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# **CAR und TCR bilden unabhängige Synapsen ohne Kreuzaktivierung, können jedoch in der T-Zell-Aktivierung kooperieren**

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Herrn Univ.-Prof. Dr. Hinrich Abken  
Herrn Dr. Andreas Hombach  
Frau Dr. Astrid Holzinger

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## Übersicht der Publikationen:

**Barden M**, Holzinger A, Velas L, *et al.* CAR and TCR form individual signaling synapses and do not cross-activate, however, can co-operate in T cell activation. *Frontiers in Immunology* 2023; 14. <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1110482>.

Diese Publikation ersetzt die Kapitel Material und Methoden, sowie Ergebnisse. Teile der Zusammenfassung, Einleitung und Diskussion sind im Wortlaut aus der Publikation übernommen worden. Aus der Publikation übernommene Abbildungen sind im Einzelnen gekennzeichnet.

Die den Abbildung 1,2 und 5A der Publikation zugrundeliegenden Experimente sind durch Herrn Dr. Andreas Hombach und mit technischer Unterstützung der Assistentinnen Frau Birgit Hops, Frau Petra Hoffmann und Frau Danuta Chrobok am Zentrum für Molekulare Medizin Köln (ZMMK) durchgeführt und gemeinsam mit mir ausgewertet worden. Jurkat76-Zellen wurden von Frau Dr. M.H.M. Heemskerk zur Verfügung gestellt.

Die in Abbildung 3 und 5B der Publikation dargestellten Westernblotexperimente sind durch Frau Dr. Astrid Holzinger und mit technischer Unterstützung der Assistentinnen Frau Charlotte Schenkel, Frau Linda Otzelberger, Frau Anja Pavlica und Frau Dorothea Weber-Steffens am Leibniz-Institut für Immuntherapie (LIT) in Regensburg durchgeführt und gemeinsam mit mir ausgewertet worden.

Die in Abbildung 4 A-E der Publikation dargestellten Experimente wurden von mir mit Unterstützung von Herrn Lukas Velas an der TU Wien, Österreich, durchgeführt. Der Versuchsplan für die mikroskopiebasierten Experimente wurde von mir selbstständig erarbeitet und in Diskussion mit Herrn Prof. Hinrich Abken, Herrn Dr. Andreas Hombach, Herrn Prof. György Vereb, Herrn Dr. Árpád Szöör, Herrn Prof. Gerhard Schütz, Frau Marianna Mezösi-Csaplár und Herrn Dr. Lukas Velas optimiert. Die Mikroskopieaufnahmen wurden von mir mit der Hilfe von Herrn Dr. Lukas Velas an einem für den Versuchsaufbau angepassten TIRF-Mikroskop durchgeführt. Die Methode zur Auswertung der Bilddaten wurde von mir gemeinsam mit Herrn Dr. Lukas Velas erarbeitet und mit individualisiertem Python Code ausgeführt.

Die in Abbildung 4 F-H, sowie in Abbildung S1 (Anhang), und Video S1-3 (Anhang) der Publikation dargestellten Experimente wurden von Frau Marianna Mezösi-Csaplár an der Universität Debrecen, Ungarn, durchgeführt und ausgewertet. Die betreffenden Daten und wissenschaftlichen Ergebnisse sind Teil der Dissertation von Frau Marianna Mezösi-Csaplár und sind deshalb vom Ergebnisteil meiner Dissertation ausgenommen.

Abbildung 6 der Publikation wurde von mir in Diskussion mit allen beteiligten Autoren als schematische Darstellung der Bündelung der experimentellen Ergebnisse erstellt. Die grafische Ausarbeitung erfolgte mit BioRender.com.

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Köln, den 20.03.2023

Unterschrift:



## **Danksagung**

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Den offenen Fragen.

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## Abkürzungsverzeichnis

Gebräuchliche Abkürzungen sowie SI-Basiseinheiten wurden nicht gesondert aufgeführt.

<b>BCMA</b>	B-cell maturation antigen/B-Zell-Reifungsantigen
<b>CAR</b>	chimärischer Antigen Rezeptor
<b>CD</b>	Cluster of Differentiation
<b>CLL</b>	chronisch lymphatische Leukämie
<b>DAG</b>	Diacylglycerol
<b>DNA/DNS</b>	deoxyribonucleic acid/Desoxyribonukleinsäure
<b>Fc</b>	fragment crystallisable
<b>FcεRI</b>	hochaffiner IgE-Rezeptor
<b>FDA</b>	food and drug agency
<b>IFN-γ</b>	Interferon-gamma
<b>IL-2</b>	Interleukin-2
<b>K<sub>D</sub></b>	Gleichgewichtsdissoziationskonstante
<b>KLRG-1</b>	Killerzellen-Lektin-ähnlicher Rezeptor der Unterfamilie G, Mitglied 1
<b>LAT</b>	Linker for activation of T cells
<b>LCK</b>	lymphozytenspezifischen Tyrosinkinase
<b>LFA-1</b>	lymphozytenfunktions-assoziiertes Antigen 1
<b>MAPK</b>	mitogen-aktivierte Proteinkinase
<b>MHC</b>	Haupthistokompatibilitätskomplex
<b>MTOC</b>	microtubule organizing center
<b>PDZ</b>	Proteininteraktionsdomäne
<b>PKC</b>	Proteinkinase C
<b>Rev-CAR</b>	reversed CAR, Adapter CAR System
<b>scFv</b>	single chain fragment of variable region
<b>SMAC</b>	supramolekulares Aktivierungscluster
<b>TCR</b>	T-Zell-Rezeptor
<b>TIRFM</b>	interne Totalreflexionsfluoreszenzmikroskopie
<b>TM</b>	Transmembrandomäne
<b>TRUCK</b>	T cells redirected for antigen-unrestricted cytokine-initiated killing
<b>ZAP-70</b>	zeta-assoziiertes Protein 70
<b>4-1BB</b>	CD137, kostimulatorische Domäne des CARs



## 1. Zusammenfassung

Adoptive T-Zell-Therapien mit chimären Antigenrezeptoren (CARs) zeigen langfristige Erfolge in der Behandlung fortgeschrittener hämatologischer Tumorerkrankungen; die Therapie solider Tumore stellt jedoch eine größere Herausforderung dar und ist noch in der Entwicklung. Die Aufklärung des Mechanismus der CAR-vermittelten T-Zell-Aktivierung ist eine wichtige Voraussetzung, um CAR T-Zellen so gestalten zu können, dass sie die Hürden solider Tumore überwinden.

