered that HS supplies nutrients and growth factors to the T-cell culture that more closely imitates the human microenvironment. However, as the composition of HS and FBS depend on the donor, product-to-product-inconsistency is a major concern (17). Furthermore, the risk of contamination of HS and FBS is also a critical aspect. FBS could theoretically transmit bovine spongiform encephalopathy (BSE) and viral pathogens. Moreover, HS also carries the risk of viral contamination (17,18). Therefore, serum must be tested in depth prior to its use in GMP manufacturing, thus leading to increased costs.

As a result, there is a trend towards the use of defined medium formulations with or without chemically defined animal (xeno)-free components for the production of CAR T-cells. Serum-free medium does not contain serum but can include purified or synthetic ingredients. Smith *et al* (19) revealed similar expansion rates for T-cells cultured in xeno-free medium compared to media supplemented with HS or FBS. Furthermore, the T-cells expanded in xeno-free medium exhibited a higher percentage of central memory T-cells and the growth of lentivirus-mediated gene transduced T-cells was comparable (19). Coeshott *et al* (20) used xeno-free medium to expand T-cells in a functionally closed, automated bioreactor system in a large scale. The phenotyping of expanded T-cells displayed high rates of central memory T-cells compared to before seeding.

The aim of the present study was to compare the effects of four different xeno-free T-cell culture media (TCM) to the standard media (RCF) containing 10% FBS for the production of a third-generation CD19 CAR T-cell product with respect to CAR T-cell transduction efficiency, expansion, viability, cytotoxicity and phenotype (Table I). CTS™ OpTmizer™ Pro SFM, Thermo Fisher Gibco™ (TF) is a serum-free medium (SFM), whereas Prime-XV™ T Cell CDM, Fujifilm™ (FF) is a chemically defined, animal component-free medium. LymphoONE™ T-Cell Expansion Xeno-Free Medium, Takara Bio™ (TB) contains pharmaceutical-grade HS albumin and recombinant human insulin. Similarly, TCM GMP-Prototype, CellGenix™ (CG) includes HS albumin and recombinant human insulin and transferrin.

Materials and methods

Ficoll separation. The isolation of peripheral blood mononuclear cells (PBMCs) was performed for 5 healthy donor buffy coats provided by the blood bank of the University Hospital of Mannheim (DRK-Blutspendedienst Baden-Württemberg-Hessen) by separation with Ficoll [Ficolite-H (Human), Linaris Biologische Produkte GmbH]. PBMCs were cryopreserved with 90% FBS (Thermo Fisher Scientific, Inc.) and 10% dimethyl sulfoxide (DMSO; SERVA Electrophoresis GmbH) until the CAR T-cell manufacturing process. Ethical approval and written consent to participate was obtained. Ethical approval was obtained from the Medical Faculty of the University of Heidelberg (reference no. S-254/2016).

Production of retroviral vector. To produce the third-generation retroviral vector SFG.CAR.CD19/CD28/4-1BB/CD3ζ, plasmids were kindly provided by Professor Malcolm Brenner (Baylor College of Medicine, Houston, TX, USA). 293T cells (DSMZ, cat. no. ACC 635) were co-transfected with the specific retroviral vector plasmid carrying the gene of interest (3.75 μg), the gag-pol plasmid PegPam3 (3.75 μg) and the envelope plasmid RDF (2,5 μg). 293T cells were cultured in Iscove's modified Dulbecco's medium (IMDM) GlutaMax-I (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 0,1 mM sodium pyruvate (Thermo Fisher Scientific, Inc.). For transfection, 470 μl IMDM GlutaMax-I and 30 μl GeneJuice Transfection Reagent (Merck KGaA) were added to the plasmids and incubated for 15 min at room temperature. The suspension was then added in a dropwise manner to the 100-mm dish. Following 48 h of incubation at 37˚C and 5% CO_2, the retroviral supernatant was collected, and new medium was added to the dishes. Following 72 h of incubation in total, the collection process was repeated. The retroviral supernatant was stored at -80˚C.

T-cell activation. The thawing and activation of PBMCs was performed on day 0 of the transduction process. A total of $1x10^6$ PBMCs per well were activated in a non-tissue culture 24-well plate (Corning, Inc.) coated with anti-CD3 (clone: OKT3; cat. no. 317301; BioLegend; dilution, 1 μg/ml;) and anti-CD28 (clone: CD28.2; cat. no. 302901; BioLegend; dilution, 1 μg/ml) monoclonal antibodies. The PBMCs were cultured in CTS™ OpTmizer™ Pro SFM (TF; Thermo Fisher Scientific, Inc.), Prime-XV T Cell CDM (FF; FUJIFILM Wako Pure Chemical Corporation), LymphoONE™ T-Cell Expansion Xeno-Free Medium (TB; Takara Bio, Inc.), TCM GMP-Prototype (CG; CellGenix Inc.) or in CAR medium (RCF) containing 45% Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Inc.), 45% Eagle's Ham's amino acids (EHAA) Clicks medium (FUJIFILM Wako Pure Chemical Corporation), 10% FBS and 2 mmol/l Gluta-MAX™ Supplement (Thermo Fisher Scientific, Inc.). A total of 10 ng/ml of IL-7 (R&D Systems, Inc.) and 5 ng/ml of IL-15 (R&D Systems, Inc.) were added to all media.

Transduction. The cells were transduced on day 2 using the vector SFG.CAR.CD19/CD28/4-1BB/CD3ζ. The wells for the CAR T-cells were coated with RetroNectin (Takara Bio, Inc.) at a concentration of 7 μg/ml and stored overnight at 4˚C. On the day of transduction, the wells were washed, and the retroviral supernatant was added to the wells. The plates were then centrifuged at 2,000 x g for 90 min at room temperature. Activated T-cells were harvested and $1x10^5$ cells per well were

Table I. Media composition.

Basal medium	CTS™ OpTmizer™ Pro SFM, Thermo Fisher Gibco™ (TF)	Prime-XV™ T Cell CDM, Fujifilm™ (FF)	LymphoONE™ T-Cell Expansion Xeno-Free Medium, (TB)	TCM GMP-Prototype, CellGenix™ (CG)	45% Click's Medium (EHAA), Fujifilm™ (RCF)
Other medium	-	-	-	-	45% RPMI
Supplement	CTS™ OpTmizer™ T-Cell Expansion Supplement, Thermo Fisher Gibco™	-	-	-	-
Serum	-	-	-	-	10% FBS
L-glutamine	2 mM	N/A[a]	Yes	N/A[a]	2 mM
Pharmaceutical-grade human proteins	Yes, e.g., albumin	No	Albumin	Albumin	-
Recombinant human proteins	No	Yes, e.g., albumin	Insulin	Insulin and transferrin	-
Antibiotics	-	-	+/-Streptomycin	-	-

[a]No information is available.

seeded in the corresponding medium in the 24-well plate. The plates were centrifuged at 500 x g for 4min at room temperature and cultured at 37˚C 5% CO_2 until day 5.

Cell expansion and viability. Following transduction, the CAR T-cells and non-transduced T-cells were cultured in the respective media with the addition of cytokines. Feeding was performed every 2-3 days adding fresh medium with cytokines. On day 12, the expansion and viability of the CAR T-cells and non-transduced cells were determined by counting the cells with Trypan blue (Merck KGaA) in a counting chamber (NanoEnTek, Inc.). All cell lines were tested by polymerase chain reaction (PCR) to be mycoplasma-free.

