Journal of Immunological Methods 528 (2024) 113667

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Magnetic CAR T cell purification using an anti-G4S linker antibody

Dennis Christoph Harrer [a,b,*], Sin-Syue Li [b,c], Marcell Kaljanac [b], Valerie Bezler [b], Markus Barden [b], Hong Pan [b], Wolfgang Herr [a], Hinrich Abken [b]

[a] Department of Internal Medicine III - Hematology and Medical Oncology, University Hospital Regensburg, Regensburg, Germany
[b] Leibniz Institute for Immunotherapy, Div. Genetic Immunotherapy, Regensburg, and Chair Genetic Immunotherapy, University Regensburg, Germany
[c] Division of Hematology, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

ARTICLE INFO

Keywords:
CAR
Magnetic separation
Isolation
Purification
MACS

ABSTRACT

Chimeric antigen receptor (CAR) redirected T cells are successfully employed in the combat against several hematological malignancies, however, are often compromised by low transduction rates making refinement of the CAR T cell products necessary. Here, we report a broadly applicable enrichment protocol relying on marking CAR T cells with an anti-glycine$_4$-serine (G4S) linker antibody followed by magnetic activated cell sorting (MACS). The protocol is broadly applicable since the G4S peptide is an integral part of the vast majority of CARs as it links the VH and VL recognition domains. We demonstrate the feasibility by using the canonical second generation CARs specific for CEA and Her2, respectively, obtaining highly purified CAR T cell products in a one-step procedure without impairing cell viability. The protocol is also applicable to a dual specific CAR (tandem CAR). Except for CD39, T cell activation/exhaustion markers were not upregulated after separation. Purified CAR T cells retained their functionality with respect to antigen-specific cytokine secretion, cytotoxicity, and the capacity to proliferate and eliminate cognate tumor cells upon repetitive stimulation. Collectively, the one-step protocol for purifying CAR T cells extends the toolbox for preclinical research and specifically for clinical CAR T cell manufacturing.

1. Introduction

Over the last decade, chimeric antigen receptor (CAR) T cells grew into a vital asset of cancer immunotherapy with an expanding field of applications (June, 2023; June and Sadelain, 2018). Predicated on durable complete remissions in patients with various B-cell derived malignancies, FDA and EMA approval was granted for the treatment of patients with defined refractory hematological malignancies including aggressive B cell lymphoma, multiple myelomas, and acute lymphoblastic leukemia (Abken, 2021; Holzinger and Abken, 2022). CAR T cell therapy attained remarkable success in the battle against these diseases; however, the overwhelming majority of patients with solid tumors yet did not benefit from CAR T cell infusions while preclinical research directed at generating novel CAR T cell products is raising hope for a therapeutic breakthrough in the near future (Ruella et al., 2023). A major obstacle for both pre-clinical research and clinical application is the broad variance in CAR T cell numbers in the final cell product making comparison of data very difficult. Hence, a standardized CAR T cell purity would make pre-clinical and specifically clinical studies easier to compare and hopefully more efficient.

Usually, T cells are equipped with CARs by viral or non-viral gene transfer technologies yielding a broad gamut of transduction rates between individual T cell donors (Harrer et al., 2023). For some preclinical and clinical applications, an enrichment of CAR T cells to a level of $\geq$90% CAR$^+$ cells is beneficial. As for those CARs that harbor a CH2-CH3 hinge domain derived from the IgG1 antibody as extracellular spacer, we previously introduced a purification protocol based on labeling CAR T cells with a biotinylated goat F(ab)2 anti-IgG antibody followed by magnetic activated cell sorting (MACS) (Harrer et al., 2022). However, as the majority of CAR products do not possess an IgG1 CH2-CH3 hinge there is a need for alternative purification methods (Fujiwara et al., 2020). Recently, addition of the N3 and N4 domains derived from the low-affinity p75 chain of the human nerve growth factor receptor (NGFR) to the CAR architecture was reported to allow efficient enrichment of CAR T cells (Bister et al., 2022). Purified CAR T cells equipped with those peptide domains displayed robust functionality against

* Corresponding author at: Department of Internal Medicine III - Hematology and Medical Oncology, University Hospital Regensburg, Regensburg, Germany.
E-mail address: dennis.harrer@ukr.de (D.C. Harrer).

https://doi.org/10.1016/j.jim.2024.113667
Received 9 January 2024; Received in revised form 30 March 2024; Accepted 1 April 2024
Available online 2 April 2024

hematological blasts. By a similar approach, Biester et al. incorporated a truncated version of human CD34 into the hinge domain of a conventional CD19-specific CAR (Bister et al., 2021). Via binding of the QBend-10 monoclonal antibody to the CD34 domain and subsequent magnetic cell sorting, CD19-specific CAR T cells were enriched to >95% purity (Bister et al., 2021). CAR T cells with CD34-modified hinges did not suffer from functional impairments and did not mediate off-target activity (Bister et al., 2021). Alternatively, indirect purification strategies for antigen-specific T cells based on targeting a co-expressed marker, such as truncated, non-signaling NGFR or EGFR, were proposed (Dragon et al., 2020; Griffioen et al., 2008). However, co-expression of an additional protein along with the CAR will lead to an increased vector size which is commonly associated with inferior transduction efficiencies (Sweeney and Vink, 2021).

Here we established a CAR T cell purification protocol that is applicable to a broad variety of CARs, independently of their specificity, spacer and signaling domains, and does not need further modifications to the hinge domain or co-expression of additional markers. We took advantage that the vast majority of CARs utilize a single chain fragment of variable region (scFv) antibody as targeting domain. The scFv is composed of the variable domains of the antibody heavy chain (VH) and light chain (VL) that are connected by a short flexible linker peptide in the VH-VL or VL-VH orientation (Zhang et al., 2023). Thus far, the four-time iteration of four glycines and one serine ($glycine_4$-serine, G4S) and the whitlow/218 (GSTSGSGKPGSGEGSTKG) peptide emerged as the most common linking peptides for scFvs (Mazinani and Rahbarizadeh, 2022). The G4S linker is commonly used in CARs targeting solid tumor antigens, such as HER2 and CEA (Mazinani and Rahbarizadeh, 2022), while the whitlow/218 linker peptide is predominantly used in CAR constructs targeting hematological malignancies, e.g., in CD19 CAR products (Kochenderfer et al., 2012). Based on the frequently used G4S linker, we developed a novel CAR T cell isolation approach relying on marking CAR T cells with a PE-labeled anti-G4S linker antibody followed by purification by magnetic activated cell sorting. The approach allows for high-grade CAR T cell enrichment in a one-step procedure without impairing viability or functionality.

