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Optimizing Chimeric Antigen Receptor (CAR)-Modified T Cells for Solid Tumor Therapy: Exploring Molecular Interactions and Differentiation Mechanisms

by Marianna Mezősi-Csaplár

Supervisors: Dr. György Vereb, Dr. Árpád Szöőr

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DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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By Marianna Mezősi-Csaplár, biochemical engineer

Supervisors: György Vereb, PhD, DSc
Árpád Szöőr, PhD

Doctoral School of Pharmaceutical Sciences, University of Debrecen

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on February 26, 2024, at 1:00 pm.

1. Introduction

In recent decades, the genetic modification of a patient's T lymphocytes by incorporating chimeric antigen receptors (CARs) became a particularly promising strategy in antitumor immunotherapy. First-generation CARs consist of an extracellular, tumor-specific recognition domain fused to an effector endodomain – derived from the CD3ζ subunit of the T cell receptor complex (TCR) – which induces the elimination of the identified tumor cell by the CAR T lymphocyte. To improve the activation and expansion of the reprogrammed cells, an additional costimulatory endodomain has been incorporated into the CAR backbone, bringing forth the second generation of CARs. Notably, CARs expressing CD28 and 41BB costimulators have transformed the treatment landscape for therapy-resistant leukemias and lymphomas. In contrast, CAR T cell therapy for patients with solid tumors so far has shown limited benefits, and a trial of a third-generation CAR combining the CD28 and 41BB costimulatory domains regrettably resulted in the death of a patient.

Given these considerations, it is imperative to identify critical parameters of the therapeutic strategy that influence the efficacy and safety profile of CAR T products. CAR activity is fundamentally determined by how the molecular mechanisms associated with its functional components are integrated into the physiological processes of T cells. The choice of costimulatory domain(s) may affect immediate molecular events as well as short- and long-term cytotoxic activity and expansion. In addition, CAR T cell manufacturing conditions exert significant influence over the latter. Therefore, our investigation focused on the impact of CD28 and 41BB costimulatory domains on the cell surface organization and diffusion kinetics of solid tumor targeting CARs spanning across generation I to III. Our findings draw correlations between primary receptor characteristics, immune synapse formation, and the efficacy of early cytolytic signaling. Additionally, we explore the effects of T cell differentiation dynamics on in vitro efficacy, expansion, exhaustion, and in vivo tumor control.

2. Background

2.1. Immuno-oncology

Decades of research have uncovered the prowess of the immune system in identifying and eradicating malignant cells through diverse mechanisms. Among these immunological defenses is the production of antibodies specific for tumor-associated antigens (TAAs). Upon binding to tumor cells, these antibodies instigate antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (Complement Dependent Cytotoxicity, CDC), as well as other mechanisms, to orchestrate the elimination of malignant cells. If tumor-specific somatic mutation-derived neoantigens are presented on the surface of tumor cells as peptides bound to the major histocompatibility complex (MHC), T lymphocytes discern and eliminate them through their T-cell receptors (TCR). However, immune surveillance is a double-edged sword: while swiftly eliminating vulnerable targets, it inadvertently fosters the survival of tumor cells adept at developing resistance through successive transformative stages.

Therapeutic interventions seek to reinstate the capability of the immune system to orchestrate a potent antitumor response by selectively targeting and strengthening natural defense mechanisms. In clinical practice, various methods have been developed to activate the immune system in a tumor-specific manner. Therapeutic use of monoclonal antibodies (mAbs) direct the ADCC and CDC immune defense mechanisms toward malignant cells, while adoptive immunotherapies, such as treatments based on tumor-infiltrating lymphocytes (TIL), enhance the efficacy of tumor-specific T cell immune responses. Acknowledging the pivotal success of immune checkpoint inhibitors (ICIs) is also inevitable. These monoclonal antibodies disrupt T-cell inhibitory signals by blocking specific checkpoint proteins, preventing the premature exhaustion and anergy of tumor reactive T cells.

While mAb, TIL, and ICI therapies have achieved significant breakthroughs in treating malignant tumors, each strategy is uniquely constrained by various tumor defense mechanisms. Malignant cells may build resistance against mAb therapies by

diminishing the expression of target antigens, impeding immune responses aimed at their eradication, and orchestrating signaling pathways that circumvent apoptosis while promoting proliferation. Additionally, solid tumors can defend themselves against mAb treatment by constructing an extracellular matrix (ECM) containing mucin-4 (MUC4), hyaluronic acid, and its receptor (CD44), impenetrable by antibodies. The efficacy of TIL and ICI treatments can be limited by the decreased expression of Major Histocompatibility Complex (MHC) on the tumor site and the small number of tumor-reactive T cells. However, the abundance of yet non-tumor-reactive T lymphocytes in the body presents an opportunity for a transformative solution. Reprogramming these T cells for targeted tumor assault offers a way to combine the merits of each previously presented therapeutic system while mitigating their disadvantages. This revolutionary approach is known as Chimeric Antigen Receptor (CAR) T cell therapy, which integrates the antigen specificity of monoclonal antibodies with the robust antitumor efficacy of T cells.

CAR T cells are derived from the patient's own blood and genetically engineered to express the synthetic receptor capable of activating T cell effector functions following the targeted recognition of tumor-specific antigens. The main steps of the therapeutic procedure are the isolation and activation of the patient's T cells, followed by the integration of the transgene encoding the chimeric antigen receptor into the genome, and the autologous transfer of the CAR T cells. Results from preclinical models have demonstrated that CAR T cells, even when confronted with a limited number of cell surface tumor antigens, demonstrate adeptness in recognizing and eradicating tumors. Consequently, they exhibit a heightened resilience against resistance through diminished antigen expression compared to monoclonal antibodies.

2.2. CAR T cell therapy

2.2.1. CAR structure and evolution

During the evolution of CAR T cell-based immunotherapies, the design of the artificial receptor has traversed a multi-stage refinement process. First-generation CARs relied solely on the two basic functional units of the receptor – an antibody-like ectodomain recognizing the membrane-associated tumor antigens of cancer cells and an effector endodomain of TCR origin triggering the cytotoxic T cell response.