Cell lines. The Burkitt lymphoma cell line, Daudi (cat. no. ACC 78), obtained from the German Collection of Microorganisms and Cell Culture (DSMZ) was used as CD19-positive target cell line. As a CD19-negative target cell line, the chronic myeloid leukemia cell line, K-562 (cat. no. ACC 10), also obtained from DSMZ, was used. The target cells were cultured in RPMI (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine at 37˚C and 5% CO_2 and the cell concentration were hold at 0.1-1x10^6/ml. The cells were thawed 1 week prior to the experiments. The cells were washed and cultured in the corresponding media when added to the experiments.

Immunophenotyping. Flow cytometry was performed on a BD™ LSRII Flow Cytometry Cell Analyzer (BD Biosciences). For immunophenotyping, the following monoclonal antibodies were used: CD3 (UCHT1; no. 300424; 2/100 μl), CD4 (OKT4; cat. no. 317408; 2/100 μl), CD8 (SK1; cat. no. 344732; 2/100 μl), CD14 (63D3; cat. no. 367112; 2/100 μl), CD27 (M-T271; cat. no. 356418; 2/100 μl), CD45RO (UCHL1; cat. no. 304228; 2/100 μl), CCR7/CD197 (G043H7; no. 353214; 2/100 μl), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)/CD366 (F38-2E2; cat. no. 345008; 2/100 μl), programmed cell death protein 1 (PD-1; A17188B; cat. no. 621616; 2/100 μl) and lymphocyte-activation gene 3 (LAG-3)/CD223 (7H2C65; cat. no. 369212; 2/100 μl), all from BioLegend, and CD45RA (MEM-56; cat. no. MHCD45RA17; 2/100 μl) from Thermo Fisher Scientific, Inc. In addition, 7-aminoactinomycin (7-AAD; BD Biosciences; no. 555816; 5/100 μl) was used to detect dead cells. To stain the CD19. CAR, a goat anti-human $F(ab)_2$ IgG (H+L) antibody (Jackson ImmunoResearch Europe, Ltd.; cat. no. 109-116-088; 0.5/100 μl) conjugated with PE was used. Compensation was based on PBMCs and controls including a non-transduced control and fluorescence minus one control were realized.

Intracellular staining. Cytokine release was measured on day 13 of CAR T-cell manufacturing. A total of 2x10^5 CAR T-cells were co-cultured with 4x10^5 Daudi or K562 cells for 6 h at 37˚C and 5% CO_2 following the addition of 1X Brefeldin A (BioLegend) and CD107a (H4A3; BD Biosciences). After the incubation time the plate was stored at 4˚C overnight as previously described (21). NEAR-IR (Thermo Fisher Scientific, Inc.) staining was performed at 4˚C for 30 min to distinguish viable and dead cells. For surface marker staining, CD3 (same as above), CD4 (same as above), CD8 (same as above), CD20 (2H7; cat. no. 302310; BioLegend; 2/100 μl) and CAR antibody (same as above) were used. For fixation and permeabilization, the FoxP3 Staining Buffer Set (Miltenyi Biotec GmbH) was

used. Intracellular cytokine staining was performed using TNF-α (Mab11; cat. no. 562783; BD Biosciences; 1/100 μl) and IFNγ (4S.B3; cat. no. 502546; BioLegend; 2/100 μl) antibodies. Multi-parametric cytometry was performed using a LSRII flow cytometer (BD Biosciences). Unstimulated control and unspecific-stimulated control served as negative controls.

Co-culture. Day 12 of the CAR T-cell manufacturing process was regarded as day 0 of co-culture. A total of $0.15x10^5$ CAR T-cells or non-transduced T-cells were co-cultured in an effector-to-target cell ratio of 1:2 in the corresponding medium. Flow cytometry was performed on days 5, 10, 15, 20, 25 and 30. On days 5, 10, 15, 20 and 25, $3x10^4$ Daudi cells were added to the remaining wells. The antibodies CD3 (same as above), CD4 (same as above), CAR (same as above), CD45RA (same as above), CCR7 (G043H7, cat. no. 353226; BioLegend; 2/100 μl), CD20 (same as above), PD-1 (29F.1A12; cat. no. 135224; BioLegend; 2/100 μl), TIM-3 (same as above) and LAG-3 (11C3C65; cat. no. 369318; BioLegend; 2/100 μl) were used for staining.

Chromium release assay. The tumor cell lines, Daudi and K-562, were incubated with ^{51}Cr (Hartmann Analytic) for 2 h at 37˚C and 5% CO_2. The ratio of effector to target cells was 30:1, 10:1, 3:1 and 1:1. A total of $5x10^3$ target cells were co-cultured with the effector cells in each well for 4 h at 37˚C and 5% CO_2; 1% Triton X-100 (Merck KGaA) served as a maximum release control. For spontaneous release control, cell culture medium was used. Ultima Gold (PerkinElmer, Inc.) was added to the co-culture supernatant, and the vials were measured using a 1414 WinSpectral liquid scintillation counter (PerkinElmer, Inc.). All experiments were performed in triplicate. To calculate the specific lysis caused of the CAR T-cells, the following formula was used: [(experimental release-spontaneous release)/(maximum release-spontaneous release)] x100.

Extraction of genomic DNA. Genomic DNA was obtained from cryopreserved CD19.CAR T-cells. They were cryopreserved at day 14 of CAR T-cell culture in freezing medium composed of 10% DMSO (SERVA Electrophoresis GmbH) and 90% FBS (Thermo Fisher Scientific, Inc.). Genomic DNA was extracted using the QIAamp® DNA Blood Mini kit (Qiagen GmbH). DNA concentration was measured using UV spectroscopy (NAnoDrop OneC, Thermo Fisher Scientific, Inc.) and adjusted to 20 ng/μl.

Quantitative PCR (qPCR). For the assessment of the vector copy number of CD19.CAR T-cells, a single copy gene (SCG)-based duplex (DP)-qPCR assay (SCG-DP-PCR) was performed as described by Kunz *et al* (22). For quantification the $2^{-\Delta\Delta Ct}$ method (23) was used. The sequence of the forward primer was 5'-AGCTGCCGATTTCCAGAAGA-3' and that of the reverse primer was 5'-GCGCTCCTGCTGAAC TTCA-3'. RNaseP served as a reference gene and the Copy Number Reference Assay, RNaseP (cat. no. 4403326; Applied Biosystems) which contains RNaseP gene-specific forward primer, reverse primer, and probe (VIC/TAMRA) was used. A total of 100 ng genomic DNA were used for the two simultaneous amplifications of the CAR transgene and the SCG RNaseP supported by the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Inc.). Following conditions were set up for thermocycling: 2 min for 50˚C, 10 min for 95˚C, followed by 40 cycles of 15 sec 95˚C and 1 min 60˚C. H_2O and non-transduced cells served as negative controls.