Der CAR ist mit dem physiologischen T-Zell-Rezeptor (TCR)/CD3-Komplex koexprimiert und nutzt dessen Signaldomänen. Es ist nicht bekannt, ob die CAR-vermittelte T-Zell-Aktivierung von der Anwesenheit des TCRs beeinflusst wird und ob es eine gegenseitige Aktivierung von CAR und TCR nach Erkennung des respektiven Antigens gibt.

In dieser Arbeit wurde gezeigt, dass die Expressionsdichte des CD3 $\zeta$  CARs unabhängig vom TCR-assoziierten CD3 $\zeta$  ist. In CD3 $\zeta$  KO T-Zellen konnte das für die Ausbildung des TCR-Komplexes benötigte CD3 $\zeta$  des TCRs nicht durch das CD3 $\zeta$  des CARs ersetzt werden. Eine Aktivierung des CARs bewirkte keine Phosphorylierung des TCR-assoziierten CD3 $\zeta$ . Umgekehrt induzierte eine Aktivierung des TCRs keine Phosphorylierung des CAR CD3 $\zeta$ . Folglich gab es keine Kreuzaktivierung der CAR und TCR Signale.

Mit Hilfe von Interner Totalreflexionsfluoreszenzmikroskopie (TIRFM) und „fast AiryScan“-Mikroskopie wurde nachgewiesen, dass CAR und TCR auf Membranebene unabhängig voneinander eigene Kontaktregionen mit ihrem Zielantigen bilden. Nach Erkennung des respektiven Zielantigens können CAR und TCR jedoch in der Signalgebung kooperieren und Effektorfunktionen induzieren. Dies ermöglicht Boolesche UND-Funktionen bei der kombinatorischen Antigenerkennung durch CAR und TCR.

Die hier generierten Daten deuten darauf hin, dass das tonische Signal eines TCR die CAR-vermittelte T-Zell-Aktivierung und das langfristige Überleben der T-Zelle unterstützen könnte. Der endogene TCR ist somit potentiell relevant für eine langfristige Aufrechterhaltung der T-Zell-Funktionen.

## 2. Einleitung

Mit Hilfe chimärer Antigenrezeptoren (CAR) können T-Zellen gezielt gegen tumorspezifische Oberflächenmerkmale ausgerichtet werden. Die reprogrammierte Immunantwort führt zur Tumorzelleliminierung. CAR T-Zellen sind in der Lage, fortgeschrittene hämatologische Tumorerkrankungen wie Chemotherapie-refraktäre lymphatische Leukämien vollständig und dauerhaft zu heilen <sup>1</sup>. Carl June und Kollegen berichten in einer aktuellen Publikation (Februar 2022) über zwei Patienten mit chronisch lymphatischer Leukämie (CLL), welche sich seit Behandlung mit CD19 CAR T-Zellen im Jahr 2010 immer noch in Komplettremissionen befinden. Im peripheren Blut dieser Patienten waren mehr als ein Jahrzehnt nach Injektion noch CAR T-Zellen nachweisbar <sup>2</sup>. Die herausragenden Ergebnisse des CD19-CARs konnten mittlerweile in unabhängigen Zentren reproduziert werden <sup>3</sup>. Die Therapie solider Tumore ist bis jetzt jedoch weniger erfolgreich.

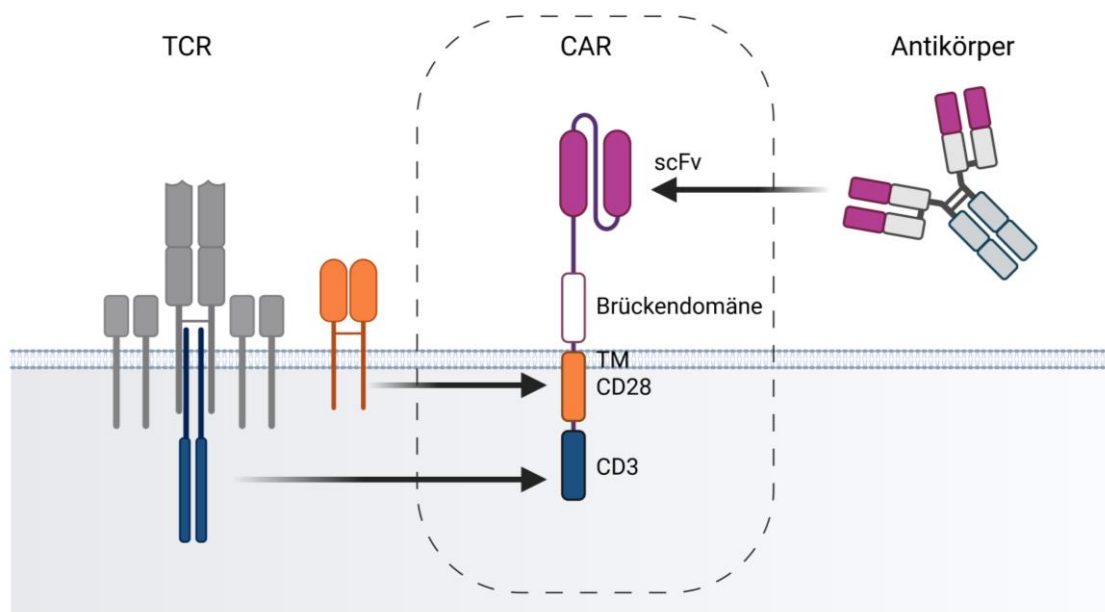
Seit 2017 wurden sechs CAR T-Zell-Therapien von der US-amerikanischen Arzneimittelaufsichtsbehörde FDA zugelassen. Alle bisher zugelassenen CAR T-Zell-Therapien basieren auf autologen T-Zellen mit intaktem endogenem TCR <sup>4</sup>. Neben dem Großteil der autologen CAR T-Zell-Therapien befinden sich erste TCR-defiziente allogene CAR T-Zell-Produkte in der klinischen Erprobungsphase <sup>4-7</sup>.

Als "Living Drugs" heben sich CAR T-Zellen deutlich von klassischen Pharmazeutika ab <sup>8</sup>. CAR T-Zellen wandern aktiv in Gewebe und verlassen sie wieder in die periphere Zirkulation, proliferieren und üben spezifische Effektorfunktionen aus. Inhibitorische Signale in der Tumorumgebung solider Tumoren reduzieren das Einwandern und die Aktivierung der CAR T-Zellen <sup>9</sup>.

CAR und TCR bedienen sich der gleichen Signalmoleküle bei der T-Zell-Aktivierung. Der Einfluss des endogenen TCR/CD3-Komplexes auf die Stabilität und Funktion des CAR und somit die CAR T-Zell-Aktivierung ist bis jetzt nicht ausreichend erforscht. Man verspricht sich, dass Erkenntnisse über eine mögliche Kreuzaktivierung und/oder Kooperation von CAR und TCR hierbei die Grundlage für die Entwicklung verbesserter CAR T-Zell-Therapien schaffen können.