First-generation CARs provided the antigen-dependent first signal, which is crucial for T cell activation. These constructs could control tumor growth in preclinical models, but they could not confer significant tumor reduction in phase I clinical trials. The reason behind their insufficient therapeutic response was that, as native T lymphocytes, CAR T cells also require a second signal provided by costimulatory receptors for the full activation of their cytolytic and proliferative capabilities. Consequently, a costimulatory domain was incorporated in the endodomain of second-generation chimeric receptors, ushering in an era of unparalleled antitumor efficacy and enduring proliferative capacity for CAR T cells. This transformative strategy marked a clinical breakthrough in treating diverse hematological disorders, yet it encountered limited success in addressing solid tumors.

In the hope of combining the benefits provided by distinct costimulators, the third generation of CARs was equipped with two costimulatory domains. During preclinical testing, they demonstrated increased persistence and antitumor activity. However, the heightened activation resulting from the synergistic functioning of the costimulatory domains proved disadvantageous: their effectiveness did not surpass second-generation CAR T cells in indications of relapsed or refractory B-cell leukemias, while their application more frequently led to the development of severe side effects.

CAR configuration exhibits considerable diversity within each generation. The prevailing extracellular component for antigen recognition is typically a single chain variable fragment (scFv) derived from an antibody, encompassing both light and heavy

chains. The CAR scFv can originate from various antibodies sharing the same specificity but possessing different epitopes and affinity. A high-affinity scFv enhances the stability of the immunological synapse (IS) formed by CARs gathering on the contact surface during the attack on the tumor. However, these CAR T cells may also damage healthy tissues expressing the targeted tumor-associated antigen to a minor extent, leading to the development of the so-called “on-target off-tumor” side effect.

The short but functionally significant building blocks of the CAR structure are the hinge (also known as spacer or linker) and transmembrane (TM) units located between the extracellular antigen-recognition and intracellular effector domains. The hinge provides flexibility to the antigen-recognition unit, while the TM domain ensures the stable membrane expression of the receptor. The most frequently employed hinges are of IgG1, IgG4, CD8, or CD28 origin, while the TM domain typically originates from CD8, CD4, or CD28. In terms of their functional effects, increasing the length and flexibility of the hinge assists the CAR in accessing difficult-to-reach tumor epitopes. However, it is crucial to maintain a sufficiently close physical distance between CAR T lymphocytes and cancer cells to exclude T cell inhibitory regulators from the immunological synapse. Beyond its primary function, the TM region can induce the dimerization of the chimeric receptor spontaneously or under stimulation. This dimerization positively impacts the efficiency of immunological synapse formation and signal transduction during early activation. The dynamics and stability of immunological synapse assembly are crucial in the progression of the cytolytic T cell response and, according to some correlational analyses, may predict the therapeutic efficacy of CAR T cells.

Following antigen recognition by the CAR scFv and the formation of the immunological synapse, the intracellular effector domain of CARs mediates the indispensable first signal for T cell activation. The effector unit, a less diverse component of the CAR structure, most commonly originates from the CD3ζ chain of the TCR complex. In most CARs, the CD3ζ unit contains three immunoreceptor tyrosine-based activation motifs (ITAMs), which, akin to native TCRs, are

phosphorylated during activation by the lymphocyte-specific protein tyrosine kinase (Lck). The subsequent steps in the signaling cascade efficiently initiate the signaling pathways leading to transcription, proliferation, and cytotoxic T cell response.

The single or double costimulatory endodomains of second and third generation CARs are positioned between TM and CD3z subunits. Both most commonly utilized costimulators, CD28 and 41BB, provide potent signals for proliferation, survival, and activation, yet they influence the effector response differently. CD28 costimulated CAR T cells exhibit a vigorous cytotoxic response but undergo rapid exhaustion, differentiating into effector memory cells with shorter persistence. Conversely, 41BB-mediated cytolysis is less intense; however, CAR T cells persist longer as a subset of central memory cells.

2.2.2. CAR T cell manufacturing

The initial phase of CAR T cell manufacturing involves isolating peripheral blood mononuclear cells (PBMCs) and initiating T cell expansion. During this process, T cells need to receive three distinct signals. The antigen-specific first signal is typically instigated by stimulating the epsilon subunit of the TCR/CD3 complex. The second, costimulatory signal is provided by activating the CD28 membrane protein. The third, cytokine signal, essential for complete T cell activation, is delivered through supplementation with IL-2, IL-7, and IL-15 cytokines.

Subsequently, the CAR-encoding transgene is incorporated into the genome of activated T cells through viral gene delivery methods. Gene transfer efficiency can be enhanced by RetroNectin, a reagent facilitating the concurrent binding of T lymphocytes and viral particles. Following the transduction efficiency and product quality assessment, the CAR T cells are ready to be employed for further experimental or therapeutic applications.

The heterogeneous peripheral T cell population, serving as the basis of the therapeutic product, contains T lymphocytes from distinct differentiational stages: naïve, central memory (CM), effector memory (EM), and terminal effector (TE) T cells.

The production of CAR T cells necessitates the activation and expansion of isolated T lymphocytes, concurrently inducing their differentiation. As T cells transition from naïve to terminal effector state, there is a gradual increase in cytolytic efficiency coupled with a concomitant decline in proliferative capacity. Although T lymphocytes from TE phenotype exhibit robust cytotoxic activity, they are incapable of further expansion and eventually undergo apoptosis or adopt a non-functional "exhausted" phenotype. The stimulation and cultivation conditions employed during ex vivo T cell expansion influence the differentiation speed, thereby defining the phenotype profile of the resulting cell product.

Another pivotal facet of CAR T cell product composition lies in the balance between $CD4^+$ helper and $CD8^+$ cytotoxic lymphocytes. During their synergistic activity, the proliferation, survival, and effector functions of $CD8^+$ CAR T cells are bolstered by cytokines released from $CD4^+$ CAR T cells. Manufacturing conditions can impact the distribution of $CD4^+/CD8^+$ proportions of CAR T cell products.