Statistical analysis. Flow cytometric analysis was performed using FlowJo 10.8 software (BD Biosciences). GraphPad Prism 9 (GraphPad Software, Inc.) was used for statistical analysis. To compare multiple groups with a single independent variable, a one-way ANOVA with a subsequent Dunnett's multiple comparisons test was used. For the analysis of multiple groups with two independent variables, a two-way ANOVA test with Dunnett's multiple comparisons test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of expansion, viability and transduction rates. The expansion of the different CAR T-cell products was assessed on day 12 following initial stimulation (Fig. 1A). No significant difference (mixed-effects analysis, P=0.1248) was observed. T-cells cultured in TF exhibited a low expansion and were excluded for further analysis due to the small cell number which was often not sufficient to reach similar cell concentrations in the experiments. The viability was comparable for all four T-cell products (Fig. 1B). The transduction efficiency (Fig. 1C) differed significantly depending on the medium (one-way ANOVA, P=0.0094) and was highest for the T-cells cultured in RPMI + Click's + FBS (RCF). Compared to the cells cultured in RCF, the transduction efficiency for the T-cells cultured in CG (Dunnett's test, P=0.0358) and TB (Dunnett's test, P=0.0167) was lower. By contrast, a trend towards a lower amount of integrated vector copies was noted for the CAR T-cells manufactured in RCF and FF media, even though the difference did not reach statistical significance (Fig. 1D). As regards the $CD4^+/CD8^+$ ratio, CAR T-cell products cultured in TB and CG contained a significantly lower population of $CD4^+$ cells than the cells cultured in RCF media (Dunnett's test, P=0.0319 and P=0.0232, respectively; Fig. 1E).

Increased IFNγ release of third-generation CAR T-cells generated in serum-free media. In order to investigate whether the type of media used for CAR T-cell production affects the secretion of stimulatory cytokines upon antigen stimulation, the different CD19 CAR T-cell products were co-cultured with $CD19^+$ Daudi cells and the intracellular expression of IFNγ and TNF-α was determined using flow cytometry at 6 h following stimulation. IFNγ secretion depended significantly on the media which was used for CAR T-cell production (Fig. 2A; one-way ANOVA, P=0.0098). CAR T-cells cultured in CG released the highest levels of IFNγ in comparison to CAR T-cells cultured in RCF (Dunnett' test, P=0.0194). The CAR T-cells produced in TB exhibited the second highest release of IFNγ (Dunnett's test, P=0.0336). The TNF-α release of the four different groups exhibited no significant difference, whereas the percentage of CAR T-cells expressing IFNγ and TNF-α was significant (one-way ANOVA, P=0.0231). CG yielded the highest percentage of cells expressing IFNγ and TNF-α (Dunnett's test, P=0.0399). The separate analysis of $CD4^+$ and

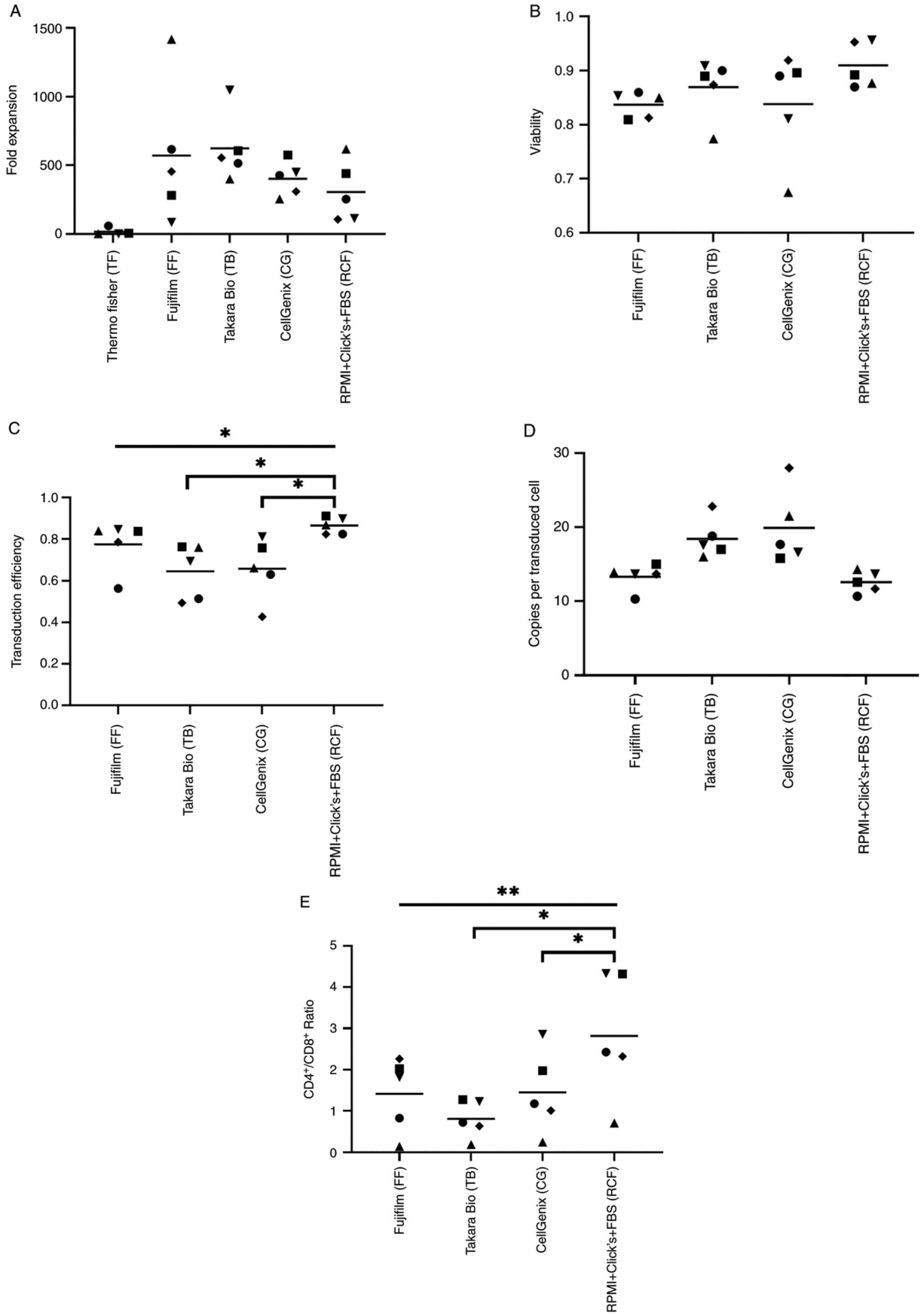

Figure 1. Expansion, viability, transduction rate, $CD4^+/CD8^+$ ratio and vector copy number of CD19.CAR T-cell culture. (A) Expansion on day 12 of T-cells cultured in five different media. (B) Viability of T-cells cultured in four different media assessed on day 12. (C) Transduction efficiency of CD19.CAR T-cell culture on day 12 of expansion in four different media. (D) Vector copy numbers of CD19.CAR T-cells cultured in four different media are shown. (E) $CD4^+/CD8^+$ ratio of 7AAD-CD19.CAR$^+$ T cells cultured in four different media. (A-E) Each symbol represents a separate donor (n=5). Short horizontal lines indicate the mean of data in each experimental group. Data were analyzed by one-way ANOVA (represented by a longer horizontal line above the data) with Dunnett's multiple comparison test (represented as brackets). $^*P<0.05$ and $^{**}P<0.01$. CAR, chimeric antigen receptor.