2. Materials and methods

2.1. Cells and reagents

Peripheral blood mononuclear cells (PBMCs) were procured by Lymphoprep centrifugation (Axis-Shield, Oslo, Norway) of blood obtained from healthy donors upon informed consent and approval by the institutional review board. Isolated PBMCs were frozen and stored at −80 °C until experimental use. T cells were cultured in RPMI 1640 medium, 1% (*w/v*) GlutaMAX (Gibco, ThermoFisher, Waltham, MA, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin (Pan-Biotech, Aidenbach, Germany), 2 mM HEPES (PAA, Palo Alto, CA, USA), and 10% (*v*/v) heat-inactivated fetal calf serum (Pan-Biotech, Aidenbach, Germany). 293 T cells (ATCC CRL-3216, American Type Culture Collection ATCC, Manassas, VA) and human pancreatic cancer cells BxPC-3 (ATCC CRL-1420) were used as target cells. Tumor cells were maintained in DMEM, 1% (w/v) GlutaMAX (Gibco, ThermoFisher), 100 IU/ml penicillin, 100 µg/ml streptomycin (Pan-Biotech), and 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, USA).

2.2. CAR T cell generation

Frozen PBMCs were defrosted and activated on the same day with the anti-CD3 monoclonal antibody (mAb) OKT-3, the CD28 mAb 15E8 and IL-2 (1000 IU/ml). Recombinant IL-2 (200 IU/ml) was supplemented on days 2, 3, and 4 after activation. Retroviral transduction was performed as previously described in detail (Golumba-Nagy et al., 2017). Viral particles were added on day +2 and day +3 after PBMC stimulation. On day four of activation (day +4), CAR T cells were stained for CAR expression and subjected to CAR T cell purification by magnetic CAR T cell separation. Untransduced cells were generated by activation of PBMCs and subsequent expansion in the presence of IL-2, but without retroviral transduction. The expression cassettes encoding the CEA-specific CAR (Hombach et al., 2001), the anti-CD30/CEA CAR, (Hombach et al., 2019) and the HER-2 specific CAR (Chmielewski et al., 2004b) were previously reported.

2.3. Magnetic CAR T cell separation

Four days after activation, three million T cells were resuspended in 50 µl MACS buffer (PBS supplemented with 1% (*v*/v) heat-inactivated fetal calf serum) and labeled with 1.5 µl of PE-labeled anti-G4S linker antibody (clone E7O2V, Cell Signaling Technology, Danvers, MA, USA). Following five minutes incubation at 4 °C, cells were washed twice with 2 ml of MACS buffer, resuspended in 80 µl of MACS buffer and labeled with 20 µl of anti-PE MicroBeads (Miltenyi, Bergisch-Gladbach, Germany) for 15 min at 4 °C. Then, cells were washed one with 2 ml MACS buffer, resuspended in 500 µl of MACS buffer, and loaded onto a MS MACS column (Miltenyi Biotec), which had been equipped with a pre-separation filter and placed into a magnetic holder. Afterwards, the filter was washed once with 500 µl MACS buffer. The filter was discarded, and the MS column was washed three times with 500 µl MACS buffer. Next, the MS column was removed from the magnet and cells were eluted twice by adding 1 ml MACS buffer and pressing cells through the column using a plunger. Finally, cells were resuspended in medium for further use.

2.4. Flow cytometry

Cells were incubated with antibodies at 4 °C for 15 min for surface staining. The viability dye eFluor 780 (ThermoFisher, Waltham, MA, USA) was employed for live/dead discrimination. Fluorescent-minus-one (FMO) controls were used for gating. CAR expression was detected using the PE-labeled anti-G4S linker antibody acquired from Cell Signaling Technology. For some experiments as indicated in the figure legends, the goat F(ab')2 anti-human IgG-PE antibody and the goat F(ab')2 anti-human IgG-FITC antibody were purchased from SouthernBiotech to detect the CAR. The FITC-conjugated anti-CD3 (clone BW 264/56) and PE-conjugated anti-CD25 (clone 4E3) were obtained from Miltenyi Biotec. The following antibodies were purchased from Biolegend: PE-conjugated anti-CD39 (clone A1), PerCP-Cy5.5-conjugated anti-LAG3 (clone 11C3C65), and PerCP-Cy5.5-conjugated anti-TIGIT (clone A15153G). The following antibodies were purchased from BD Biosciences: BV421-conjugated anti-TIM3 (clone 7D3) and BV421-conjugated anti-PD-1 (clone EH12.1). Immunofluorescence was measured using a BD FACSLyric cytometer equipped with the FACSuite software (BD Biosciences). Data were analyzed using the FlowJo software version 10.7.1 Express 5 (BD Biosciences).

2.5. Cytokine secretion

Target cells were maintained in 96 well round-bottom plates (1×10^5 cells/well) overnight, before adding untransduced T cells or CAR T cells (1×10^5 cells/well). Following 48 h of co-culture, IL-2 and IFN-γ in culture supernatants were determined by ELISA as previously reported (Hombach et al., 2019).

2.6. Cytotoxicity assay

Untransduced T cells or CAR T cells (0.125–10 $\times$ 10^4 cells/well) were co-cultivated for 48 h in 96 well round bottom plates together with target cells (each 1×10^4 cells/well) at the indicated effector to target ratios. The XTT-based colorimetric assay employing the "Cell Proliferation Kit II" (Roche Diagnostics, Mannheim, Germany) was used to analyze specific cytotoxicity. Viability of tumor cells was calculated as

mean values of six wells containing only tumor cells subtracted by the mean background level of wells containing medium only. The percentage of viable tumor cells in experimental wells was determined as follows: viability (%) = [OD(experimental wells - corresponding number of T cells)]/[OD(tumor cells without T cells - medium)] × 100. Cytotoxicity (%) was defined as 100 - viability (%).

2.7. Repetitive stimulation assay

GFP-labeled BxPC-3 cells were seeded in 12 well plates (0.1×10^6 cells per well). After 24 h, untransduced T cells (0.1×10^6 T cells per well) or CAR T cells (0.1×10^6 CAR T cells per well) were added. Three days later (Round 1, R1), T cells and tumor cells were harvested, cells were washed with PBS and resuspended in 1 ml medium. Then, an aliquot of 100 µl was used for cell counting (live GFP^+ tumor cells and