2.2.3. Clinical application of CAR T cell therapies

Over the past decade, several phase II clinical trials have been conducted, evaluating the efficacy and safety of various CAR T cell products targeting B-cell leukemias and lymphomas. The primary therapeutic targets included CD19, expressed on both native and malignant B cells, CD22, and the B-cell maturation antigen (BCMA). Noteworthy breakthroughs were achieved in clinical investigations featuring second-generation CAR T cell treatments, achieving complete remission for 40-80% of patients. To date, the US Food and Drug Administration (FDA) has granted regulatory approval for four CD19-specific and two BCMA-specific CAR T therapies.The aforementioned achievements of CAR T cells have opened avenues for their application in treating solid tumors, which, however, poses a more complex challenge compared to hematological disorders. Solid tumors often lack TAAs that can serve as a specific target for CAR T cells. Furthermore, the expansion and persistence of gene-edited T cells are frequently inadequate for eliciting an effective antitumor response. Despite these

obstacles, approximately 200 clinical trials are currently underway involving CAR T cells targeting solid tumors of diverse organs – such as the brain, pancreas, lung, esophagus, stomach, breast, colon, and ovary.

The earliest and most extensively explored therapeutic target to date is HER2, exhibiting elevated expression on the surface of various tumor cells, including breast, lung, stomach, colon, glia, and pancreatic cancers. Initial investigations into HER2-CAR T therapy involved a clinical trial targeting metastatic colorectal carcinoma, utilizing high doses of third-generation CAR T cells. Regrettably, following the infusion of therapeutic cells, a severe "cytokine storm" ensued, resulting in the immediate fatality of the patient. This unfortunate incident impeded HER2-CAR T cell clinical trials for several years, prompting the adoption of a lower dosage and a safer second-generation CAR construct in renewed trials. In a phase I clinical trial of patients suffering from glioblastoma, the infusion of CD28 costimulated HER2-CAR T lymphocytes was well-tolerated, exhibiting an absence of severe treatment-related toxicities. However, within the cohort of 16 evaluable patients, only one demonstrated a partial tumor response. As per the available published data, a noteworthy outcome has been observed in one patient who achieved complete recovery through HER2-specific CAR T cell therapy. A child suffering from refractory soft tissue sarcoma accompanied by bone metastases underwent seven cycles of CAR T cell therapy utilizing CD28 costimulation, resulting in the complete eradication of the tumor by the tenth week after the last infusion.

3. Aims

The success of CAR T cells in addressing B-cell leukemias and lymphomas rests upon the meticulous optimization of the therapeutic system, ranging from the design of the construct to the phenotypic profile of the cell formulation. However, clinical experience with CAR T cells directed at solid tumors has thus far fallen short of expectations. The modest tumor responses observed in clinical trials suggest that HER2-CAR T therapy requires refinement to augment cytolytic activity and ensure sustained long-term expansion. Therefore, our research objective was to identify intervention points that could potentially elevate the efficacy of CAR T cells in the treatment of HER2-positive solid tumors.

In our experimental system, we sought to answer the following questions:

- To what extent do the molecular composition, cell surface arrangement, and membrane diffusion dynamics of the chimeric receptor impact the short-term antitumor efficacy of CAR T cells?

- What T cell phenotypic profile yields the optimal combination of prolonged expansion and efficient effector response for CAR T cells directed at HER2-positive solid tumors?

4. Materials and methods

4.1. Cell lines

The retroviral vectors encoding the HER2-CAR were generated using the HEK293T viral packaging cell line. In vitro assessment of CAR T cell antitumor activity involved the MDA-MB-468 cell line exhibiting stable expression of transduced HER2 (MDA-HER2), alongside the $HER2^+$ N87 and JIMT-1 cell lines, and their luciferase-expressing variants (MDA-HER2.ffLuc, N87.ffLuc, and JIMT-1.ffLuc). $HER2^-$ MDA/MDA.ffLuc cell lines were used as controls. Cells were cultured in DMEM medium supplemented with 2 mmol/l GlutaMAX, 10% fetal calf serum (FBS) and antibiotics, except for JIMT-1/JIMT-1.ffLuc, which were cultured in a 1:1 ratio of Ham's F12 mixed with DMEM with the addition of 2 mmol/l GlutaMAX, 20% FBS, 300 U/l insulin and antibiotics. In vivo evaluation of CAR T cell antitumor efficacy utilized a JIMT-1/JIMT-1.ffLuc tumor xenograft model. All cell lines were maintained in a humidified atmosphere with 5% carbon dioxide at 37°C.

4.2. CAR T cell production

4.2.1. PBMC isolation

To produce HER2-CAR T cells PBMCs were isolated from the peripheral blood of healthy donors using Ficoll gradient centrifugation at 1200 rpm for 10 minutes at room temperature. The isolated T cells were cryopreserved in liquid nitrogen in a solution containing 10% dimethyl sulfoxide, 10% RPMI-1640 medium, and 80% FBS.

4.2.2. Structural composition of the employed CARs

We have used four distinct CAR designs. The molecular configuration of the first-generation CAR comprised a HER2-specific FRP5-derived scFv, an IgG1 "short hinge" (SH), a TM derived from CD28, and a CD3z effector domain (.z CAR). The second-generation CAR incorporated in the .z CAR either a CD28 or a 41BB (CD28.z, 41BB.z CAR) costimulatory domain. The third generation CAR contained both of these costimulation domains (CD28.41BB.z CAR).

4.2.3. Retrovirus Production

Retroviral particles were generated by transient transfection of HEK293T cells with the HER2-CAR-encoding pSFG retroviral vectors, Peg-Pam-e plasmid containing the sequence for MoMLV gag-pol, and pMax.RD114 plasmid containing the sequence for the viral envelope protein, using jetPrime transfection. After incubation for three days, the retrovirus-containing supernatant was purified using a sterile syringe filter with a pore diameter of 0.22 μm and stored at −80°C.