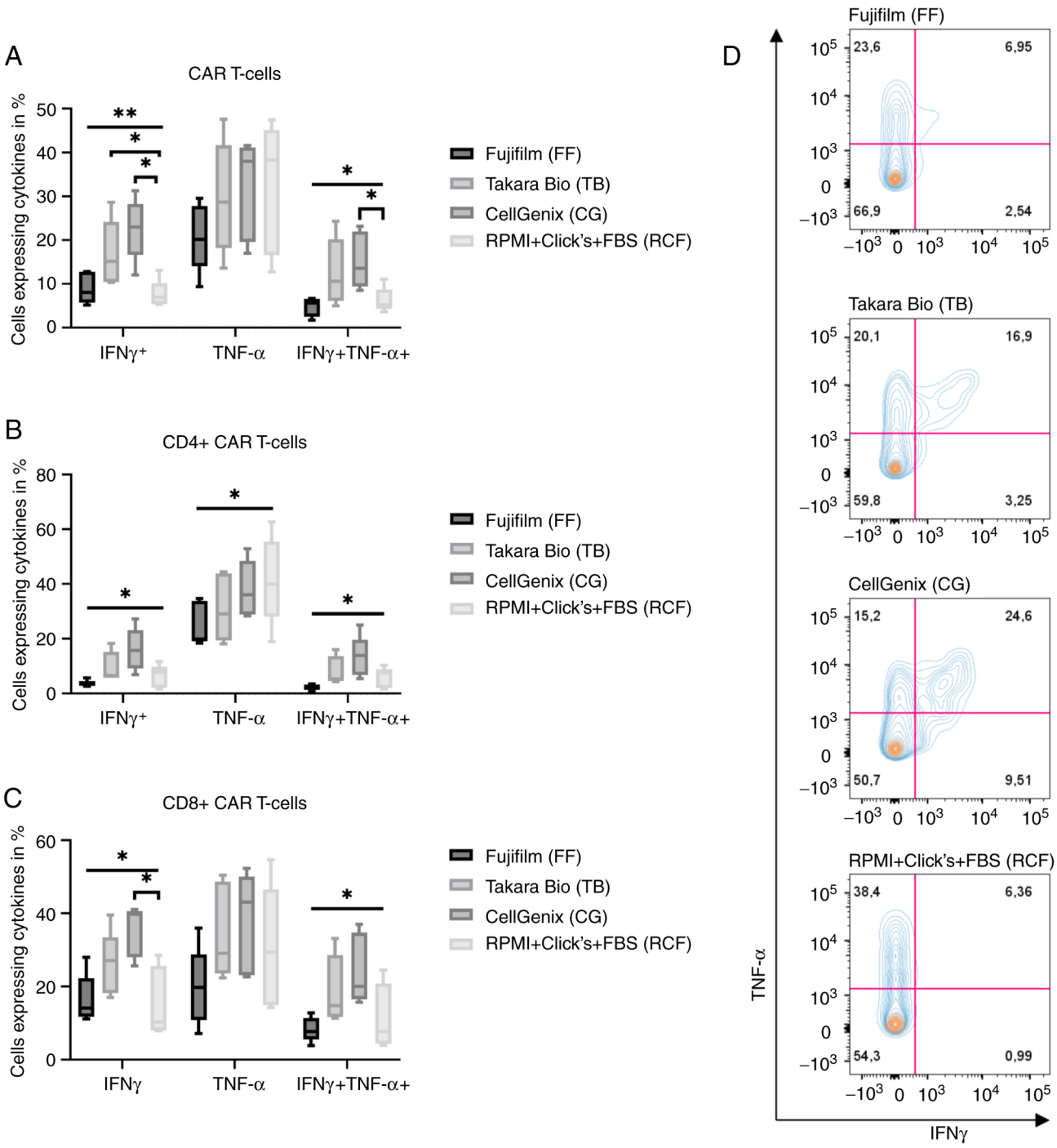

Figure 2. Intracellular staining of CD19.CAR T-cells cultured in four different media. (A-C) Cells expressing IFNγ, TNF-α or IFNγ and TNF-α are shown as a percentage of all CAR T-cells, CD4+ CAR T-cells or CD8+ CAR T-cells. Box and whisker plots represent median, upper and lower quartiles and minimum and maximum (n=5). The line indicates a comparison using one-way ANOVA, whereas the brackets indicate comparisons using Dunnett's test. *P<0.05 and **P<0.01. (D) Representative data of one healthy donor from five independent experiments are shown. CAR T-cells were assessed using anti-TNF-α and anti-IFNγ antibodies by flow cytometry. Plots are gated on live CAR T-cells. CAR T-cells stimulated with CD19+ tumor cells are shown in blue, whereas non-transduced T-cells are visualized as a control in orange. CAR, chimeric antigen receptor.

CD8+ CAR T-cells revealed a similar result. The IFNγ release of CD4+ CAR T-cells differed, depending on which medium was used for production (one-way ANOVA, P=0.0167; Fig. 2B). The highest percentage of IFNγ-expressing cells were the cells cultured in CG. The findings for IFNγ- and TNF-α-positive cells in CD4+ CAR T-cells were similar. The distribution of these cells depending on the medium was significant (one-way ANOVA, P=0.0173), and the CD4+ CAR T cells cultured in CG expressed the highest levels of IFNγ and TNF-α. Additionally, the expression of TNF-α in CD4+ CAR T-cells exhibited significant differences (one-way ANOVA, P=0.0248). The cells produced in RCF had the highest expression, whereas CD4+ CAR T-cells produced in FF had the lowest level. In addition, the distribution of IFNγ-releasing cells of CD8+ CAR T-cells was significant (one-way ANOVA, P=0.0264; Fig. 2C) and again, CAR T-cells produced in CG had the highest