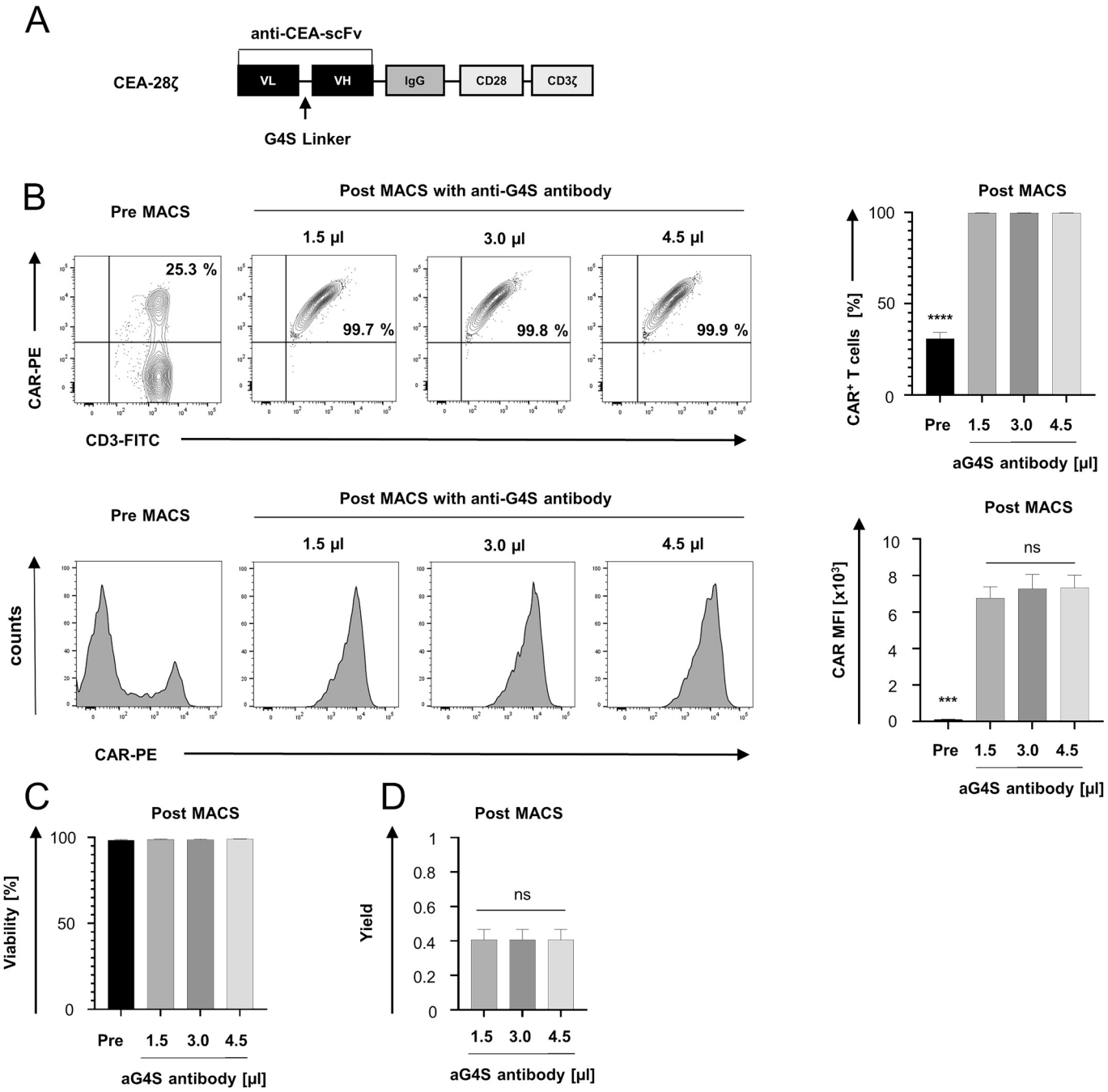

Fig. 1. Establishing of magnetic CAR T cell separation using the PE-labeled anti-G4S linker antibody. **(A)** Schematic outline of the CAR indicating the binding site of the anti-G4S linker antibody E7O2V. **(B)** CAR expression by T cells detected before magnetic cell separation (Pre MACS) and immediately after magnetic cell separation (Post MACS). Pre MACS CAR expression was detected by staining with the PE-labeled anti-G4S linker antibody. Magnetic CAR T cell separation was carried out as indicated in the Materials and Methods using the PE-labeled anti-G4S linker antibody. To avoid interference with blocked binding sites after previous labeling for the magnetic cell separation, Post MACS CAR expression was detected by staining with a PE-labeled goat F(ab')2 anti-human IgG antibody, which binds to the IgG1 CH2-CH3 hinge of the CAR. In the FACS panels, one representative donor out of three is shown. **(C)** Viability of CAR T cells before and after magnetic cell separation. **(D)** Average yield of CAR T cells after magnetic cell separation. All data represent means ± SEM of three donors, p values were calculated by Student's t-test, ns indicates not significant, *** $p \leq 0.001$, **** $p \leq 0.0001$.

live CD3$^+$ T cells) by flow cytometry using counting beads ("Count-Bright", ThermoFisher). The remaining 900 µl were added to a new 12 well plate with 0.1 × 10^6 BxPC-3 cells to start the second round (R2) of stimulation. After four days, the procedure was repeated for round 3 (R3).

2.8. Statistical analysis

Statistical analysis was carried out utilizing GraphPad Prism, Version 9 (GraphPad Software, San Diego, CA, USA). *P* values were determined by Student's *t*-test; ns indicates not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

3. Results

3.1. Magnetic CAR T cell separation using a PE-labeled anti-G4S linker antibody

Aiming to develop a purification protocol for CAR T cells without the need for additional modifications of the CAR expression vector, we exploited the G4S linker sequence present in the majority of published CAR constructs as a purification target for CAR T cells (Hombach et al., 2019; Ma et al., 2023; Mao et al., 2022). Recently, the PE-labeled monoclonal antibody E7O2V that specifically binds to the G4S linker peptide became commercially available. In order to establish an experimental protocol for magnetic CAR T cell separation using the PE-labeled anti-G4S linker antibody, we sorted T cells engineered with a well-characterized CAR with a CEA-specific scFv tethered together by a G4S linker (Fig. 1A). Retroviral gene transfer yielded a transduction rate of approximately 25% CAR+ T cells as measured by staining with the PE-labeled anti-G4S linker antibody (Fig. 1B). Next, we modified a previously published CAR T cell purification approach in that we stained with the PE-labeled anti-G4S linker antibody and subsequently sorted stained CAR T cells by magnetic cell separation with anti-PE microbeads. Testing of dilutions of the PE-labeled anti-G4S linker antibody revealed high-grade (≥ 99% CAR positive cells) enrichment of CAR T cells with no difference between the applied dilutions (Fig. 1B). Moreover, magnetic cell separation did not compromise viability of CAR T cells (Fig. 1C). The recovery of CAR T cells after magnetic cell separation amounted to roughly 40% indicating some loss of CAR T cells during the isolation process (Fig. 1D). Since higher concentrations of the PE-labeled anti-G4S linker antibody did not improve the yield, we used 1.5 µl of PE-labeled anti-G4S linker antibody for further experiments. Collectively, we demonstrated the feasibility of high-grade CAR T cell enrichment using a magnetic cell separation protocol and applying the PE-labeled anti-G4S linker antibody.

We validated the isolation protocol with two additional CAR constructs. First, we transduced T cells with a dual specific CAR containing two separate G4S linkers that connect the VH and VL chains of the CD30-specific scFv and of the CEA-specific scFv, respectively (Fig. 2A). The transduction rate of the CD30/CEA-specific CAR was similar to the CEA-specific CAR approximately 25% (Fig. 2B). Making use of magnetic CAR T cell separation using a PE-labeled anti-G4S linker antibody, the CD30/CEA-specific CAR could be purified to nearly 100% CAR$^+$ cells. Moreover, no drop in viability after magnetic separation was observed (Fig. 2C). Second, we used a HER2-specific CAR, also harboring a G4S linker, to further corroborate the feasibility of magnetic CAR T cell separation using the PE-labeled anti-G4S linker antibody (Fig. 2D).