4.2.4. T cell stimulation and culture protocols, CAR transduction

Two distinct methods were employed for CAR T cell production in our experimental procedures. In the *antiCD3-antiCD28/RPMI* protocol, T cell division was stimulated in non-tissue culture 24-well plates precoated with 1 μg/mL anti-CD3e and anti-CD28 antibody in RPMI medium supplemented with 2 mmol/L GlutaMAX, 10% FBS, and antibiotics (complete RPMI). On the second day, further expansion of T cells was prompted by adding 10 ng/ml human interleukin-7 (IL-7) and 5 ng/ml human interleukin-15 (IL-15). On the third day, retroviral vectors containing the CAR construct were spinned onto 24-well plates coated with 20 μg/ml RetroNectin through centrifugation at 4000 rpm for 2 hours. T cells were then incubated in the retrovirus-coated culture dish for 3 days, maintaining a presence of 10 ng/ml IL-7 and 5 ng/ml IL-15 in complete RPMI medium. Subsequently, the CAR T cells were cultured in a fresh RPMI medium containing 10 ng/ml IL-7 and 5 ng/ml IL-15 until they were utilized. Simultaneously, non-transduced (NT) T cells were generated using the same protocol, serving as controls for our measurements.

In the *antiCD3-RetroNectin/LymphoONE* protocol, T cell division was induced overnight in 24-well plates precoated coated with 1 μg/ml anti-CD3e antibody and 20 μg/ml RetroNectin reagent in LymphoONE medium. All other conditions for T cell transduction were the same as described in the antiCD3-antiCD28/RPMI protocol.

4.3. Immunofluorescent staining and flow cytometry

Expression of HER2-specific CARs was verified by indirect labeling with a HER2-Fc fusion protein and Alexa Fluor 488 (A488) conjugated secondary IgG. The helper and cytotoxic lymphocyte ratios of CAR T cell products were determined from CD4 and CD8 expression using FITC conjugated anti-human CD4 and Alexa Fluor 647 (A647) conjugated anti-human CD8 double labeling, respectively. $CD4^+$ cells were defined as the $CD4^+$, $CD8^-$ quadrant, $CD8^+$ cells were defined as the $CD8^+$ $CD4^-$ quadrant population. $CD4^+$, $CD8^+$ double positive and $CD4^-$, $CD8^-$ double negative cells were excluded from the analysis. To investigate the distribution of memory phenotypes, the expression of CCR7 and CD45RA receptors was determined by direct double labeling with FITC conjugated anti-human CD197 (CCR7) and APC-conjugated anti-human CD45RA monoclonal antibodies. Naïve cells were identified as $CCR7^+$ and $CD45RA^+$, T_{CM} cells as $CCR7^+$, $CD45RA^-$, T_{EM} cells as $CCR7^-$ and $CD45RA^-$, T_{TE} cells as $CCR7^-$, $CD45RA^+$ populations. Labeling with antibodies and HER2-Fc recombinant protein was performed at a final concentration of 10 μg/ml for 10 min on ice. A minimum of 10^4 cells were analyzed in each measurement using a NovoCyte flow cytometer and the NovoExpress software.

4.4. Microscopy

Microscopic measurements were performed using an LSM 880 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with an AiryScan/AiryScan Fast imaging unit. Our experiments employed a water immersion objective (C-Apochromat, 1.2 NA; 40×). The diamino-phenylindole (DAPI) dye was excited by a 405 nm laser diode, while A488 and the green fluorescent protein (GFP) at 488 nm, and phycoerythrin (PE) at 543 nm by an argon ion laser. The A647 dye was excited by a HeNe laser at 633 nm. The images were analyzed using the ImageJ/Fiji software.

4.4.1. Analysis of immune synapse activation by confocal laser scanning microscopy

For studying the formation and activation of the CAR immune synapse (IS) a total of 3×10^4 N87 target cells were plated on eight-well chambered tissue culture-treated slides overnight. Following 15 min of co-culturing with 2×10^5 CAR lymphocytes, the cells were fixed in 1% formaldehyde for 10 min at 37°C and labeled with 10 µg/mL A488-conjugated anti-HER2 antibody and, after permeabilization, with either A647-conjugated anti-p-CD3-z or PE-conjugated anti-p-Lck for 30 minutes on ice. The cells were washed with PBS, then in PBS containing 10 µg/mL DAPI, then washed in PBS again. Images were captured in confocal laser scanning mode. The emission of fluorescent dyes was detected utilizing a 32-element GaAsP photomultiplier, with ranges adjusted to the emission maxima of the dyes. The recordings were performed in frame switch mode.

4.4.2. Characterization of CAR membrane organization by Airyscan microscopy

We studied the cell membrane organization of CARs in resting CAR T cells. CAR T cells were incubated in serum-free RPMI at 37°C for one hour and washed with PBS at 4°C. Cells were labeled with 5 µg/ml A647 conjugated monomer HER2 for 10 min on ice, washed, and then resuspended in 10 mM glucose-PBS. Cells were incubated at 37°C throughout the assay. 600 nm thick optical slices were captured of the apical membrane surface of labeled CAR T cells in AiryScan mode. This mode, distinguished by a special detection principle, offers higher resolution and an improved signal-to-noise ratio compared to conventional confocal microscopy. For the detection of A647 emission, a 660 nm long-pass filter was used. Intensity-based segmentation of the images was employed to determine the average size and intensity of individual CAR clusters and the average number of receptor clusters per unit membrane surface area.

4.5. Measurement of CAR membrane diffusion by fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is based on the measurement of stochastic fluctuations in the position and density of fluorescently labeled diffusing particles in the observation volume excited by a focused laser beam. FCS studies of

living cell membranes involve detecting intensity fluctuations in the intersection of the focused laser beam with the cell membrane. By fitting an appropriate diffusion model to the temporal autocorrelation function G(τ), derived from mathematically transforming the measured fluctuations, one can estimate the local concentration and diffusion characteristics of the fluorescently labeled molecules and/or receptors.

For the determination of CAR membrane diffusion by FCS, the preparation, labeling, and measurement conditions of CAR T cells were consistent with the details provided in section 4.4.2. The length of the measurements was 100 seconds per cell and the autocorrelation curves were calculated for 10 s sub-intervals. A triplet corrected model function describing the free diffusion of one component in 3D and one component in 2D was fitted to the autocorrelation curves. The component exhibiting 3D diffusion characteristics was identified as the dissociated monomer A647-HER2, while the component with 2D diffusion was identified as the mobile CAR fraction.