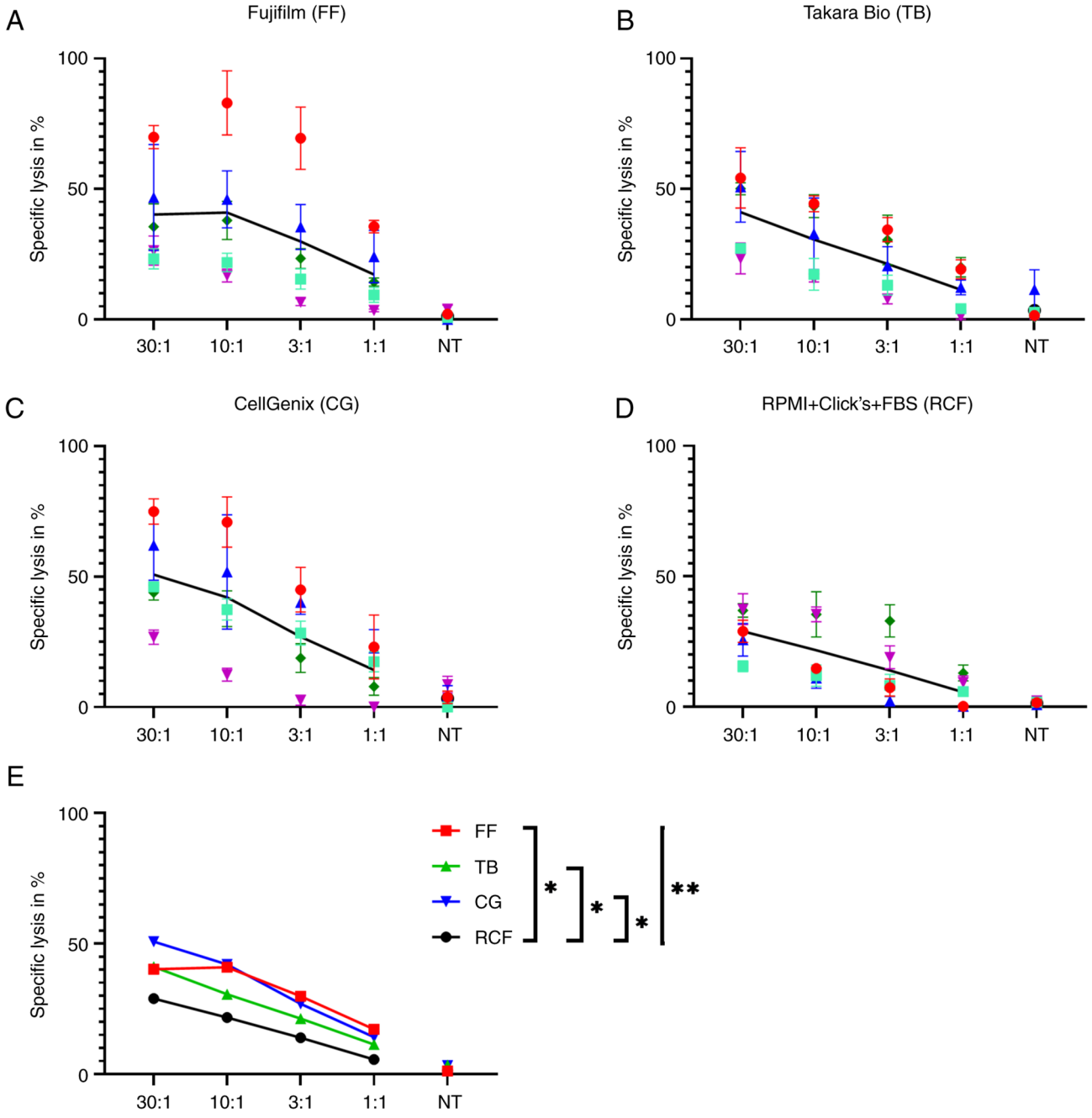

Figure 3. Chromium release assay. (A-D) Data of five unique donors of chromium release assay are shown. Each color/symbol represents a separate donor. Experiments were performed in triplicate and are represented as the mean ± SD. The mean value of all five donors is shown as a connecting line. The ratio of effector to target cells was 30:1, 10:1, 3:1 and 1:1 and non-transduced cells (NT) were used as a control. The results of chromium release assays for each respective medium are shown. (E) The mean value is shown as a connecting line for each medium separately. Data were analyzed using one-way ANOVA (represented by a longer vertical line) with Dunnett's multiple comparison test (represented as brackets). *P<0.05 and **P<0.01.

percentage of IFNγ-positive CD8+ CAR T-cells (Dunnett's test, P=0.0450). The difference in IFNγ- and TNF-α-positive cells was likewise significant (one-way ANOVA, P=0.0393). The representative flow cytometry patterns in each medium plotted for IFNγ and TNF-α and gated for CAR T-cells are presented in Fig. 2D.

Serum-free media increase the short-term cytotoxicity of third-generation CAR T-cells. The chromium release assay was performed to investigate the short-term cytotoxicity of the different CAR T-cell products, which can be reached within 4 h. The difference of the percentage of killed tumor cells in the chromium release assay was significant on average for all four effector-to-target-cell ratios (Fig. 3). The P-value in a one-way ANOVA test was P=0.0071. The CAR T-cells produced and cultured in CG exhibited the highest cytotoxicity (CG vs. RCF: Dunnett's test, P=0.0182). In addition, the CAR T-cells cultured in FF exhibited a high cytotoxicity (FF vs. RCF: Dunnett's test, P=0.0482) followed by the CAR T-cells cultured in TB (TB vs. RCF: Dunnett's test, P=0.0428). The CAR T-cells cultured in RCF exhibited the lowest cytotoxicity. Consequently, the lowest rate of