## 2.1. Generationen der chimären Antigenrezeptoren

Der physiologische T-Zell-Rezeptor (TCR) ist ein Multi-Protein-Komplex, der die Signaldomänen auf mehrere Ketten aufteilt (in trans). Der klassische CAR hingegen verknüpft alle Domänen in einer einzigen Molekülkette (in cis)<sup>10</sup>. Die von einem Antikörper abgeleitete Bindedomäne ermöglicht dem CAR die MHC-unabhängige Erkennung des Zielantigens (Abb. 1)<sup>10</sup>. CARs der ersten Generation beinhalten nur das Primärsignal CD3 $\zeta$ . In der Klinik am besten erprobt sind CARs der zweiten Generation, welche neben der Signaldomäne eine kostimulatorische Domäne wie CD28 oder 4-1BB tragen<sup>11</sup>. Diese zusätzliche Kostimulation fördert eine anhaltende T-Zell-Antwort und Persistenz<sup>2</sup>. Die dritte Generation der CARs kombiniert zwei kostimulatorische Domänen. CARs der vierten Generation, sogenannte „T cells redirected for universal cytokine killing“ (TRUCKs), bieten die Möglichkeit zur Modulation des Tumormilieus und Rekrutierung weiterer Immunzellen durch induzierbare Sekretion von Zytokinen, Antikörpern oder anderen Molekülen<sup>12,13</sup>. Neue CAR-Formate basieren auf der direkten Integration von Zytokinen in das CAR-Konstrukt<sup>14</sup>. Einfügung von Interleukin-12 in die extrazelluläre Domäne des CARs verleiht der T-Zelle ähnliche Eigenschaften der natürlichen Killerzelle, welche sie zur Eliminierung antigen-negativer Tumorzellen einsetzen kann<sup>15</sup>. Um die CAR T-Zell-Therapie an die individuellen Eigenschaften eines soliden Tumors anpassen zu können, bedarf es weiterer Optimierung der CARs.

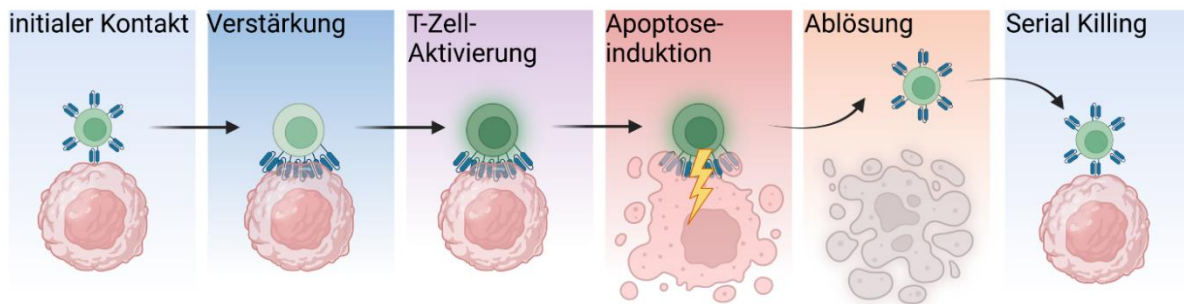


**Abbildung 1: Aufbau des CARs**

Der CAR-Prototyp setzt sich aus einer extrazellulären Einzelkettenbindedomäne (scFv), fakultativ einer extrazellulären Brückendomäne, einer Transmembrandomäne (TM) und intrazellulären Signaldomänen zusammen. Der scFv leitet sich von einem monoklonalen Antikörper (variable Region der schweren und der leichten Ig Kette) ab. Die CD3 $\zeta$  und CD28 Signaldomänen stammen aus dem TCR-Komplex der T-Zelle.

## 2.2. Funktionsweise der CAR T-Zell-Immunsynapse

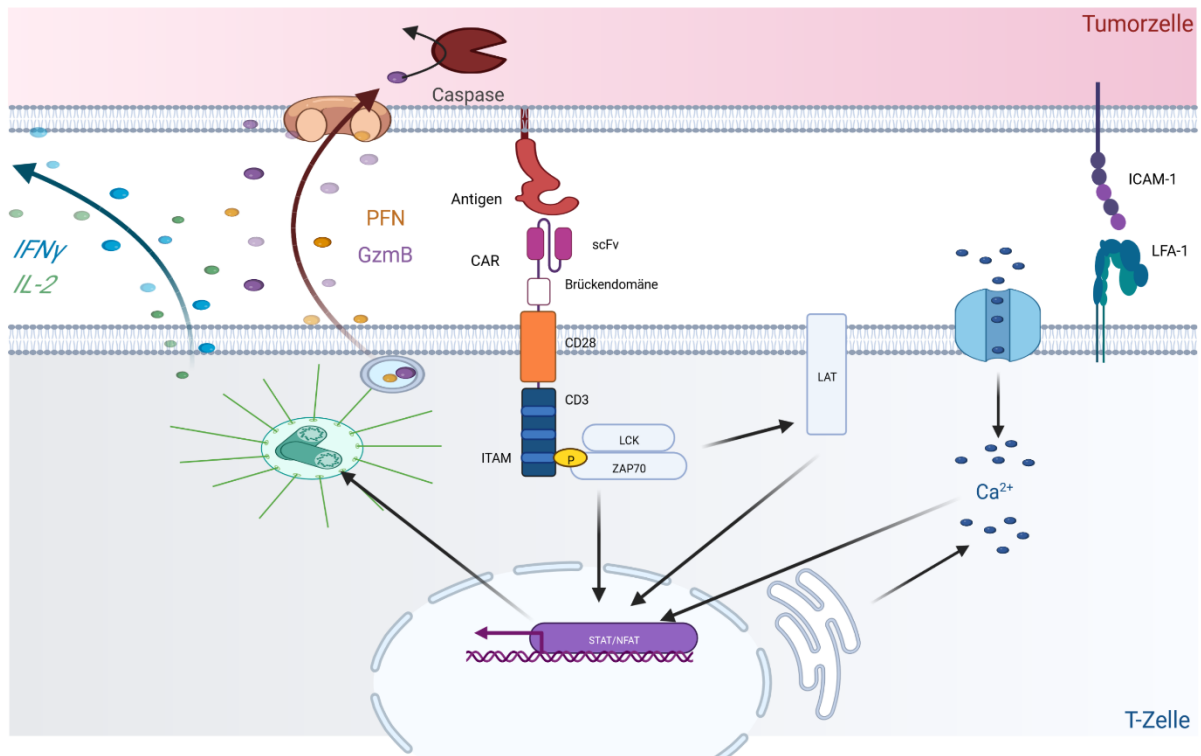
Der initiale Schritt der CAR T-Zell-Aktivierung ist die Bindung des CARs an das entsprechende Zielantigen (Abb. 2). Im Gegensatz zum TCR, welcher bereits nach Erkennung von weniger als 10 Agonisten eine T-Zell-Antwort generiert <sup>16</sup>, benötigt der CAR die Bindung an tausende Antigenmoleküle zur Einleitung der T-Zell-Aktivierung <sup>17</sup>. Die Affinität der CAR-Liganden Bindung ( $K_D$ -Werte im nanomolaren Bereich) ist höher als die Affinität der TCR-Liganden Bindung ( $K_D$ -Werte im micromolaren Bereich) <sup>18,19</sup>.