4.6. Western blot

The oligomerization state of CARs was investigated through Western blot analysis. CAR T cells were washed and lysed. Dimeric and oligomeric CARs were separated under native conditions, and monomeric receptors were separated in reducing sample buffer containing 0.1 mM dithiothreitol (DTT) on 10% SDS-PAGE polyacrylamide gels. The protein bands were transferred to polyvinylidene fluoride (PVDF) membrane using a semi-dry blotting technique. The membrane was blocked and labeled with 1 µg/ml mouse anti-human CD3z antibody overnight at 4°C. After washing, the membrane was labeled with a peroxidase-conjugated anti-mouse IgG secondary antibody and incubated in a development solution containing HRP substrate for 3 min after a repeated washing step. Images were acquired using the FluorChem Q imaging and analysis system.

4.7. CAR structure prediction

The secondary and tertiary structures of CARs were modelled using the RoseTTAFold algorithm. Prediction models with the highest confidence score were selected for analysis.

4.8. In vitro rechallenge assay

The proliferation potential of CAR T cells was evaluated using a rechallenge assay. 2×10^5 CAR T cells were plated onto 1 µg/mL HER2-Fc precoated plates. The number of CAR T cells was determined every 3.5 days by flow cytometry. The proliferation rate was calculated by dividing the cell count measured on a given day by the baseline cell count. Subsequently, the same number of cells as the baseline count was restimulated in fresh 1 µg/ml HER2-Fc covered culture dishes. On days 0, 3.5 and 10.5 of serial stimulation, flow cytometry was used to determine the CD4/CD8 and memory phenotype distribution of CAR T cell preparations as described in section 0.

4.9. Cytokine Secretion Assay

Antigen-specific activation of CAR T cells was characterized by the release of IL-2 and IFNγ cytokines. 2×10^5 CAR T cells were incubated for 24 hours in 1 µg/mL HER2-Fc precoated plates. The concentration of IL-2 and INFγ in the supernatant was determined by ELISA kits using a Synergy HT ELISA reader. Cell-free medium and NT T cells were used as controls.

4.10. Analysis of in vitro CAR T cell cytotoxicity

The cytotoxicity of CAR T cells was assessed using the tumor cell lines MDA-HER2.ffLuc, N87.ffLuc and JIMT-1.ffLuc. Target and effector cells were incubated at a 1:1 ratio for 24 h. The relative proportion of surviving tumor target cells was determined from the luminescence signal evoked by D-luciferin degradation using a Synergy HT luminometer. NT T cells and the $HER2^-$ MDA.ffLuc cell line were used as controls.

4.11. Kinetic analysis of in vitro CAR T cell cytotoxicity

The kinetics of the cytotoxic activity of CAR T cells was characterized based on electrical impedance measurements using an Electrical Cell Substrate Impedance Sensor (ECIS). JIMT-1 tumor cells were plated on slides covered with gold electrodes. The cells were cultured on the plate until confluence was achieved, a crucial step for meaningful comparisons across various treatments. The death of tumor cells results in decreased measured impedance, allowing the cytotoxic effect of CAR T cells to be studied in real time. The effector/target cell ratio was set to 1:1. Impedance was monitored for 25h. Averaged traces were normalized to impedance measured at the start of treatment, and then normalized impedances at every time point were normalized to the corresponding value in the NT T cell control.

4.12. Xenograft tumors and in vivo CAR T cell treatments

For assessing the in vivo effector functions of the CAR T cells seven-week-old female NSG mice were administered a subcutaneous (s.c.) injection in both flanks, each containing 3×10^6 JIMT-1.ffLuc or JIMT-1.ffLuc cells in 100 µL of PBS mixed with an equal volume of Matrigel. Mice received a single dose of 2.5×10^6 HER2-CAR T cells or HER2-CAR.ffLuc T cells i.v. 14 days after tumor cell inoculation. As a control, 2.5×10^6 NT or NT.ffLuc T cells were administered. Tumor growth was monitored with an IVIS Spectrum CT instrument (Perkin Elmer). Before measurement, isoflurane-anesthetized animals were injected IP with D-luciferin (150 mg/kg). Bioluminescence images were obtained and analyzed after 10 min using Living Image software Version 4.0 (Caliper Life Sciences).

4.13. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Data are presented as mean ± SD or ± SEM. Two-tailed t-test was used for comparison between two groups, and one-way ANOVA with Tukey's post hoc test was applied to compare three or more groups. For small samples, normality was confirmed using the Shapiro-Wilk test. p-values < 0.05 were considered statistically significant.

5. Results

5.1. Costimulatory domains differentially influence cell surface dynamics and organization of CARs and early activation of CAR T cells

The short-term effector response of CAR T cells is profoundly influenced by the capacity of chimeric receptors to accumulate at the contact surface following the recognition of tumor cells and establish a stable immunological synapse. The extracellular domains of CARs exercise established effects on this process, involving the strength of antigen binding, the physical proximity between CAR T and tumor cells, and the dimerization of CARs, however, the role of the most widely used costimulatory endodomains has not been elucidated yet. Therefore, in the first stage of our research project, we characterized the role of the CD28 and 41BB costimulatory endodomains in the molecular structure, cell surface organization and membrane diffusion dynamics of first, second and third-generation CARs. In the next step, we examined the impact of these fundamental receptor properties on the formation of the immunological synapse, early cytolytic signaling, and the short-term kinetics of tumor cell elimination.

The first generation .z, second generation CD28.z and 41BB.z, and the third generation CD28.41BB.z HER2-CAR T cells were produced in a retroviral transduction system using the *antiCD3-antiCD28/RPMI* protocol. Following receptor-specific fluorescent labeling, transduction efficiency was determined by flow cytometry. Between 75-95% of the cells exhibited CAR expression, no significant difference was observed among the four different constructs. Receptor oligomerization was characterized by Western blot analysis. Our findings revealed that approximately 90% of .z and CD28.z CARs are present in dimeric form, while 40-60% of CARs incorporating the 41BB costimulation domain form oligomers. Following this, we examined the antigen-independent organization of CARs at the level of cell membrane structures using Airyscan super-resolution microscopy and digital image analysis. According to our results, all four tested CARs exhibited a segregated cell surface distribution: approximately 75% of the chimeric receptors localized in high-density clusters, immobile on the scale of the acquisition time (2-5 s), covering one-third of the

entire apical membrane surface. However, discernible differences were identified in individual clusters' size and CAR density among the various CAR constructs. We found that while the .z and CD28.z CARs, which form predominantly dimers, were organized into a small number of large submicron clusters, the 41BB containing CARs frequently forming higher oligomers assembled into a large number of smaller clusters.