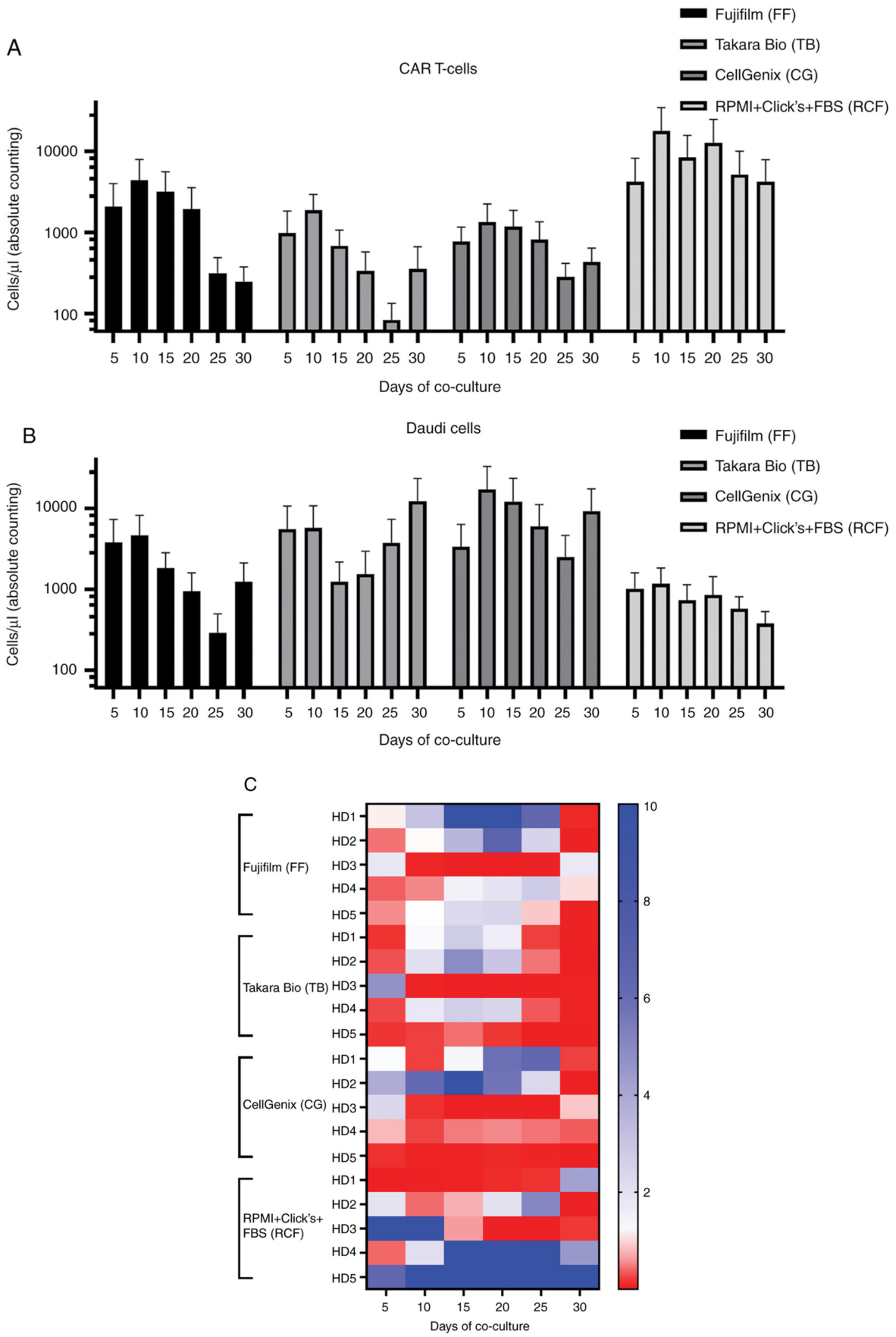

Figure 4. Cytotoxicity of CAR T-cells in a long-term co-culture. (A and B) Absolute cell counts of (A) CAR T-cells and (B) Daudi cells following co-culture in the corresponding medium are shown. Before adding new tumor cells to the CAR T-cells every 5 days, the co-culture was measured using flow cytometry. The mean ± SEM values are displayed. (C) The ratio of CAR T-cells per μl/Daudi cells per μl at different time points of co-culture are shown in the heatmap. Blue symbolizes a ratio of >1, whereas red symbolizes a ratio of <1. Therefore, blue indicates the overgrowth of CAR T-cells and the lysis of tumor cells, while red indicates an insufficient killing of tumor cells. Data are displayed separately for every donor. CAR, chimeric antigen receptor.

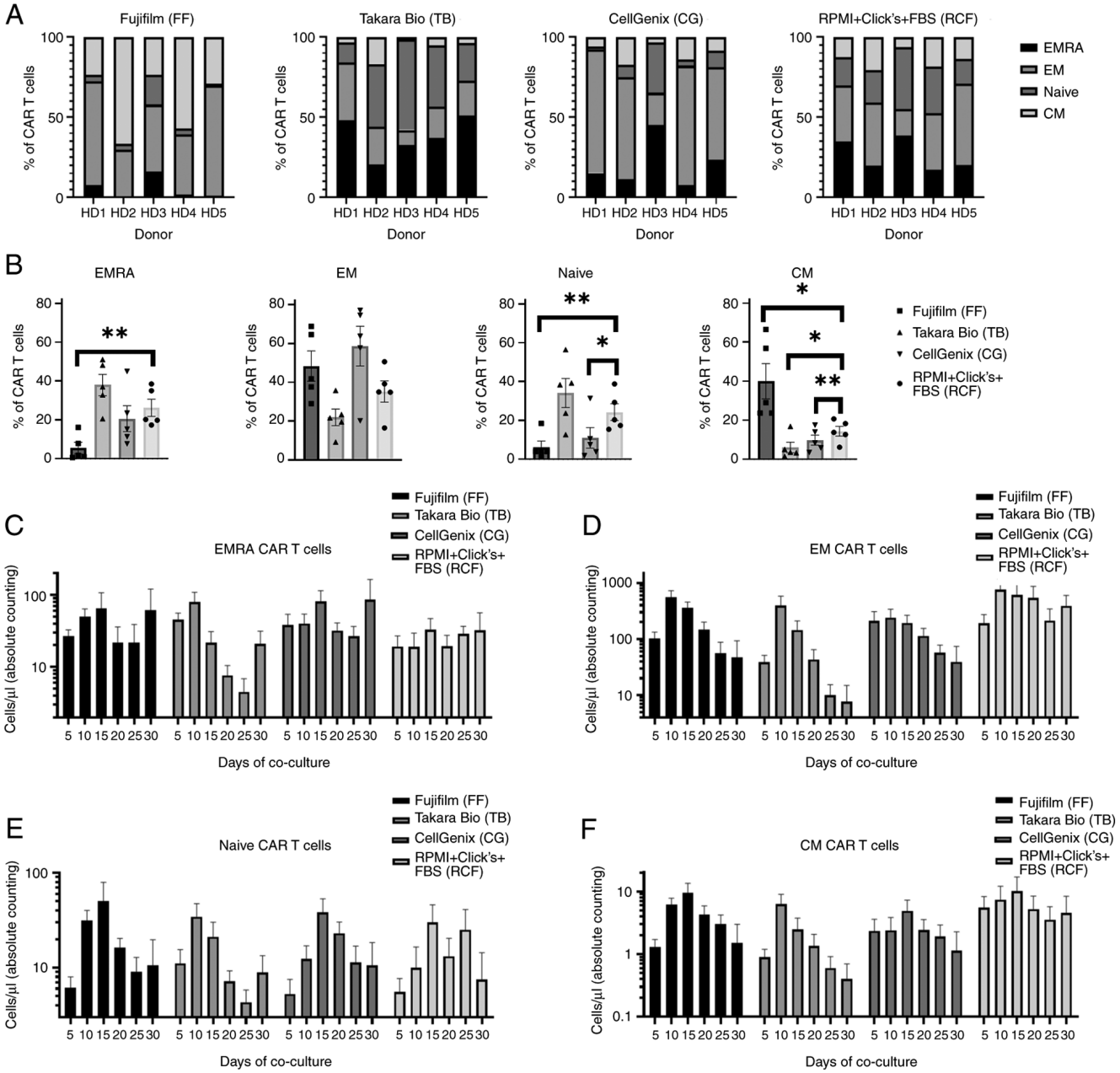

Figure 5. Phenotyping of CAR T-cells. (A) Phenotyping of CAR T-cells on day 12 of production was assessed using flow cytometry. Each donor is visualized separately. (B) Phenotyping of CAR T-cells on day 12 visualized separately for EMRA, EM, naïve and CM CAR T-cells. Data were analyzed using one-way ANOVA of all five donors followed by Dunnett's multiple comparisons test (represented as brackets). *P<0.05 and **P<0.01. Boxes show the mean and error bars indicate SEM. (C-F) Phenotyping of CAR T-cells co-cultured with tumor cells. Absolute cell count of CAR T-cells divided to the four different CAR T-cell types, EMRA, EM, naïve and CM. Boxes show the mean and error bars indicate SEM. CAR, chimeric antigen receptor; EMRA, effector memory cells re-expressing RA; EM, effector memory cells; naïve, naïve cells; CM, central memory cells.

killed tumor cells was observed with CAR T cells generated with FBS.

Serum-containing media improve the long-term functionality of CAR T-cells. Long-term functionality was tested by the co-culture of tumor cells and CAR T-cells. CAR T-cells were cultured in the corresponding medium which was also used for expansion and on days 0, 5, 10, 15, 20 and 25 of co-culture, the tumor cells were added. Assessment using flow cytometry was effective on days 5, 10, 15, 20, 25 and 30 prior to the addition of new tumor cells. The cells cultured in RCF exhibited the highest expansion of CAR T-cells (Fig. 4A) and these CAR T-cells were most efficient in eliminating tumor cells (Fig. 4B) compared to all other CAR T-cell products. The cells cultured in FF exhibited the second-best result. The absolute cell counts of cells cultured in TB and CG were comparable.

Another interesting aspect was the ratio of CAR T-cells to Daudi cells for every single co-culture. These data are presented in Fig. 4C. The quotient was highest for cells cultured in RCF followed by cells cultured in FF. Additionally, the donor-to-donor variability can be observed in Fig. 4C. Therefore, the different groups of co-culture were not compared statistically and a descriptive analysis was performed. Consequently, RCF improves the long-term functionality of CAR T-cells.