**Abbildung 2: Schritte der CAR-vermittelten Tumorzelleliminierung**

Die Antigenerkennung, Rezeptoraggregation und Ausbildung der Kontaktregion werden als Initiation der CAR-vermittelten Tumorzelleliminierung zusammengefasst, gefolgt von der Einschleusung zytolytischer Enzyme in die Zielzelle. Die Ablösung der CAR T-Zelle von der Tumorzelle und der fortschreitende Apoptoseprozess der Tumorzelle werden als Termination zusammengefasst. CAR T-Zellen eliminieren entweder weitere Tumorzellen und durchlaufen den beschriebenen Prozess erneut (Serial Killing) oder sterben durch Apoptose.

Nach Bindung des Zielantigens wird der Kontakt durch Aggregation des Immunrezeptors verstärkt. Es kommt zur Bildung von „Microclustern“. Die Immunrezeptor Tyrosin-basierten Aktivierungsmotive (ITAMs) der CD3 $\zeta$  Domäne rekrutieren die Kinase ZAP70 zur Zellmembran (Abb. 3). Die Aktivierung des TCRs induziert eine Phosphorylierung des Linker for activation of T cells (LAT) und verstärkt die Ausbildung von TCR-Microclustern <sup>20</sup>. Im Gegensatz zum TCR ist LAT für die Ausbildung der immunologischen Synapse des CARs nicht zwingend erforderlich <sup>21</sup>. Die kostimulatorische Domäne im CAR der zweiten und dritten Generation trägt möglicherweise zum LAT-unabhängigen CAR-Signaling bei <sup>21</sup>. Die CD28-Transmembrandomäne des CARs vermittelt eine Heterodimerisierung mit endogenem CD28 <sup>22</sup>. Homodimere des endogenen CD28 können wiederum CAR-Clustering herbeiführen und somit das LAT-unabhängige CAR-Signaling verstärken.



**Abbildung 3: Effektorfunktionen nach CAR-vermittelter T-Zell-Aktivierung.**

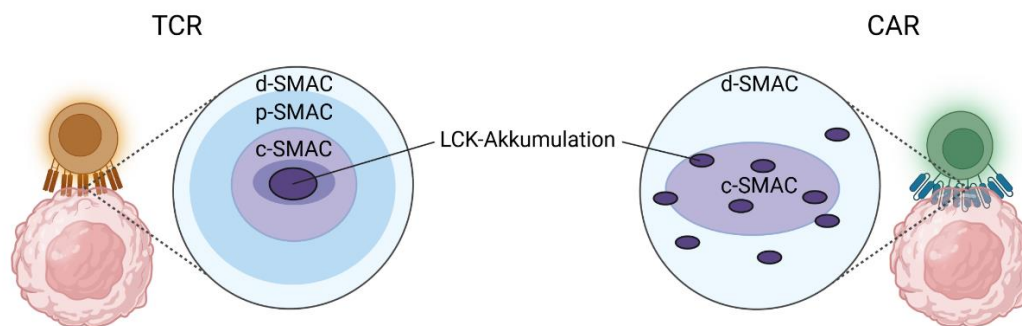
Die Antigenerkennung durch den CAR initiiert die Aktivierungskaskade der T-Zelle. Diese resultiert u.a. in der Freisetzung von Zytokinen, Sekretion zytolytischer Granula, Umstrukturierung der Aktinfilamente und Kalzium-Influx.

Die nachgeschaltete Signaltransduktion führt zu Kalzium-Influx, MAPK-Aktivierung und Aktin-Remodelling. Die inhibitorisch wirkende Tyrosinphosphatase CD45 wird durch die Annäherung der Zellmembranen aus der Kontaktregion verdrängt und die T-Zell-Aktivierung initiiert <sup>23,24</sup>. Die Polarisierung des T-Zell-Zentrosoms, welches auch als Mikrotubuliorganisierendes Zentrum (MTOC) bezeichnet wird, resultiert in der Beförderung zytolytischer Granula der T-Zelle zur Kontaktregion. Bei der Ausbildung der TCR-Immunsynapse vermitteln das Lipid Diacylglycerol (DAG) als Second Messenger und die Proteinkinase C (PKC) die Rekrutierung des Motorproteins Dynein <sup>25,26</sup>; dies hängt räumlich und zeitlich von der F-Aktin-Depletion im zentralen supramolekularen Aktivierungscluster (c-SMAC) ab <sup>27</sup>. Dynein richtet die Mikrotubuli neu aus und das Zentrosom wird zur Immunsynapse bewegt. Das Zentrosom wird vom Golgi-Apparat, dem endosomalen Kompartiment, und lytischen Granula mit Perforin (PFN) und Granzym begleitet. Da das F-Aktin-depletierte c-SMAC in der CAR Synapse kleiner ist als in der TCR-Synapse, ist die Dynein-Rekrutierung möglicherweise weniger effizient.

Zytolytische T-Zellen eliminieren Zielzellen meistens durch Freisetzung von Perforin und Granzym. Perforin und Granzyme werden durch Exozytose in den interzellulären Spalt ausgeschüttet. Perforin durchlöchert die Zielzellmembran und ermöglicht das Eindringen von

Granzym in die Zielzelle. Die Serinprotease Granzym B (GzmB) induziert in den Zielzellen Apoptose, indem sie Caspase und Substrate des Caspase-Signalwegs spaltet. T-Zellen können ebenfalls über alternative Wege wie die Interaktion von Fas-Ligand und Fas-Rezeptor Apoptose auslösen. Zelltod durch Apoptose resultiert in einer gezielten Beseitigung der betroffenen Zelle durch Fragmentierung und anschließende Phagozytose. Nach Ablösung von der Tumorzelle kommt es entweder zur Eliminierung weiterer Tumorzellen, dem sogenannten „Serial Killing“<sup>28</sup>, oder es folgt Anergie, Erschöpfung oder Zelltod der CAR T-Zelle.