Although chimeric receptors with different costimulatory domains formed cell surface structures with distinct morphologies, a consistent observation across all tested constructs was that approximately one-fourth of CARs resided outside the clusters. Recognizing that the accumulation of CARs in the immunological synapse may involve not only pre-assembled receptor aggregates but also mobile receptor components, our subsequent investigation delved into the membrane diffusion kinetics of CARs with different costimulatory domains using fluorescence correlation spectroscopy (FCS). According to our findings, the .z CAR showed significantly slower, whereas the CD28.41BB.z CAR showed significantly faster diffusion in the T cell membrane than the second generation CD28.z or 41BB.z constructs. We correlated these findings with the molecular structure of the constructs modeled by the RoseTTAFold deep-learning-based protein structure prediction algorithm. The predicted models indicated that with the addition of one or two costimulatory endodomains, the CD3z domain is increasingly shifted away from the inner membrane surface. We discerned that the lateral diffusion of CARs proportionally increases as the distance of the CD3z domain diverges further away from the CD3ζ position within the native TCR.

Like native $CD8^+$ T lymphocytes, CAR T cells can only perform their cytolytic functions if a stable synaptic connection is established upon recognition of the target antigen. The native immunological synapse is a highly organized membrane structure. TCRs associated with MHC-peptide complexes accumulate in the center of the IS, activating the phosphatidylinositol and tyrosine kinase signaling pathways, leading to transcription, proliferation, and cytotoxic T cell responses. The structure is stabilized by rearranging the cytoskeletal network and an adhesion molecule ring formed at the edge of the contact surface. In contrast, the CAR IS relies on chimeric receptors not

only for transducing the activation signal, but also for providing stable adhesion. Preformed CAR clusters with high receptor density and nanometer-scale diameter may serve as pre-assembled activation and adhesion centers during IS formation. However, according to our results, the clusters cover only one-third of the CAR T cell membrane surface. Mobile receptor components located outside of the clusters might also influence and facilitate the shaping of the CAR synapse and signal transduction.

Moving forward, we examined the relationship between the efficacy of signal transduction within the immunological synapse and the CARs' membrane organization and mobility. HER2-CAR T cells, featuring distinct costimulatory domains, were incubated on a plate covered by tumor cells for 15 minutes. We fixed the cells and quantified the two initial steps of CAR-mediated signal transduction – CD3z and Lck phosphorylation – within the immunological synapse using confocal microscopy and digital image analysis. According to our findings, both CD28.41BB.z, characterized by high mobility and small clusters and .z CARs, which exhibit slow diffusion and aggregate into large clusters, more effectively induced CD3z phosphorylation compared to the moderately mobile CD28.z and 41BB.z CARs. Similarly, in terms of Lck phosphorylation, both the large .z clusters and the mobile CD28.41BB.z oligomers proved advantageous: the former favored the building of more extensive synapses, while the latter was associated with increased Lck phosphorylation.

The kinetics of tumor-specific killing efficiency was determined by electric cell-substrate impedance sensing. Even though short-term activation upon first target cell engagement was most pronounced in T cells redirected by non-costimulated .z and third-generation CD28.41BB.z CARs, based on the measured impedance changes, longer-term cytotoxic efficacy was found to be grossly different for these two species. First-generation .z CARs induced the most robust target cell killing, while CARs containing 41BB costimulatory endodomains performed the worst. Combining these results with the findings on clustering trends and diffusion dynamics, we can conclude that the larger, higher-density preformed clusters of .z and CD28.z CARs were the most beneficial for tumor elimination during the first 25 h.

5.2. The phenotypic composition of CAR T cell products determine the long-term efficacy of anti-tumor activity

The phenotype profile of CAR T cell products, including memory composition and CD4/CD8 ratio, affect clinical efficacy after the adoptive transfer. In the realm of CAR T therapies directed against leukemias and lymphomas, both preclinical and clinical observations highlight that less differentiated cell products − characterized by a naïve and central memory phenotype profile − provide more favorable antitumor efficacy. However, in the case of solid tumors, the impact of differentiation status and CD4/CD8 distribution on product quality is unclear. According to previous findings, the LymphoONE medium, fine-tuned for T cell expansion, coupled with RetroNectin based pre-stimulation may slow T cell differentiation during the manufacturing process of CAR T cells.

During our investigation, we produced retrovirally transducted CD28.z and 41BB.z HER2-CAR T cells employing the *antiCD3-antiCD28/RPMI* and *antiCD3-RetroNectin/LymphoONE* protocols. We evaluated transduction efficiency, memory phenotype composition, and the balance between helper and cytotoxic CAR T lymphocytes four days after CAR transduction using flow cytometry. No notable differences in transduction efficiency or CAR expression were observed among cell products. Nevertheless, the *antiCD3-RetroNectin/LymphoONE* approach markedly slowed T cell differentiation, leading to a significant enrichment of CM phenotype CAR T cells. Conversely, cell products prepared with the *antiCD3-antiCD28/RPMI* protocol displayed an advanced degree of differentiation, resulting in a higher proportion of EM lymphocytes. We determined by ELISA the release of IFNγ and IL-2 cytokines, indicating the activation of effector cells following stimulation with immobilized HER2 targets. EM phenotype-enriched HER2.CD28.z and HER2.41BB.z CAR T cells produced more IFNγ and IL2 than their CM-enriched counterparts, although this only reached statistical significance for 41BB.z CAR T cells. Next, we investigated the antitumor activity of HER2-CAR T cells in a firefly luciferase (ffLuc) activity-based cytotoxicity assay at an E:T ratio of 1:1 using luciferase-expressing tumor target cells.