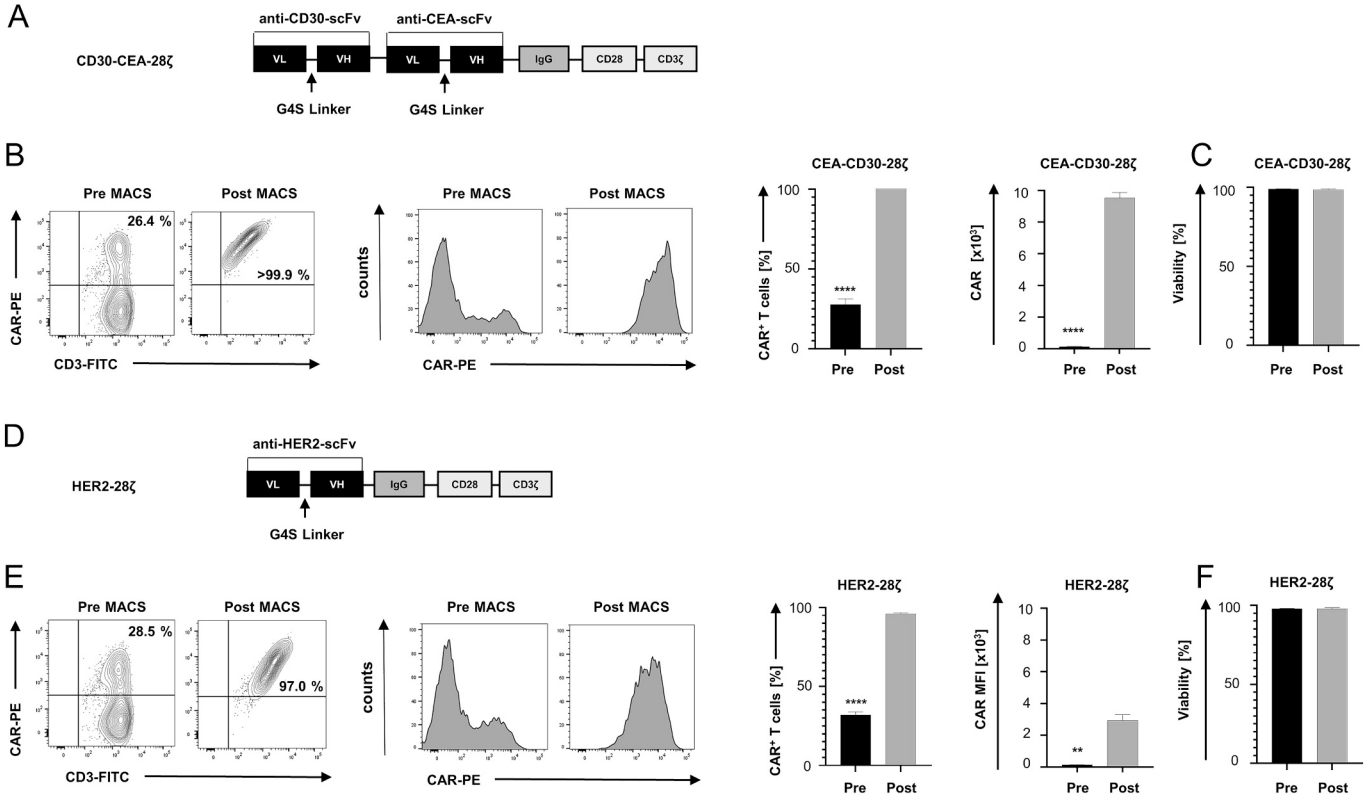

Fig. 2. Validation of magnetic CAR T cell separation using the PE-labeled anti-G4S linker antibody. **(A + D)** Schematic outline of the CAR design indicating the binding site of the anti-G4S linker antibody E7O2V. **(B + E)** CAR expression by T cells detected before magnetic cell separation (Pre MACS) and immediately after magnetic cell separation (Post MACS). Pre MACS CAR expression was detected by staining with the PE-labeled anti-G4S linker antibody. Magnetic CAR T cell separation was carried out using 1.5 µl of the PE-labeled anti-G4S linker antibody. Post MACS CAR expression was detected by staining with a PE-labeled goat F(ab')2 anti- IgG antibody, which binds to the hinge of the CAR. For FACS panels. One representative donor out of three is shown. Data represent means ± SEM of three donors, *p* values were calculated by Student's *t*-test, ns indicates not significant, **** $p \leq 0.0001$. **(C + F)** Viability of CAR T cells before and after magnetic cell separation. Data represent means ± SEM of three donors, p values were calculated by Student's t-test, ns indicates not significant, ** $p \leq 0.01$, **** p ≤ 0.0001.

While about 28% of T cells were transduced with the HER2-specific CAR (Fig. 2E), subsequent labeling with the PE-labeled anti-G4S linker antibody followed by magnetic cell separation yielded a pure population of 97% CAR$^+$ cells (Fig. 2E). Like the other CAR constructs, no impairment in viability was registered after magnetic separation (Fig. 2F). In aggregate, we corroborated the feasibility of the MACS based CAR T cell enrichment protocol with CD30/CEA tandem CAR T cells and with HER2-specific CAR T cells.

3.2. Upregulation of CD39 following magnetic CAR T cell separation

Binding of the anti-G4S antibody to the scFv of CAR T cells and subsequent magnetic cell separation can potentially result in CAR T cell activation through CAR cross-linking. To investigate the issue, we recorded the expression of various surface markers upregulated in response to T cell activation before and 24 h after magnetic cell separation. For this, we used CEA-specific CAR T cells as described in Fig. 1A. Whereas the expression of CD25, TIM3, PD-1, TIGIT, and LAG3 did not alter after magnetic cell separation, the expression of CD39 on CAR T cells was significantly upregulated (Fig. 3 and supplemental Fig. S1). While persistently elevated expression of the ectonucleotidase CD39 is implicated in the process of T cell exhaustion (Canale et al., 2018; Jiang et al., 2023), evidence is accumulating to see CD39 as a marker for T cell activation in non-exhausted, functionally active T cells (Chow et al., 2023). Noteworthy, apart from elevated CD39 expression, the tested markers related to T cell activation/exhaustion were not upregulated in response to magnetic CAR T cell separation.

3.3. CAR T cell purification does not impair CAR T cell functionality

To assay the functionality after G4S antibody mediated CAR T cell separation, we interrogated CAR-triggered upregulation of CD25 and cytokine secretion upon CAR specific antigen engagement. CAR T cells targeting CEA were enriched by magnetic CAR T cell separation and co-cultured with CEA$^-$ 293 T cells or CEA$^+$ BxPC-3 pancreatic cells.