Während der physiologische TCR mit seiner Zielzelle nach einem geordneten Schema von supramolekularen Aktivierungsklustern eine TCR-Synapse<sup>29</sup> ausbildet (Abb. 4), ist die Kontaktregion des CARs weniger strukturiert. Die Akkumulation der lymphozytenspezifischen Tyrosinkinase (LCK) ist beim CAR im Gegensatz zum TCR disorganisiert<sup>30</sup>. Lymphozytenfunktions-assoziiertes Antigen 1 (LFA-1) ist unregelmäßig in der CAR-Immunsynapse verteilt. Durch die schwächere Adhäsion kann sich der CAR-vermittelte Kontakt schneller ablösen als der TCR-Kontakt<sup>30</sup>.



**Abbildung 4: TCR-Synapse vs. CAR-Kontaktregion**

Die TCR-Synapse ist als distales, proximales und zentrales supramolekulares Aktivierungskluster (d-, p- und c-SMAC) organisiert. Im Zentrum des c-SMACs kommt es zur LCK-Akkumulation. Die CAR-Kontaktregion ist unregelmäßiger in d- und c-SMAC aufgeteilt. LCK akkumuliert an mehreren Stellen in Mikroclustern (adaptiert von Davenport et al.<sup>30</sup>).

### 2.3. TCR-defiziente CAR T-Zellen

Der Großteil der zum jetzigen Zeitpunkt klinisch eingesetzten CAR T-Zell-Produkte basiert auf T-Zellen mit endogenem TCR. Eine Integration der CAR-kodierenden DNS in den Locus der TCR- $\alpha$ -Kette erzeugt TCR-defiziente CAR T-Zellen<sup>31</sup>. Um allogene CAR T-Zell-Therapie zu ermöglichen, wird der Einsatz von TCR-negativen T-Zellen in der CAR T-Zell-Produktion erprobt<sup>5</sup>. TCR-defiziente CAR T-Zellen zeigten jedoch in ersten Studien eine reduzierte Persistenz in vivo im Vergleich zu CAR T-Zellen mit endogenem TCR, die eine verlängerte Persistenz zeigten<sup>32</sup>. Der CAR scheint nicht vollends in der Lage zu sein, die Signalfunktionen des endogenen TCRs zu ersetzen.

## 2.4. Zielsetzung

In dieser Arbeit wird untersucht, wie sich das Vorhandensein des endogenen TCR/CD3-Komplexes auf die Stabilität und Funktion des CARs und vice versa auswirkt. Dies ist von besonderer Bedeutung, da konventionelle CAR T-Zellen einen funktionalen TCR/CD3-Komplex exprimieren und somit mit dem TCR um Signalmoleküle kompetitieren. Eine Kreuzaktivierung würde zur Aktivierung von CAR und TCR bei Erkennung nur eines der beiden respektiven Zielantigene führen.

Mit Hilfe von Interner Totalreflexionsfluoreszenzmikroskopie (TIRFM) und „Fast AiryScan“-Mikroskopie wird untersucht, ob der TCR in die CAR Synapse integriert oder eine vom CAR unabhängige Kontaktregion mit dem Zielantigen ausbildet. Des Weiteren soll untersucht werden, ob es zu einer Kreuzaktivierung des CAR und TCR kommt. Hierfür wird die Phosphorylierung der CD3 $\zeta$  Kette analysiert, die ein frühes Ereignis in der T-Zell-Aktivierung darstellt. Außerdem wird die Sekretion von Zytokinen untersucht, die als Effektormoleküle des distalen Signalweges dienen.

Die hierdurch gewonnenen Erkenntnisse fördern die Entwicklung neuer Strategien zur Integration der Signale des TCRs und des CARs, was wiederum neue Therapieansätze durch kooperative Erkennung durch TCR und CAR auf derselben T-Zelle ermöglicht.

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# CAR and TCR form individual signaling synapses and do not cross-activate, however, can co-operate in T cell activation

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In engineered T cells the CAR is co-expressed along with the physiological TCR/CD3 complex, both utilizing the same downstream signaling machinery for T cell activation. It is unresolved whether CAR-mediated T cell activation depends on the presence of the TCR and whether CAR and TCR mutually cross-activate upon engaging their respective antigen. Here we demonstrate that the CD3ζ CAR level was independent of the TCR associated CD3ζ and could not replace CD3ζ to rescue the TCR complex in CD3ζ KO T cells. Upon activation, the CAR did not induce phosphorylation of TCR associated CD3ζ and, vice versa, TCR activation did not induce CAR CD3ζ phosphorylation. Consequently, CAR and TCR did not cross-signal to trigger T cell effector functions. On the membrane level, TCR and CAR formed separate synapses upon antigen engagement as revealed by total internal reflection fluorescence (TIRF) and fast AiryScan microscopy. Upon engaging their respective antigen, however, CAR and TCR could co-operate in triggering effector functions through combinatorial signaling allowing logic "AND" gating in target recognition. Data also imply that tonic TCR signaling can support CAR-mediated T cell activation emphasizing the potential relevance of the endogenous TCR for maintaining T cell capacities in the long-term.

## KEYWORDS

immunotherapy, adoptive cell therapy, CAR, TCR, synapse

## Introduction

Chimeric antigen receptors (CARs) can be remarkably powerful in redirecting a T cell response towards defined target cells (1) while utilizing the TCR/CD3 downstream signaling machinery for triggering T cell activation upon target engagement. Most "second generation" CARs in clinical application incorporate the CD3ζ signaling chain to provide the primary

signal together with the costimulatory domain to add the second signal in order to trigger T cell activation (2–5). While this type of CAR is efficacious in clinical application, little is known whether the endogenous TCR/CD3 complex affects the stability and function of the CAR and vice versa. This is a relevant issue since conventional CAR T cells express a functionally active TCR/CD3 complex with the consequence that the CAR competes with the TCR for downstream signaling molecules (6, 7). This situation may result in a functional cross-talk between CAR and TCR upon either target recognition. The issue is also of relevance when replacing the TCR  $\alpha$ -chain locus of the endogenous TCR by the CAR encoding DNA sequence (8) thereby producing TCR-deficient CAR T cells. “Off-the-shelf” CAR T cell therapy also uses TCR<sup>+</sup> T cells for manufacturing (9). In both situations, CAR redirected T cell activation would not compete with the endogenous TCR, however, would not get “help” by tonic TCR signaling.