We found that the increased amount of effector memory phenotypes in the *OKT3-antiCD28/RPMI*-stimulated CD28.z and 41BB.z CAR T cell products induced stronger CAR-specific cytotoxicity compared to the less differentiated *antiCD3-RetroNectin/LymphoONE* CAR T cell products.

Our subsequent objective was to explore if the in vitro persistence of effector cells is influenced by their differentiation state. HER2-CAR T cells were restimulated bi-weekly on immobilized HER2-Fc molecules in a rechallenge assay. We found that CM-enriched HER2-CAR T cells had a reduced proliferative capacity compared to EM-enriched CAR T cell populations. In parallel, we characterized the memory composition and CD4/CD8 ratio of the CAR T cells after 3.5 and 10.5 days of continuous stimulation. According to our results, in *antiCD3-Retronectin/LymphoONE*-generated CAR T cell products, CAR-specific antigen stimulation reduced the frequency of $CD4^+$ T cells and restrained a significant portion of CM T cells from EM-directed differentiation. In contrast, *antiCD3-antiCD28/RPMI* CAR T cells almost completely differentiated into effector memory and terminal effector T cells.

In the final set of experiments, we focused on comparing the antitumor activity and expansion/persistence of EM- and CM-enriched CAR T cell products in vivo using a $HER2^+$ JIMT-1 xenograft model, in which either tumor cells or CAR T cells were genetically modified to express firefly luciferase. EM-enriched CD28.z and 41BB.z CAR T cells showed greater antitumor activity than their CM-enriched counterparts, improving overall survival. However, only the EM-dominant CD28.z CAR T cell product, characterized by a balanced CD4/CD8 distribution, achieved complete tumor eradication. NT T cells had no antitumor activity, confirming specificity. EM-enriched CD28.z CAR T cells expanded faster and for a longer time than CM-enriched T cell products. In accordance with this result, EM-enriched 41BB.z CAR T cells also outperformed their less differentiated counterparts.

6. Discussion

The inherent complexity of autologous immune cells poses a major challenge in optimizing their clinical application. The CAR-mediated tumor-specific cytotoxic effect, the long-term persistence of therapeutic cells, and the severity of side effects associated with the treatment depend on the synergy between the synthetic receptor and the physiological functions of T cells. Like any natural membrane receptor, CARs can form complex cell surface structures. Due to their chimeric nature, CARs can interact with the native partners of each of their protein domains. Thus, CAR activation signal upon target-specific stimulation is intertwined with the endogenous signaling processes of the lymphocyte. The success of CAR T therapies for B-cell leukemias and lymphomas was founded on optimizing this complex therapeutic system, ranging from the CAR design to the phenotypic profile of the heterogeneous cell product. However, clinical experiences with CAR T cells targeting solid tumors currently fall behind expectations, compelling their refinement to augment cytolytic activity and ensure sustained expansion.

The long-term therapeutic efficacy of CAR T cells depends mainly on the costimulatory endodomains integrated into the receptor constructs. Second-generation CARs, especially HER2-specific CARs utilizing a CD28 costimulatory domain, exhibited promising antitumor efficacy in preclinical animal models but failed to elicit a therapeutic response in clinical trials. Our findings on HER2-CAR T cells incorporating the 41BB.z costimulatory domain indicate their limited antitumor activity in HER2-positive xenograft models. Contrary to initial expectations, third-generation CAR T cells combining CD28 and 41BB costimulation in the clinic did not enhance cytotoxic efficacy and long-term expansion. Instead, their administration led to a fatal adverse event in a clinical trial targeting HER2-positive metastatic colorectal cancer, emphasizing the need for a thorough exploration of CAR functionality before progressing to the clinical phase.

Beyond their impact on killing efficacy and signal transduction pathways, we sought to unravel the influence of costimulatory domains on the behavior of chimeric receptors at the molecular level. We studied the molecular structures, cell surface

organization, and mobility of HER2-specific CAR constructs belonging to the first, second and third generations, each featuring distinct endodomains. Subsequently, we correlated these parameters with assembly and signaling activity of the immune synapse and early cytolytic activity. Initially, we demonstrated that first-generation .z and second-generation CD28.z CARs predominantly form dimers, aggregating into fewer yet larger surface clusters. In contrast, a substantial portion of 41BB.z and CD28.41BB.z CARs exists in oligomeric states and gather into significantly smaller yet more numerous membrane clusters. This observation is partly explicable by the structural prediction models of CAR constructs, suggesting that incorporating 41BB costimulatory endodomains results in a unique tertiary structure that facilitates the antigen-independent oligomerization of CARs. However, these findings do not exclude the possibility of other molecular mechanisms, such as scFv-mediated cross-linking, in the antigen-independent aggregation.

Our primary observation regarding the mobility of CARs suggests that the lateral diffusion of chimeric receptors accelerates proportionally as the CD3z effector endodomain moves further away from the membrane surface. Literature data indicate a significant increase in the cell surface mobility of the TCR/CD3 complex when its association with present in the plasma membrane is inhibited, supporting the hypothesis that the native interaction of CD3z effector domain with phosphoinositides may contribute to the observed changes of lateral diffusion in the case of CAR constructs as well.

Balagopalan and colleagues demonstrated that TCR nanoclusters in the resting T cell membrane enhance signaling following stimulation. Based on these findings, we hypothesized that pre-assembled receptor clusters and the mobility of receptors outside these clusters might influence the formation of the immunological synapse and the kinetics of activation in signaling pathways upon the interaction between CAR T effector cells and tumor cells. Therefore we investigated the accumulation of CARs and early activation using confocal microscopy, employing specific labeling for p-CD3z and p-Lck. Quantitative analysis of the recordings indicated that .z and CD28.41BB.z

constructs induced stronger CD3z and Lck phosphorylation than CD28.z and 41BB.z CARs, suggesting that cluster pre-formation and high receptor mobility may both be advantageous during early activation. However, concerning the cytotoxic effect, the 41BB-containing HER2-CARs, forming fragmented cluster structures, proved to be the least effective during the first 25 hours of co-culturing with tumor cells.