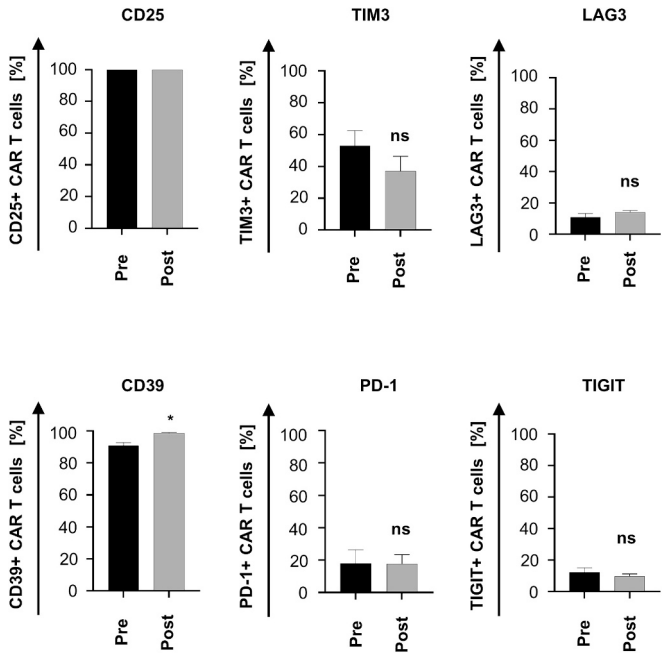

Fig. 3. Phenotypic analysis of CEA-specific CAR T cells before and 24 h after magnetic cell separation. CAR T cells were detected with a FITC-labeled goat F(ab')2 anti- IgG antibody and further characterized with regard to CD25, CD39, TIM3, PD-1, TIGIT, and LAG3 expression. Data represent means ± SEM of three donors, *p* values were calculated by Student's *t*-test, ns indicates not significant, * $p \leq 0.05$.

Untransduced T cells served as controls. After 48 h of co-culture with BxPC-3 tumor cells, an antigen-triggered upregulation of CD25 was observed on CAR T cells but not on untransduced T cells (Fig. 4A and supplemental Fig. S2). Co-culture T cells with CEA$^-$ 293 T cells did not induce CD25 upregulation. Moreover, in response to CEA$^+$ BxPC-3 tumor cells, an antigen-triggered secretion of IFN-γ and IL-2 was detected in the culture supernatant of CAR T cells but not of untransduced T cells (Fig. 4B). No background cytokine secretion towards 293 T cells was recorded. In addition, CEA-specific CAR T cells exhibited robust cytotoxicity across different effector to target ratios towards CEA$^+$ BxPC-3 tumor cells (Fig. 4C). No significant background cytotoxicity against CEA$^-$ 293 T cells was recorded. Untransduced T cell did not mediate tangible specific cytotoxicity towards BxPC-3 cells (Fig. 4C).

We aimed to assess the long-term functional performance of CAR T cells during repetitive antigen challenge. To this end, we stimulated unmodified T cells and CEA-specific CAR T cells with CEA$^+$ BxPC-3 cells for three consecutive rounds with each round lasting three to four days. While non-modified T cells did not show T cell expansion or elimination of tumor cells, CEA-specific CAR T cells evinced T cell proliferation and robust tumor cell killing (Fig. 4D). Collectively, magnetic CAR T cell separation did not impair CAR T cell functionality with regard to cytokine release and redirected cytotoxicity, both key functions in a productive anti-tumor response.

4. Discussion

Isolation of CAR T cells by magnetic cell separation to high purity without cellular activation remains challenging. The current state of technologies requires modifications of the CAR hinge region (Bister et al., 2022; Bister et al., 2021) or co-expression of an inert targetable marker. Our goal was to develop an enrichment protocol for a broad variety of CAR T cells without additional modification of the existing constructs. Therefore, we utilized the very recently commercially available antibody E7O2V with binding specificity for the G4S linker, which is frequently used as part of the scFv in CARs. The antibody was used to label CAR T cells for subsequent purification by magnetic activated cell sorting. Our approach fostered a fast one-step CAR T cell enrichment to high purity (>99%), high cell viability, and applicability to different CAR T cells including mono- and bispecific CARs. Extensive functional testing after isolation did not reveal any impairment regarding CAR T cell functionality. By these properties, the described CAR T cell isolation approach represents a so far not available addition to the toolbox of adoptive cell therapy for pre-clinical and clinical applications.

The G4S linker, that serves as a target in the marking and isolation protocol, is an essential moiety in a wide variety of CAR constructs, predominantly in those targeting antigens of solid tumors. The CEA-specific CAR employed to establish the isolation protocol harbors a CEA-specific scFv, termed BW431/26, to target CEA-expressing cancer cells as documented in a plethora of preclinical studies (Chmielewski et al., 2004b; Chmielewski and Abken, 2017; Hombach et al., 2001). Of note, several other published CEA-specific CAR constructs also harbor the G4S linker in their scFv (Kronig et al., 2023). As an additional example, we demonstrated successful purification of T cells expressind the trastuzumab-derived HER-2 specific 4D5 scFv CAR (Chmielewski et al., 2004a). A substantial portion of other Her-2 specific CAR constructs also harbor the G4S linker in their scFv (Xu et al., 2021) rendering the respective CAR T cells also amenable to the isolation protocol. As further examples, G4S linkers are used in CAR constructs specific for GD2 (Zimmermann et al., 2020), mesothelin (Uslu et al., 2023), and CD30 (Hombach et al., 1999). We assume the protocol is generally applicable to the broad panel of CARs with a G4S linker in their scFv. We further confirmed the feasibility the purification protocol with a more complex CAR design, the CD30/CEA bi-specific CAR. This CAR construct is currently being prepared for clinical evaluation (Hombach et al., 2019). In contrast to these examples, the FDA-