The TCR associated CD3 $\zeta$  chain is crucial for regulating the stability of the entire TCR complex and experiences a rapid turn-over on the T cell membrane independently of the other TCR chains (10). The impact of the TCR associated CD3 $\zeta$  chain on the CD3 $\zeta$ -based CAR with respect to expression and function was so far not addressed. Mutual co-regulation of the TCR and CAR would have substantial consequences for both CAR- and TCR-mediated T cell activation. This became most recently obvious when CAR T cells with genetically deleted TCR experienced reduced persistence *in vivo* compared to CAR T cells with the endogenous TCR (11). On the other hand, TCR<sup>+</sup> CAR T cells showed superior persistence implying that the CAR cannot fully substitute for the TCR in sustaining downstream functional capacities.

We asked whether the TCR affects a CD3 $\zeta$  CAR, and vice versa, in T cell activation on the membrane level of chain phosphorylation and on the downstream level of effector functions. We revealed that TCR and CAR are co-regulated on the T cell surface and can complement in providing downstream T cell activation. However, there is no cross-phosphorylation of CAR and TCR CD3 $\zeta$  signaling chains. Accordingly, the TCR is not recruited into the CAR synapse as revealed by TIRF and fast AiryScan microscopy. Such lack of cross-signaling allows Boolean logic “AND” gating during combinatorial antigen recognition through the TCR and CAR.

## Materials and methods

### Cell lines and cell culture

The murine T cell hybridoma line MD45 was described elsewhere (12). The human Jurkat T cell line (ATCC TIB-152), the N87 (ATCC CRL-5822) and the CA19-9<sup>+</sup> and CA19-9<sup>-</sup> human tumor cell lines LS174T (ATCC CCL-188), H498 (ATCC CCL-254), H716 (ATCC CCL-251) and A375 (ATCC CRL-1619) were obtained from ATCC. Jurkat 76 cells lacking endogenous TCR expression (13) were kindly provided by Dr M.H.M. Heemskerk, Leiden, The Netherlands. Cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated FCS. The N87 human gastric carcinoma cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM GlutaMAX, 10% (v/v) FCS and

antibiotics. HEK293T cells are human embryonic kidney cells that express the SV40 large T antigen (14). Anti-CD3 mAb OKT3 and anti-CD28 mAb 15E8 were purified by affinity chromatography from supernatants of OKT3 hybridoma (ATCC CRL 8001) and 15E8 hybridoma cells (kindly provided by Dr. R. van Lier, Red Cross Central Blood Bank, Amsterdam, The Netherlands), respectively. The anti-BW431/26 idiotype antibody BW2064 was described earlier (15). Recombinant ErbB2-Fc protein was purchased from R&D Systems, Wiesbaden, Germany. The PE-conjugated and the AF647-conjugated F(ab')<sub>2</sub> goat anti-human IgG antibody, goat anti-human IgG-UNLB antibody, goat anti-mouse IgG human ads-UNLB and rabbit anti-goat IgG (H+L)-UNLB antibody were purchased from Southern Biotechnology, Birmingham, AL, USA. PE-conjugated anti-CD3 $\zeta$  mAb clone 6B10.2 and AF647-conjugated anti-TCR  $\alpha/\beta$  mAb clone IP26 was purchased from BioLegend, San Diego, CA, USA. Fluorochrome-conjugated anti-human CD3 mAb was purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. Fluorochrome-conjugated isotype controls were purchased from BD Biosciences, San Diego, CA, USA. Matched antibody pairs for capture and detection of human IFN- $\gamma$  and IL-2 were purchased from BD Biosciences. Recombinant IL-2 was obtained from Endogen, Woburn, MA, USA. Alkaline phosphatase conjugated streptavidin was purchased from Roche Diagnostics, Mannheim, Germany. Peroxidase-labeled goat anti-human IgG Fc antibody and peroxidase-labeled anti-mouse IgG Fc antibody were purchased from Dako, Hamburg, Germany. Anti-actin antibody (clone 1A4) was purchased from Thermo Fisher Scientific, Dreieich, Germany. AF647-conjugated transferrin receptor monoclonal antibody (MEM-75) was purchased from Invitrogen, Regensburg, Germany.

### Genome editing of Jurkat cells

Deletion of CD3 $\zeta$  in Jurkat cells was performed by CRISPR/Cas9 mediated genome editing utilizing the CD3 $\zeta$  CRISPR/Cas9 ko plasmid coding for a human CD3 $\zeta$  guide RNA and the CD3 $\zeta$  homology directed repair (HDR) plasmid for site specific integration of a puromycin resistance gene (both Santa Cruz Biotechnology, Dallas, TX, USA). Briefly, 5 × 10<sup>6</sup> Jurkat cells were transfected with 2  $\mu$ g of each plasmid DNA utilizing the MACSfectin transfection system (Miltenyi Biotec) according to the manufacturer's recommendations. Two days after transfection cells were further cultured in presence of 250 ng/ml puromycin (Sigma Aldrich, Taufkirchen, Germany). Puromycin resistant subclones were established and tested for expression of CD3 $\zeta$  by flow cytometry and Western blot analysis.

### Preparation of human T cells

Peripheral blood lymphocytes were obtained from healthy donors by Ficoll density centrifugation (Ethic approval 01-090 Cologne; Ethic approval 21-2224-101 Regensburg). T cells were initially activated by OKT3 (100–200 ng/ml) and 15E8 (50–100 ng/ml) antibodies and IL-2 (400–1,000 U/ml) and further cultured in the presence of IL-2 (100–500 U/ml).

## Engineering and expression of CARs

Cloning and expression of CAR constructs were described previously (5, 16–19). MD45 T hybridoma cells with stable expression of  $\zeta$ - and  $\gamma$ -chain CARs were generated as follows: The DNA for  $\zeta$ - and  $\gamma$ -chain CARs in pRSV (50–100  $\mu$ g) was transfected into  $2 \times 10^7$  MD45 T cells by electroporation (one pulse, 250 V, 2400  $\mu$ F) using a gene pulse electroporator (BioRad, Munich, Germany). After culture for two days, transfected cells with CAR expression were selected in the presence of G418 (2 mg/ml; Gibco, Eggenheim, Germany). For expression of CARs in peripheral blood T cells and Jurkat cells all CARs were cloned into the same retroviral expression vector as previously described (20). Transduction of T cells was previously described (5, 20, 21). Briefly, peripheral blood T cells were activated with anti-CD3 (100–200 ng/ml) and anti-CD28 (50–100 ng/ml) antibodies and IL-2 (400–1,000 U/ml). Cells were transduced on day 2–3 by co-cultivation with virus producing 293T cells or, alternatively, with  $\gamma$ -retrovirus containing supernatants. Retroviruses were transiently produced by 293T cells upon transfection with vector DNA and plasmids encoding the GALV envelope and MMLV derived gag/pol (21). For transient expression in non-lymphoid cells, CAR encoding DNAs were transfected in 293T cells. CAR expression was monitored by flow cytometry using an antibody against the common extracellular IgG1 Fc domain.