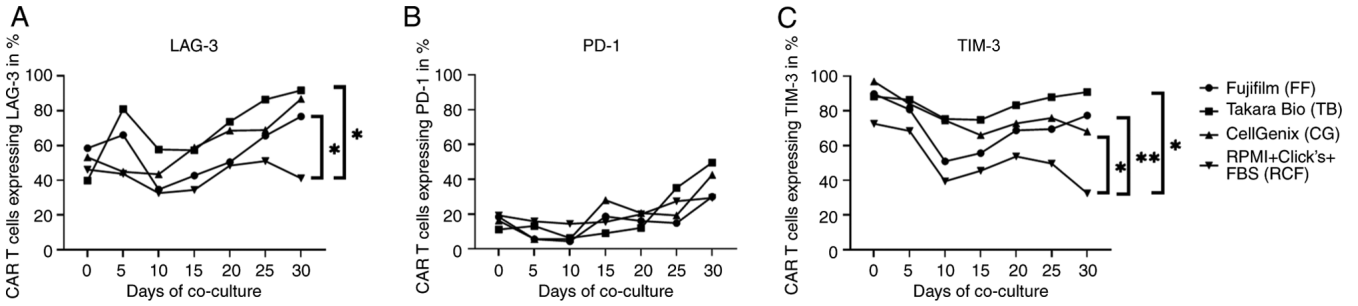

Figure 6. Exhaustion markers of CAR T-cells. The levels of exhaustion markers (A) LAG-3, (B) PD-1 and (C) TIM-3 in CAR T-cells co-cultured with tumor cells are shown. Graphs show the mean values. Data were analyzed using a two-way ANOVA followed by Dunnett's multiple comparison test. P-values of Dunnett's multiple comparison test at day 30 are represented as brackets. *P<0.05 and **P<0.01. CAR, chimeric antigen receptor; LAG-3, lymphocyte-activation gene 3; PD-1, programmed cell death protein 1; TIM-3, T-cell immunoglobulin and mucin-domain containing-3.

CAR T-cells cultured in serum-free media exhibit a unique phenotype. The first phenotyping of CAR T-cells was completed using flow cytometry on day 12 of CAR T-cell production (Fig. 5A and B). On day 12, the CAR T-cells produced and cultured in FF exhibited a high percentage of central memory CAR T-cells in comparison to the CAR T-cells cultured in RCF (one-way ANOVA, P=0.0087; Dunnett's test, FF vs. RCF, P=0.0470). The CAR T-cells cultured in TB and CG exhibited a lower percentage of central memory than CAR T-cells cultured in RCF (one-way ANOVA, P=0.0087; Dunnett's test, TB vs. RCF: P=0.0210 and CG vs. RCF: P=0.0092, respectively). The distribution of naïve CAR T-cells did not differ significantly between the CAR T-cells cultured in TB and RCF; however, the CAR T-cells cultured in CG and FF exhibited a significantly lower percentage of naïve CAR T-cells (one-way ANOVA, P=0.0014; Dunnett's test, CG vs. RCF: P=0.0468 and FF vs. RCF: P=0.0029, respectively). The percentage of effector memory CAR T-cells cultured in FF, TB and CG did not differ significantly in comparison to that of the CAR T-cells cultured in RCF (one-way ANOVA, P=0.0172; Dunnett's test, not significant). Only the CAR T-cells cultured in FF exhibited a significantly lower percentage of terminally differentiated effector memory cells (EMRA) in comparison to the CAR T-cells cultured in RCF (one-way ANOVA, P=0.0098; Dunnett's test, FF vs. RCF, P=0.0010). Of note is also the balanced distribution of all four CAR T-cell subtypes when cultured in RCF.

The phenotypic characteristics of the CAR T-cell products were also determined upon repeated antigen stimulation during the co-culture assay. The assessment of phenotypes was repetitively performed for the entire co-culture (Fig. 5C-F). The CAR T-cells cultured in FF and RCF exhibited the highest percentage of undifferentiated cells; i.e., naïve and central memory cells. The CAR T-cells cultured in RCF had the highest percentage of effector memory cells, whereas the CAR T-cells cultured in CG and FF had the highest cell count of EMRA CAR T-cells.

Serum-containing medium reduces the exhaustion of third-generation CAR T-cells during long-term co-culture. The expression of surface markers associated with T-cell exhaustion, such as lymphocyte-activation gene 3 (LAG-3), programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) were evaluated using flow cytometry on day 12 of CAR T-cell production, which corresponds to day 0 of co-culture and every 5 days after each antigen stimulation thereafter (Fig. 6). The baseline values prior to the start of the co-culture were similar for all different CAR T-cell products. In general, a trend towards an increase in the percentage of LAG-3-, PD-1- and TIM-3-expressing T-cells was observed for all CAR T-cell products with repetitive antigen stimulation. However, the CAR T-cells generated in RCF exhibited a significantly lower expression of LAG-3 and TIM-3 at day 30 than the CAR T-cells cultured in FF, TB and CG (LAG-3: Dunnett's test, P=0.0205, P=0.0306 and not significant, respectively; TIM-3: Dunnett's test, P=0.0034, P=0.0159 and P=0.0294, respectively). By contrast, we didn't find any difference in the level of PD-1 expression between the different groups.

Discussion

It was recently demonstrated that CAR T-cell therapy is a promising approach which can be used to treat patients with refractory and relapsed hematological malignancies (1-5). Therefore, an increasing number of pharmaceutical companies and academic institutions entered the GMP manufacturing process of CAR T-cells. FBS and HS are widely used, leading to the intensive testing of the different batches to reduce lot-to-lot inconsistency. To overcome this time- and cost-consuming test procedure, serum-free media may represent a potential solution.

Serum-free media often include pharmaceutical-grade and/or recombinant human proteins. Pharmaceutical-grade human proteins are obtained from pooled human plasma (24). The more donors are included, the higher is the lot-to-lot consistency. However, differences between the different batches cannot completely be excluded. Furthermore, the purification process and several manufacturing steps lead to the structural alternations of human proteins. Olsen *et al* (25) reported an impaired binding capacity of pharmaceutical-grade HS albumin for drugs and named the stabilizers caprylic acid and N-acetyl-DL-tryptophan as probable reason. Park *et al* (26) examined the crystal structure of pharmaceutical-grade HS albumin, which had bound fatty acids and tryptophan; they concluded a reduced biorelevance of HS albumin.

By contrast, recombinant proteins are produced under well-defined conditions, although the purification process also

Table II. Characteristics and rating of different media.

Characteristic	Fujifilm (FF)	Takara (TB)	CellGenix (CG)	RPMI + Click's + FBS (RCF)
Expansion	3	4	2	1
Viability	1	3	2	4
Transduction efficiency	3	1	2	4
$CD4^+/CD8^+$ ratio	3	4	2	1
Vector copy number	3	2	1	4
Intracellular staining	1	3	4	2
Chromium release assay	3	2	4	1
Co-culture	3	2	1	4
Phenotype	4	3	1	2
Exhaustion	3	1	2	4

All tested media were subjected to a rating for the parameters listed in the left column: From 4 points for optimal performance to 1 point for the least favorable performance. For expansion, viability and transduction efficiency the highest values were considered as favorites. The $CD4^+/CD8^+$ ratio and the vector copy number were considered the better the lower the value was. The $INF\gamma^+TNF\text{-}\alpha^+$ CAR T-cells were rated for intracellular staining. The cytotoxicity in the chromium release assay and co-culture suited as further characteristic. The phenotyping was rated with the aid of the percentage of naïve and central memory CAR T-cells. To evaluate the exhaustion, the percentage of $LAG\text{-}3^+$ CAR T-cells was used, the lower the percentage the better. CAR, chimeric antigen receptor; LAG-3, lymphocyte-activation gene 3.

alters the protein structure. The structure of recombinant HS albumin was found to be of the same pharmaceutical grade as human serum albumin (27-29). Consequently, Prime-XV™ T Cell CDM, Fujifilm™ (FF) stands out due to the exclusive use of recombinant human serum proteins. FF will have the highest lot-to-lot consistency with the same structural quality. CTS™ OpTmizer™ Pro SFM, Thermofisher Gibco™ (TF), LymphoONE™ T-Cell Expansion Xeno-Free Medium, Takara Bio™ (TB) and TCM GMP-Prototype, CellGenix™ (CG) all include pharmaceutical-grade HS albumin, which is not completely chemically definable.