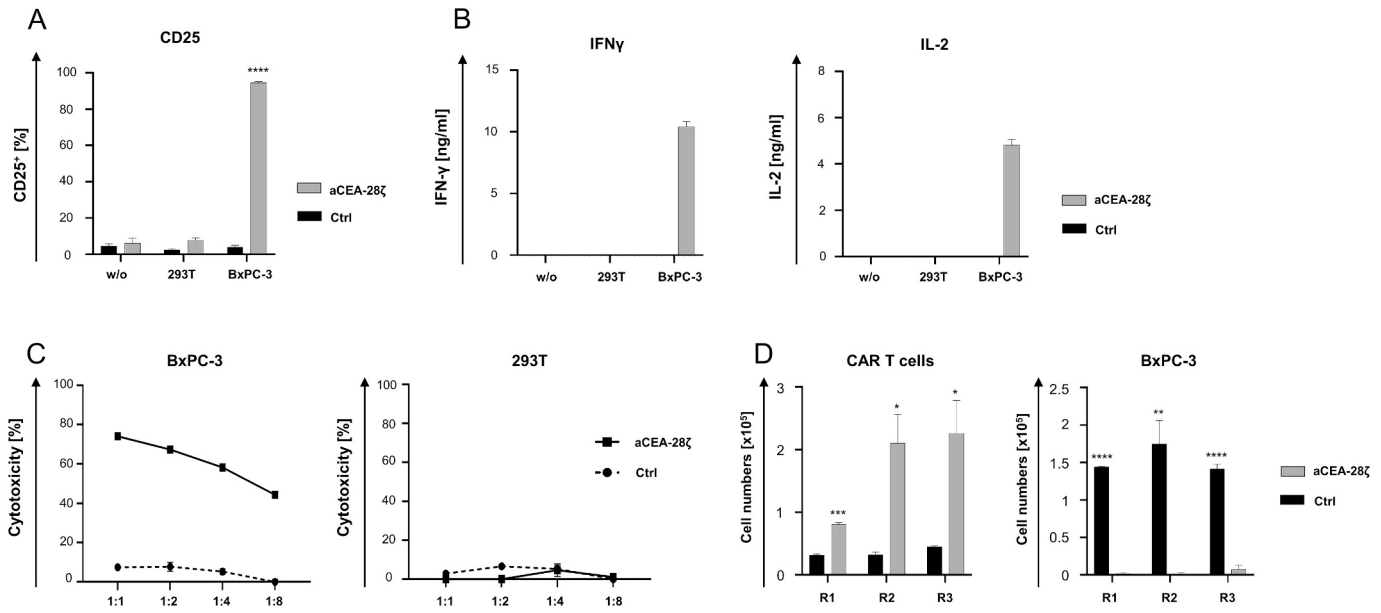

Fig. 4. Magnetic CAR T cell separation does not impair CAR T cell functionality. CAR T cells were enriched by magnetic CAR T cell separation using the PE-labeled anti-G4S linker antibody. After a 48-h expansion with IL-2, CAR T cells were depleted from cytokines via medium change. Untransduced cells were expanded with IL-2, but not retrovirally transduced. **(A)** Upregulation of CD25 on CEA-specific CAR T cells (grey bars) and on untransduced T cells (black bars) after a 48-h co-culture with medium (w/o), CEA^- 293 T cells, and CEA^+ BxPC-3 cells. **(B)** ELISA-based detection of IFN-γ (left panel) and IL-2 (right panel) in the supernatant after a 48-h co-culture of CEA-specific CAR T cells (anti-CEA-28ζ CAR, grey bars) or untransduced T cells (Ctrl, black bars) with medium (w/o), 293 T cells, and BxPC-3 cells. **(C)** Killing capacity of CEA-specific CAR T cells (anti-CEA-28ζ CAR, solid line) or untransduced T cells (Ctrl, dotted line) upon a 24-h co-culture with CEA^+ BxPC-3 cells (left panel) and CEA^- 293 T cells (right panel) was measured at the indicated effector to target ratios via an XTT-based colorimetric assay. **(D)** 1×10^5 CEA-specific CAR T cells (anti-CEA-28ζ CAR, grey bars) or 1×10^5 untransduced T cells (Ctrl, black bars) were repetitively stimulated for three rounds (R1-R3) with GFP-labeled CEA^+ BxPC-3 cells (1×10^5 tumor cells at the start of each round). After each round, T cells (live $CD3^+$) (left panel) and BxPC-3 cells (live GFP^+) (right panel) were enumerated by flow cytometry. Data represent means ± SEM of three donors, *p* values were calculated by Student's *t*-test, ns indicates not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** indicates $p \leq 0.0001$.

approved CAR constructs that bind CD19 or BCMA on malignant B cells lack the GS4 linker necessitating alternative approaches to enrich the respective CAR T cells, such as FACS sorting by means of anti-idiotypic antibodies (Gahvari et al., 2023; Neelapu et al., 2017) or labeled CD19 antigen (Schanda et al., 2021). In addition, the whitlow/218 linker peptide predominantly used in CD19 CAR products could be exploited as a selection marker for CAR T cells similar to the G4S linker by using the commercially available Whitlow/218 linker antibody. A corresponding CAR purification protocol involving the whitlow/218 linker antibody could be developed on the foundation of our approach.

Previous efforts to enrich CAR T cells by magnetic cell isolation predominantly depended on targetable markers inserted into the hinge domain of the CAR constructs (Casucci et al., 2018). In one approach, N3- and N4-hinged CAR T cells were generated by inserting truncated sequences of the extracellular domain of the nerve growth factor receptor (NGFR). Both the 120 amino acid N3 and the 162 amino acid N4 were detected by the ME20.4 anti-NGFR antibody attached to microbeads for magnetic cell separation (Bister et al., 2022). The expression of N3- and N4-hinged CAR T cells was like conventional CAR T cells. Magnetic CAR T cell separation using the anti-NGFR microbeads and MACS columns yielded in a population of ≥90% CAR^+ cells (Bister et al., 2022). Subsequent functional testing of cytotoxicity and cytokine secretion did not show an inferiority of N3- and N4-hinged CD19-specific CAR T cells in comparison to CD8-hinged CAR T cells. Moreover, N3- and N4-hinged CD33-specific CAR T cells were equally efficacious in clearing AML from inoculated NSG mice (Bister et al., 2022). Similarly, a 99 amino acid CAR hinge derived from the human CD34 molecule was used to isolate CAR T cells. This hinge, termed C6 hinge, which encompassed a 16 amino acid long epitope specifically recognized by the QBend-10.53 antibody, enabled GMP-compliant magnetic cell separation of CAR T cells with high purity and with similar functionality as conventional CD8-hinged CAR T cells (Bister et al., 2021). Despite feasibility, hinge modification of CAR T cells holds some disadvantages, such as a potential impairment of CAR expression, increase in tonic signaling, changes to antigen recognition and consequently sensitivity in T cell activation, and eliciting human antibody responses.

After magnetic CAR T cell separation, CD39 was upregulated on CAR T cells while the expression levels of CD25, TIM3, PD-1, TIGIT, and LAG3 were not altered. We assume that staining with the anti-G4S antibody and subsequent magnetic cell separating caused some extend of CAR cross-linking resulting in partial CAR T cell activation. CD39, also known as ectonucleotide triphosphate diphosphohydrolase 1 (ENTPD1), is an ectoenzyme in T cells which is instrumental in the stepwise degradation of adenosine triphosphate (ATP) into immunosuppressive adenosine (Jiang et al., 2023). Generally, T cells with CD39 expression are associated with functional T cell exhaustion indicated by a diminished cytotoxicity, a reduced proliferative capacity, and a low production of inflammatory cytokines (Canale et al., 2018). Thus, we extensively interrogated CAR T cell functionality after magnetic cell separation and found no impairments regarding in vitro cytotoxicity, cytokine secretion and proliferation despite increased CD39 levels. Recently, CD39 expression in T cells was also related to T cell activation, tumor reactivity, and clonal expansion with CD39 serving as a potential marker of activated tumor-reactive $CD8^+$ T cells in tumors (Chow et al., 2023). Accordingly, CAR T cell functionality was not impaired in an in-vitro "stress test", predicated on repetitive stimulation with tumor cells, which rapidly induces functional exhaustion (Harrer et al., 2023). Even under challenging "stress-test" conditions, purified CAR T cells showed robust elimination of tumor cells obviating concerns about CAR T cell exhaustion originating from CD39 expression after magnetic cell separation. Collectively, we assume that elevated CD39 expression after magnetic cell separation did not prevent CAR T cell functionality.

In general, CAR T cell manufacturing in clinical trials tends to avoid high transduction rates due to clinical risks, such as genotoxicity.

Following the presented CAR purification approach, a CAR product with a high proportion of CAR^+ cells could be generated without incurring elevated risks of genotoxicity. However, whether the proportion of CAR^+ cells in CAR T cell products really correlates with a better clinical outcome in cancer patients, has not been comprehensively investigated so far.

In aggregate, our data establish a magnetic CAR T cell separation procedure using a PE-labeled anti-G4S antibody as a tool for fast and robust isolation of CAR T cells, independently of the CAR specificity and without the need of a co-expressed marker molecule. This novel isolation approach holds promise to facilitate preclinical CAR T cell research with the potential to be translated towards application in a GMP-compliant CAR T cell manufacturing process.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of Regensburg (21–2224-101 Regensburg).