## Immunofluorescence and flow cytometry

The CAR on the cell surface of engineered T cells was detected by FITC- or PE-labeled antibodies against the human IgG1 Fc domain and T cells were identified with fluorochrome-labeled anti-CD3 antibodies which recognize an epitope located on the  $\epsilon$ -chain of the CD3 complex, respectively. Flow cytometry was performed using a FACScan<sup>TM</sup> cytofluorometer equipped with the FACScan<sup>TM</sup> research software type-B (BD Bioscience), a FACSCanto II flow cytometer equipped with the FACSDiva software (BD Bioscience), and FACSLytic flow cytometer equipped with FACSuite software (BD Bioscience). To monitor expression of the  $\zeta$ -chain, cells were permeabilized and fixed utilizing the Cytofix/Cytoperm<sup>TM</sup> reagent kit (BD Bioscience) prior to incubation with the PE-conjugated anti-CD3 $\zeta$  mAb (2  $\mu$ g/ml).

## Pulse chase labeling of CARs

CAR engineered cells ( $5 \times 10^7$  cells/ml) were washed twice in cold PBS, pH 7.6, and incubated with 100  $\mu$ g/ml biotin- $\epsilon$ -amidocaproate-N-hydroxy-succinimidester (Sigma-Aldrich) for 60 min on ice. Cells were washed three times in RPMI 1640 medium, 10% (v/v) FCS, and incubated with or without the anti-IgG antibody (1  $\mu$ g/ml) at 37°C to cross-link the CAR. Aliquots of cells ( $10^7$  cells) were spun down at different time points and lysed by adding 100  $\mu$ l lysis buffer (1% (v/v) NP40, 150 mM NaCl, 50 mM Tris/HCl, pH 8, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide. After 30 min on ice, the lysates were cleared by centrifugation. Nuclei free supernatants (100  $\mu$ l) were stored at -20°C. Lysates were added to microtiter wells coated with

anti-IgG antibody (1  $\mu$ g/ml) and incubated for 2 h at room temperature. The bound biotinylated CAR was detected by alkaline phosphatase conjugated streptavidin (1:10,000). The reaction product was developed with pNPP (Sigma-Aldrich).

## SDS PAGE and western blot analysis

For analysis of protein half-life on T cell surface, protein synthesis of CAR transfected cells ( $5 \times 10^7$ /ml) was blocked by culture in the presence of cycloheximide (10  $\mu$ g/ml). Cells were lysed ( $5 \times 10^7$ ) at different time points, lysates separated by SDS-PAGE in 8% (w/v) polyacrylamide gels under non-reducing conditions and subsequently blotted onto a PVDF membrane (Thermo Fisher Scientific). The membrane was probed with the peroxidase-labeled goat anti-human IgG Fc antibody to detect the CAR (1:10,000). For loading control blots were stripped and probed with an anti-actin antibody (0.5  $\mu$ g/ml) and peroxidase-labeled anti-mouse IgG Fc antibody (1:5,000). Bands were visualized by chemoluminescence utilizing the "ECL Western blotting detection system" (Amersham Biosciences, Freiburg, Germany). Intensity of bands was densitometrically quantified utilizing the ImageJ software. Data were presented as percent of the intensity at time 0. To monitor expression of endogenous CD3 $\zeta$  chain, lysates of non-modified and CD3 $\zeta$  genome edited Jurkat cells were separated by SDS-PAGE in 12% (w/v) polyacrylamide gels under reducing conditions, blotted and probed with the anti-CD3 $\zeta$  mAb (clone 4B10, Thermo Fisher Scientific). Bound antibodies were detected by a peroxidase-conjugated anti-mouse IgG antibody (Sigma Aldrich) at 1:5,000 dilution. Membranes were stripped and re-probed with the peroxidase-labeled anti- $\beta$ -actin antibody (Santa Cruz Biotechnology) at 1:20,000 dilution. Bands were visualized by chemoluminescence. To monitor expression of phosphorylated CD3 $\zeta$ , cells were resuspended in RIPA buffer and protein concentrations were determined by ROTI-Quant (Carl Roth, Karlsruhe, Germany). For Western blot analysis, lysates were electrophoresed by SDS-PAGE in 4–12% (w/v) Bis-Tris gels under reducing conditions, blotted and probed with the anti-phospho-CD247 (CD3 zeta) (Tyr142) mAb (clone EM-54, Thermo Fisher Scientific) at 1:1,000 and detected by the peroxidase-labeled anti-mouse IgG1 ( $\gamma$ -chain specific) antibody (Sigma-Aldrich) at 1:10,000 dilution. Membranes were stripped and re-probed with peroxidase-labeled anti- $\beta$ -actin antibody (Santa Cruz Biotechnology) at 1:20,000 dilution. Bands were visualized by chemoluminescence (ChemiDoc Imaging System, BioRad).

## Total internal reflection fluorescence microscopy

All images were recorded using a home-built setup based on an Olympus IX73 (Japan) microscope body equipped with a high NA objective (Carl Zeiss, alpha-plan apochromat, 1.46 NA, 100x, Germany), 488 nm and 640 nm excitation lasers (OBIS Laser box, Coherent, USA), a quad dichroic mirror (Di01-R405/488/532/635, Semrock, USA) and an emission filter (ZET405/488/532/642m, Chroma, USA). The emission path was split into two color



channels using a dichroic mirror (H 643 LPXR superflat, Chroma, USA) and emission filters (650/SP BrightLine HC Shortpass, Semrock, USA; 690/70 H Bandpass, AHF, Germany); the two color channels were imaged onto the same EM-CCD camera (Ixon Ultra, Andor, UK). Prior to measurements, CAR engineered T cells were labeled with either anti-TCR $\alpha/\beta$  AF647-conjugated full antibody, AF647-conjugated F(ab')<sub>2</sub> goat anti-human IgG antibody or AF647-conjugated anti-transferrin receptor (TfR) monoclonal antibody, and seeded on glass slides coated either with recombinant HER2 protein or the anti-CD3 antibody OKT3. Cells were fixed 20 minutes post seeding and imaged by TIRF microscopy upon illumination at 488 nm for CAR-GFP and 640nm for TCR-AF647, for CAR-AF647, or for TfR-AF647. Data analysis was performed with custom Python code (version 3.6) utilizing the following libraries: numpy, mpl\_toolkits, scipy, sdt, pandas, matplotlib, seaborn (22–24). The code is available upon request from the corresponding author. Data analysis was performed on regions of interest which included exclusively pixels above a user-defined threshold in at least one of the two color channels. To quantify the size of the contact region, the number of selected pixels was determined and multiplied by the pixel size of 160x160 nm<sup>2</sup>. To quantify the extent of CAR and TCR co-localization, the Pearson's correlation coefficient was calculated via  $r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}}$ , where  $x$  and  $y$  denote the intensity per pixel, and  $\bar{x}$  and  $\bar{y}$  the corresponding average.