Optimal phenotype composition stemming from the differentiation processes of T lymphocytes has also contributed significantly to the success of CAR T therapies targeting hematological disorders. In our research, employing various stimulation and culturing methodologies, we generated CD28.z and 41BB.z HER2-CAR T cells with distinct phenotype profiles. The *antiCD3-antiCD28/RPMI* protocol yielded highly differentiated cell products, predominantly exhibiting the EM phenotype. In contrast, CAR T cell products generated through the *antiCD3-RetroNectin/LymphoONE* protocol exhibited a restricted differentiation process, resulting in an enrichment of CM lymphocytes.

Our functional in vitro studies confirmed that CAR T cell products containing a higher proportion of effector memory lymphocytes secreted significantly larger amounts of IFNγ and IL-2 cytokines in the presence of immobilized target antigens. Moreover, these EM-dominant CAR T cells elicited a stronger CAR-specific antitumor effect than their slowly differentiating counterparts. Furthermore, EM-dominant CAR T cells at an advanced differentiation stage demonstrated higher long-term proliferative potential upon repeated stimulation with immobilized target antigens. Consistent with these in vitro observations, CAR T cell products enriched in the EM phenotype efficiently infiltrated and eliminated tumor xenografts, exhibiting greater expansion than their CM-rich counterparts.

These findings appear to contradict the conclusions drawn from studies aiming to decipher the optimal phenotype composition of CD19-specific CAR T cells. Both preclinical and clinical investigations have shown that a heightened prevalence of naive and central memory T cell subtypes plays a pivotal role in endowing CD19-CAR T cells with robust tumor-specific cytolytic activity and the potential for sustained persistence

over extended periods, even spanning years. This phenomenon can be attributed to many factors, encompassing the distinct tissue localization tendencies of various T lymphocyte phenotypes – while activated effector memory cells predominantly occupy peripheral tissues, central memory lymphocytes maintain their residence within the bloodstream. In addition, leukemic blasts are readily accessible and relatively defenseless to circulating CAR T cells, which starkly contrasts the complex tumor microenvironment (TME) of solid tumors. Thus, it appears that the decreased cytolytic activity of CM-enriched CAR T cells is sufficient for the eradication of leukemic blasts, but insufficient to overcome the immunosuppressive TME of solid tumors, which requires a stronger T cell activation.

Although both CD28.z and 41BB.z EM-dominant CAR T cell products exhibited superior antitumor activity compared to their CM-dominant counterparts in our in vivo experiments, only CD28.z CAR lymphocytes characterized by a mature phenotype and a balanced CD4/CD8 distribution, as observed in our in vitro studies, achieved complete tumor eradication. Numerous studies emphasized that the synergistic antitumor potential of $CD4^+$ helper and $CD8^+$ cytotoxic CAR T cells positively influence overall antitumor efficacy. Therefore, our findings corroborate the concept that employing CAR T therapeutic cell products with a balanced CD4/CD8 ratio holds promise for enhanced efficacy against solid tumors.

7. Conclusions

In conclusion, our results from molecular and functional imaging suggest that chimeric antigen receptors incorporating 41BB are homooligomerized and that these oligomers form a larger number of smaller-sized clusters in the membrane of resting T cells. Lateral diffusion of CARs decreases proportionally as the CD3z effector domain is positioned further away from the inner surface of the membrane, owing to the insertion of one or more costimulatory domains. This suggests that the interaction of CD3z with phosphoinositides seen in the native TCR/CD3 complex might take place in chimeric receptors containing the CD3z effector domain. Confocal microscopy analysis of immunological synapses showed that both small clusters of highly mobile CD28.41BB.z and large preformed clusters of less mobile .z CAR induced more efficient proximal signaling than CD28.z or 41BB.z CARs of intermediate mobility. However, electric cell-substrate impedance sensing revealed that the CD28.41BB.z CAR performs the worst in sequential short-term elimination of adherent tumor cells, while the .z CAR is superior to all others. We conclude that the molecular structure, membrane organization, and mobility of CARs are critical design parameters that correlate with the formation of the immune synapse and the efficiency of target cell elimination.

Another possible aspect of optimizing CAR T therapy targeting solid tumors is the investigation of the coalescence of costimulatory domain-mediated activation signals with native T cell functions. We demonstrated that the method of CAR T cell maunfacturing influences the phenotypic composition of the resulting heterogeneous cell products and confirmed the significant impact of these characteristics on the function of CAR T cells. According to our findings, the more differentiated CAR T cell products enriched in the effector memory phenotype exhibit stronger in vitro cytotoxicity and proliferative potential compared to cell populations where differentiation was restricted during the manufacturing stage. Furthermore, we observed that 41BB costimulation and the use of RetroNectin during T cell expansion led to a

significant decrease in the proportion of $CD4^+$ cytotoxic CAR T cells. Our preclinical model demonstrated that differentiation toward the effector memory phenotype and a balanced $CD4^+/CD8^+$ ratio confer the most potent expansion and cytolytic efficacy for CAR T cells targeting $HER2^+$ solid tumors. This realization emphasizes that optimizing CAR T cell product manufacturing and determining the ideal phenotypic profile based on the type and antigen profile of the treated tumor are essential prerequisites for successful clinical trials.

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List of publications related to the dissertation

1. Barden, M., Holzinger, A., Velas, L., **Mezősi-Csaplár, M.**, Szöőr, Á., Vereb, G., Schütz, G., Hombach, A. A., Abken, H.: CAR and TCR form individual signaling synapses and do not cross-activate, however, can co-operate in T cell activation.
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2. **Mezősi-Csaplár, M.**, Szöőr, Á., Vereb, G.: CD28 and 41BB Costimulatory Domains Alone or in Combination Differentially Influence Cell Surface Dynamics and Organization of Chimeric Antigen Receptors and Early Activation of CAR T Cells.
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3. **Mezősi-Csaplár, M.**, Szöllősi, J., Gottschalk, S., Vereb, G., Szöőr, Á.: Cytolytic Activity of CAR T Cells and Maintenance of Their CD4+ Subset Is Critical for Optimal Antitumor Activity in Preclinical Solid Tumor Models.
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