Funding

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) to D.H. grant 324392634—TRR221, and by the Else-Kröner Fresenius Foundation to D.H. and the "Overseas Research Grant" from National Cheng Kung University Hospital to S.L. Bavarian Cancer Research Center (BZKF), BZKF-Young Scientist Fellowship grant to D.H.

CRediT authorship contribution statement

Dennis Christoph Harrer: Data curation, Conceptualization. **Sin-Syue Li:** Data curation. **Marcell Kaljanac:** Data curation. **Valerie Bezler:** Data curation. **Markus Barden:** Data curation. **Hong Pan:** Data curation. **Wolfgang Herr:** Conceptualization. **Hinrich Abken:** Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Charlotte Schenkel for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2024.113667.

References

Abken, H., 2021. Building on synthetic immunology and T cell engineering: a brief journey through the history of chimeric antigen receptors. Hum. Gene Ther. 32, 1011–1028. https://doi.org/10.1089/hum.2021.165.

Bister, A., Ibach, T., Haist, C., Smorra, D., Roellecke, K., Wagenmann, M., Scheckenbach, K., Gattermann, N., Wiek, C., Hanenberg, H., 2021. A novel CD34-derived hinge for rapid and efficient detection and enrichment of CAR T cells. Molecul. Therapy Oncolyt. 23, 534–546. https://doi.org/10.1016/j.omto.2021.11.003.

Bister, A., Ibach, T., Haist, C., Gerhorst, G., Smorra, D., Soldierer, M., Roellecke, K., Wagenmann, M., Scheckenbach, K., Gattermann, N., Wiek, C., Hanenberg, H., 2022. Optimized NGFR-derived hinges for rapid and efficient enrichment and detection of CAR T cells in vitro and in vivo. Molecul. Therapy Oncolyt. 26, 120–134. https://doi.org/10.1016/j.omto.2022.05.012.

Canale, F.P., Ramello, M.C., Núñez, N., Araujo Furlan, C.L., Bossio, S.N., Gorosito Serrán, M., Tosello Boari, J., Del Castillo, A., Ledesma, M., Sedlik, C., Piaggio, E., Gruppi, A., Acosta Rodríguez, E.A., Montes, C.L., 2018. CD39 expression defines cell exhaustion in tumor-infiltrating CD8(+) T cells. Cancer Res. 78, 115–128. https://doi.org/10.1158/0008-5472.CAN-16-2684.

Casucci, M., Falcone, L., Camisa, B., Norelli, M., Porcellini, S., Stornaiuolo, A., Ciceri, F., Traversari, C., Bordignon, C., Bonini, C., Bondanza, A., 2018. Extracellular NGFR spacers allow efficient tracking and enrichment of fully functional CAR-T cells co-expressing a suicide gene. Front. Immunol. 9, 507. https://doi.org/10.3389/fimmu.2018.00507.

Chmielewski, M., Abken, H., 2017. CAR T cells releasing IL-18 convert to T-bet(high) FoxO1(low) effectors that exhibit augmented activity against advanced solid tumors. Cell Rep. 21, 3205–3219. https://doi.org/10.1016/j.celrep.2017.11.063.

Chmielewski, M., Hombach, A., Heuser, C., Adams, G.P., Abken, H., 2004a. T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. J. Immunol. 173, 7647–7653. https://doi.org/10.4049/jimmunol.173.12.7647 (Baltimore, Md. : 1950).

Chmielewski, M., Hombach, A., Heuser, C., Adams, G.P., Abken, H., 2004b. T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. J. Immunol. 173, 7647–7653. https://doi.org/10.4049/jimmunol.173.12.7647 (Baltimore, Md. : 1950).

Chow, A., Uddin, F.Z., Liu, M., Dobrin, A., Nabet, B.Y., Mangarin, L., Lavin, Y., Rizvi, H., Tischfield, S.E., Quintanal-Villalonga, A., Chan, J.M., Shah, N., Allaj, V., Manoj, P., Mattar, M., Meneses, M., Landau, R., Ward, M., Kulick, A., Kwong, C., Wierzbicki, M., Yavner, J., Egger, J., Chavan, S.S., Farillas, A., Holland, A., Sridhar, H., Ciampricotti, M., Hirschhorn, D., Guan, X., Richards, A.L., Heller, G., Mansilla-Soto, J., Sadelain, M., Klebanoff, C.A., Hellmann, M.D., Sen, T., de Stanchina, E., Wolchok, J.D., Merghoub, T., Rudin, C.M., 2023. The ectonucleotidase CD39 identifies tumor-reactive CD8(+) T cells predictive of immune checkpoint blockade efficacy in human lung cancer. Immunity 56, 93–106.e6. https://doi.org/10.1016/j.immuni.2022.12.001.

Dragon, A.C., Zimmermann, K., Nerreter, T., Sandfort, D., Lahrberg, J., Klöß, S., Kloth, C., Mangare, C., Bonifacius, A., Tischer-Zimmermann, S., Blasczyk, R., Maecker-Kolhoff, B., Uchanska-Ziegler, B., Abken, H., Schambach, A., Hudecek, M., Eiz-Vesper, B., 2020. CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation. J. Immunother. Cancer 8. https://doi.org/10.1136/jitc-2020-000736.

Fujiwara, K., Tsunei, A., Kusabuka, H., Ogaki, E., Tachibana, M., Okada, N., 2020. Hinge and transmembrane domains of chimeric antigen receptor regulate receptor expression and signaling threshold. Cells 9. https://doi.org/10.3390/cells9051182.

Gahvari, Z., Brunner, M., Schmidt, T., Callander, N.S., 2023. Update on the Current and Future Use of CAR-T to Treat Multiple Myeloma. Eur. J. Haematol. https://doi.org/10.1111/ejh.14145.

Golumba-Nagy, V., Kuehle, J., Abken, H., 2017. Genetic modification of T cells with chimeric antigen receptors: a laboratory manual. Human. Gene. Therapy Method. 28, 302–309. https://doi.org/10.1089/hgtb.2017.083.

Griffioen, M., van Egmond, H.M., Esther Barnby-Porritt, H., van der Hoorn, Menno A.W. G., Hagedoorn, R.S., Kester, M.G.D., Schwabe, N., Willemze, R., Falkenburg, J.H.F., Heemskerk, M.H.M., 2008. Genetic engineering of virus-specific T cells with T-cell receptors recognizing minor histocompatibility antigens for clinical application. Haematologica 93, 1535–1543. https://doi.org/10.3324/haematol.13067.

Harrer, D.C., Schenkel, C., Bezler, V., Kaljanac, M., Hartley, J., Barden, M., Pan, H., Holzinger, A., Herr, W., Abken, H., 2022. CAR triggered release of Type-1 interferon limits CAR T-cell activities by an artificial negative autocrine loop. Cells 11. https://doi.org/10.3390/cells11233839.