The present study observed a notably low expansion rate in TF. According to the manufacturer, this medium is better suited for workflows >15 days. Thus, this was excluded as the experimental workflow used herein was too short for the use of TF.

The transduction procedure and expansion of CAR T-cells needs to be further improved with respect to media formulations to achieve enhanced antitumor activity (18). Herein, no significant difference was observed between the CAR T-cell cultures with four different culture media regarding expansion, viability and vector copy numbers. However, the transduction rate was lower in CAR T-cell cultures with TB and CG, thus demonstrating that FF is better suited for the transduction process than TB and CG.

In vitro potency assays reflect the biological activity of CAR T-cell products and have to be performed for the quality control of GMP production (30,31). They are currently used to evaluate the functionality of CAR T-cell products, even though it is not clear whether this is predictive of the *in vivo* antitumor activity. In the present study, CAR T-cells cultured in CG exhibited the highest short-time cytotoxicity in the chromium release assay followed by FF and TB. These results suggest a superior biological activity, whereas CAR T-cells cultured in RCF had the worst *in vitro* potency. Moreover, CAR T-cells cultured with CG exhibited the highest cytokine release followed by CAR T-cells generated in TB. CAR T-cells cultured in FF and RCF had lower cytokine levels, suggesting lower activation. However, CAR T-cells with lower levels of cytokine release were found in previous studies with lower levels of clinical toxicity and with consistently good anti-lymphoma activity (32). Therefore, CAR T-cells generated in FF appear to be advantageous, when compared to other serum-free media due to the high potency observed in the chromium release assay and low potential side-effects through lower cytokine levels. However, it should be mentioned that CAR T-cells cultured in RCF exhibited a better long-term functionality in the co-culture assay. This assay was performed with the corresponding medium which was also used for the production and expansion of CAR T-cells. This fact probably influences the outcome of the single co-cultures and highlights the importance of media providing nutrients. Nevertheless, *in vivo*, all CAR T-cell products would face the same serum-containing conditions. Therefore, the quality of the starting product is considered of particular importance.

Furthermore, previous studies have suggested an association between the frequency of CAR T-cells with a less differentiated phenotype and long-term remission rates due to enhanced persistence (33,34). Subsequently, CAR T-cell effector function and therapeutic potential is increased (35). Blaeschke *et al* (36) produced CAR T-cells with central memory and stem cell memory subtype in an automated system, and reported high expansion rates, specific cytotoxicity and cytokine expression. Apparently, CAR T-cell cultures with mainly undifferentiated CAR T-cell subtypes are not restricted as regards expansion and potency (34). In the present study, CAR T-cells cultured in TB and particularly, FF exhibited a high frequency of central memory and naïve CAR T-cells on day 12 of expansion. It can be concluded that CAR T-cells cultured in these media have a favorable phenotype,

leading to long-term antitumor activity. However, CAR T-cells cultured with FBS exhibited a superior long-term cytotoxicity when co-cultured with $CD19^+$ target cells. Additionally, CAR T-cells cultured in RCF exhibited the lowest increase of exhaustion markers. The expression of the surface markers, LAG3, PD-1 and TIM-3, is associated with the exhaustion and senescence of T-cells (37). It can thus be concluded that the use of serum-containing media during co-culture reduces the level of exhaustion of CAR T-cells. However, *in vivo*, all CAR T-cell products would experience the same conditions during repetitive antigen stimulation. Nevertheless, a comparable quality of RCF and the serum-free medium FF can be observed when considering all aspects together (Table II).

Another critical aspect which should be considered, is the wide range of costs of different media. The authors calculated a price of ~440€/l for all components of the RCF medium used herein. In comparison, TF, CG and TB are considerably more affordable (~153€/l, ~240€/l and ~255€/l). FF is the only serum-free medium which is more costly than the FBS-containing medium, with ~636€/l. Consequently, the high costs of FF and RCF are an economic disadvantage.

The use of serum, particularly FBS, is ethically and ecologically controversial. For FBS manufacturing, >1,000,000 bovine fetuses are harvested annually (38). Hence, carbon dioxide emissions should not be underestimated. Moreover, the harvest process is ethically questionable. The bovine fetal blood is collected by a cardiac puncture of the unanesthetized fetus (38).

Due to practical reasons for GMP standardization and ethical and ecological concerns, it would be desirable to replace current CAR T-cell culture media with serum-free media. However, it should be mentioned that all serum-free media provide different culture conditions with specific characteristics. Alnabhan *et al* (17) observed a reduced viability and expansion, a lower CD4/CD8 ratio and a higher frequency of differentiated cells in CAR T-cell culture. By contrast, Xu *et al* (39) reported an improved proliferation environment in serum-free media for T-cells. Thus, each serum-free medium needs to be regarded individually (Table II). The varying media formulations (Table I) lead to this diversity.

Prime-XV™ T Cell CDM, Fujifilm™ (FF) exhibited some advantages compared to TB and CG, as for example, the freedom of pharmaceutical-grade human proteins, a high cytotoxicity, a lower cytokine release and an undifferentiated phenotype. Based on these observations, it would be reasonable to implement FF in GMP CAR T-cell production.

In conclusion, the use of serum-free media can be recommended for the GMP manufacturing of CAR T-cells. Serum-free media do not lead to a loss of functionality or a slower expansion of CAR T-cells. Serum-free media are produced under very robust conditions. Given the unique characteristics of each serum-free medium, it needs to be selected for each individual purpose. Prime-XV™ T Cell CDM, Fujifilm™ (FF) proved advantageous in the manufacturing process used herein compared to the two other serum-free media.

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Availability of data and materials

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

Authors' contributions

FE, AHK, MS, AS, CMT and AKe were involved in the conceptualization of the study. FE, GJ, BN, TS and AKu were involved in the study methodology. FE and GJ performed the analysis of data. FE was involved in the formal analysis and in the investigative aspects of the study, as well as in the writing and preparation of the original draft. CMT, AS and MS were involved in the provision of financial resources and laboratory facilities. FE, AR, KB and AKu were involved in data curation. TS, AKe, AHK, BN, KB, AS and MS were involved in the writing, reviewing and editing of the manuscript. FE and GJ were involved in data visualization and creating graphs. AK and MS supervised the study. FE and AHK were involved in project administration. AKe and FE confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Medical Faculty of the University of Heidelberg (reference no. S-254/2016). Consent to participate was obtained in a written manner.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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