Harrer, D.C., Bezler, V., Hartley, J., Herr, W., Abken, H., 2023. IRF4 downregulation improves sensitivity and endurance of CAR T cell functional capacities. Front. Immunol. 14.

Holzinger, A., Abken, H., 2022. Treatment with living drugs: pharmaceutical aspects of CAR T cells. Pharmacology 107, 446–463. https://doi.org/10.1159/000525052.

Hombach, A., Heuser, C., Sircar, R., Tillmann, T., Diehl, V., Pohl, C., Abken, H., 1999. Characterization of a chimeric T-cell receptor with specificity for the Hodgkin's lymphoma-associated CD30 antigen. J. Immun. (Hagerstown, Md. : 1997) 22, 473–480. https://doi.org/10.1097/00002371-199911000-00001.

Hombach, A., Wieczarkowiecz, A., Marquardt, T., Heuser, C., Usai, L., Pohl, C., Seliger, B., Abken, H., 2001. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. J. Immunol. 167, 6123–6131. https://doi.org/10.4049/jimmunol.167.11.6123 (Baltimore, Md. : 1950).

Hombach, A.A., Rappl, G., Abken, H., 2019. Blocking CD30 on T cells by a dual specific CAR for CD30 and Colon Cancer antigens improves the CAR T cell response against CD30(−) tumors. Molecular Therapy : J. American Soc. Gene. Therapy 27, 1825–1835. https://doi.org/10.1016/j.ymthe.2019.06.007.

Jiang, X., Wu, X., Xiao, Y., Wang, P., Zheng, J., Wu, X., Jin, Z., 2023. The ectonucleotidases CD39 and CD73 on T cells: the new pillar of hematological malignancy. Front. Immunol. 14, 1110325. https://doi.org/10.3389/fimmu.2023.1110325.

June, C.H., 2023. The unlikely development of CAR T cells: a brief history and prospects for the future. Keio J. Med. 72, 26. https://doi.org/10.2302/kjm.ABSTRACT_72_1-1.

June, C.H., Sadelain, M., 2018. Chimeric antigen receptor therapy. N. Engl. J. Med. 379, 64–73. https://doi.org/10.1056/NEJMra1706169.

Kochenderfer, J.N., Dudley, M.E., Feldman, S.A., Wilson, W.H., Spaner, D.E., Maric, I., Stetler-Stevenson, M., Phan, G.Q., Hughes, M.S., Sherry, R.M., Yang, J.C., Kammula, U.S., Devillier, L., Carpenter, R., Nathan, D.-A.N., Morgan, R.A., Laurencot, C., Rosenberg, S.A., 2012. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood 119, 2709–2720. https://doi.org/10.1182/blood-2011-10-384388.

Kronig, M.-N., Wehrli, M., Salas-Benito, D., Maus, M.V., 2023. Hurdles race for CAR T-cell therapy in digestive tract cancer. Immunol. Rev. 320, 100–119. https://doi.org/10.1111/imr.13273.

Ma, R., You, F., Tian, S., Zhang, T., Tian, X., Xiang, S., Wu, H., Yang, N., An, G., Yang, L., 2023. Enhanced Efficacy of CD19/CD22 Bispecific CAR-T Cells With EAAAK Linker on B-cell Malignancies. Eur. J. Haematol. https://doi.org/10.1111/ejh.14090.

Mao, R., Hussein, M.S., He, Y., 2022. Chimeric antigen receptor engineered T cells and their application in the immunotherapy of solid tumours. Expert Rev. Mol. Med. 24, e7 https://doi.org/10.1017/erm.2021.32.

Mazinani, M., Rahbarizadeh, F., 2022. CAR-T cell potency: from structural elements to vector backbone components. Biomark. Res. 10, 70. https://doi.org/10.1186/s40364-022-00417-w.

Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., Timmerman, J.M., Stiff, P.J., Friedberg, J.W., Flinn, I.W., Goy, A., Hill, B.T., Smith, M.R., Deol, A., Farooq, U., McSweeney, P., Munoz, J., Avivi, I., Castro, J.E., Westin, J.R., Chavez, J.C., Ghobadi, A., Komanduri, K.V., Levy, R., Jacobsen, E.D., Witzig, T.E., Reagan, P., Bot, A., Rossi, J., Navale, L., Jiang, Y., Aycock, J., Elias, M., Chang, D., Wiezorek, J., Go, W.Y., 2017. Axicabtagene Ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. N. Engl. J. Med. 377, 2531–2544. https://doi.org/10.1056/NEJMoa1707447.

Ruella, M., Korell, F., Porazzi, P., Maus, M.V., 2023. Mechanisms of resistance to chimeric antigen receptor-T cells in haematological malignancies. Nat. Rev. Drug Discov. 22, 976–995. https://doi.org/10.1038/s41573-023-00807-1.

Schanda, N., Sauer, T., Kunz, A., Hückelhoven-Krauss, A., Neuber, B., Wang, L., Hinkelbein, M., Sedloev, D., He, B., Schubert, M.-L., Müller-Tidow, C., Schmitt, M., Schmitt, A., 2021. Sensitivity and specificity of CD19.CAR-T cell detection by flow cytometry and PCR. Cells 10. https://doi.org/10.3390/cells10113208.

Sweeney, N.P., Vink, C.A., 2021. The impact of lentiviral vector genome size and producer cell genomic to gag-pol mRNA ratios on packaging efficiency and titre. Molecular Therapy. Methods & Clinical Develop. 21, 574–584. https://doi.org/10.1016/j.omtm.2021.04.007.

Uslu, U., Da, T., Assenmacher, C.-A., Scholler, J., Young, R.M., Tchou, J., June, C.H., 2023. Chimeric antigen receptor T cells as adjuvant therapy for unresectable adenocarcinoma. Sci. Adv. 9, eade2526. https://doi.org/10.1126/sciadv.ade2526.

Xu, J., Meng, Q., Sun, H., Zhang, X., Yun, J., Li, B., Wu, S., Li, X., Yang, H., Zhu, H., Aschner, M., Relucenti, M., Familiari, G., Chen, R., 2021. HER2-specific chimeric antigen receptor-T cells for targeted therapy of metastatic colorectal cancer. Cell Death Dis. 12, 1109. https://doi.org/10.1038/s41419-021-04100-0.

Zhang, C., Wang, L., Zhang, Q., Shen, J., Huang, X., Wang, M., Huang, Y., Chen, J., Xu, Y., Zhao, W., Qi, Y., Li, Y., Ou, Y., Yang, Z., Qian, C., 2023. Screening and characterization of the scFv for chimeric antigen receptor T cells targeting CEA-positive carcinoma. Front. Immunol. 14, 1182409. https://doi.org/10.3389/fimmu.2023.1182409.

Zimmermann, K., Kuehle, J., Dragon, A.C., Galla, M., Kloth, C., Rudek, L.S., Sandalcioglu, I.E., Neyazi, B., Moritz, T., Meyer, J., Rossig, C., Altvater, B., Eiz-Vesper, B., Morgan, M.A., Abken, H., Schambach, A., 2020. Design and characterization of an "all-in-one" lentiviral vector system combining constitutive anti-G(D2) CAR expression and inducible cytokines. Cancers 12. https://doi.org/10.3390/cancers12020375.