## Fast AiryScan and confocal microscopy

Images were recorded with an LSM 880 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with an AiryScan/AiryScan Fast detection unit providing up to 120 nm lateral and 350 nm axial resolution (25) and a high NA water immersion objective (C-Apochromat, 1.2 NA, 40x). 488 nm and 633 nm excitation lasers were used to avoid spectral overlap, guided by a 488/543/633 nm triple dichroic mirror. Emission was detected in line switch mode through a 495-560BP/660LP dual band filter. Prior to the experiments CAR-transduced primary human T cells were labelled with either human anti-TCR $\alpha/\beta$  AF647-conjugated full antibody, AF647-conjugated F(ab')<sub>2</sub> goat anti-human IgG antibody or AF647-conjugated anti-transferrin receptor (TfR) monoclonal antibody and seeded on HER2 expressing N87 target cells plated on eight-well tissue culture-treated chambered coverslips (ibidi, Gräfelfing, Germany). Images of live anti-HER2 CAR T cells forming contacts with the tumor target were recorded in AiryScan Fast mode. The chamber was incubated at 37°C during the measurement. 3D images of entire cells were captured by optical sectioning applying 0.23  $\mu$ m step size along the z-axis. ZEN Black 2.3 software was used to process the acquired raw datasets where Wiener filter deconvolution with 3D reconstruction algorithm and automatic filter strength was applied. ZEN Blue 2.3 software was used to render 3D images for illustrative purposes. Confocal image of each analyzed cell was captured for overall orientation purposes. Differential distribution of CAR-GFP (green) and either CAR-AF647, TCR-AF647, or TfR-AF647 (red) in the synaptic contact region, the extrasynaptic membrane, and the whole cell membrane of AiryScan processed 3D images was quantified based on intensity values in the far red and in the green channels in 3D regions of interest generated

using the software ImageJ/Fiji (26) with the 3DSuite plugin (27). 3D Mean Filtering was performed on voxels of 3x3x3 pixel radius (equivalent to 0.30x0.30x0.68  $\mu$ m radius), then images were segmented based on intensity thresholding to acquire 3D regions of interest. 3D ROIs of the synaptic contact region were generated based on the CAR-GFP signal, and of the extrasynaptic membrane based on either CAR-AF647, TCR-AF647, or TfR-AF647. Each synaptic 3D ROI for all analyzed cells was manually verified based on the extent of the contact region visible in the confocal images to exclude ROIs outside the contact region. Each extrasynaptic 3D ROI for all analyzed cells was manually verified to exclude any non-contacting anti-HER2 CAR T cells in the field of view. Whole cell 3D ROIs were generated by merging the synaptic and extrasynaptic membrane ROIs. Mean intensity of the synaptic contact region, the extrasynaptic membrane, and the whole cell membrane was quantified in both green and red channels. Relative intensity values were generated by dividing the mean intensity values of the synaptic contact region and the extrasynaptic membrane by the mean intensity of the whole cell membrane. Pixel-wise correlation was quantified based on intensity values recorded for CAR-GFP (green) and either CAR-AF647, TCR-AF647, or TfR-AF647 (red) in the synaptic contact region and the extrasynaptic membrane 3D ROIs, including only pixels that were, in at least one of the channels, above the threshold determined as the intersect of intensity histograms from the cell-containing and cell-free areas, i.e. expectedly high cross-correlation of non-labeled areas was excluded from analysis. Pearson's correlation coefficient was calculated separately for each slice in the 3D images using a custom ImageJ/Fiji plugin. The development of this code will be published separately and will be available at GitHub and the Fiji updater. Average PCC values of all slices for each individual cell were calculated and their mean was plotted with SD as error bars across all cells from at least 3 independent experiments. As an exception to this procedure, for control PCC values, only one AiryScan Fast 2D slice was imaged for each unstimulated cell given the relatively high spatial mobility of unengaged lymphocytes. GraphPad Prism 5 software was used for statistical analysis.

## Activation of CAR engineered Jurkat cells

Microtiter plates were coated with anti-human IgG antibody, that binds to the CAR, and the anti-CD3 antibody OKT3 (5  $\mu$ g/ml each). CAR engineered or non-modified Jurkat T cells (5 x 10<sup>4</sup>/well) were incubated in coated plates for 48 h and IL-2 in the supernatant was determined by ELISA with a solid phase anti-human IL-2 (2  $\mu$ g/ml) capture and a biotinylated anti-human IL-2 detection antibody (0.5  $\mu$ g/ml) (BD Bioscience). The reaction product was visualized with a peroxidase-streptavidin-conjugate (1:10,000) and ABTS (Roche Diagnostics).

## Activation of CAR T cells

CAR T cells (0.32 x 10<sup>4</sup>–5 x 10<sup>4</sup> cells/well) were co-cultivated for 24–48 h in 96-well round bottom plates with tumor cells (2–5 x 10<sup>4</sup> cells/well). Supernatants were removed and tested for IFN- $\gamma$  as described below. Specific target cell lysis of CAR T cells was

determined by XTT assay as previously described (28). Viability of target cells without T cells was calculated as the OD-mean of six wells containing only tumor cells subtracted by the background OD-mean of wells with medium only. Non-specific formation of formazan by T cells was determined from ODs of triplicate wells containing exclusively T cells and in same numbers as in the corresponding experimental wells. Viability of target cells in experimental wells was calculated by: viability (%) =  $[\text{OD}(\text{experimental wells} - \text{corresponding number of T cells})] / [\text{OD}(\text{tumor cells only} - \text{medium})] \times 100$ . Cytotoxicity (%) was calculated by: cytotoxicity (%) =  $100 - \text{viability} (\%)$ . Alternatively, CAR T cells ( $2.5\text{--}5 \times 10^4$  cells/well) were incubated in 96 well microwell plates coated with the agonistic anti-CD3 (1  $\mu\text{g/ml}$ ), anti-CD28 (5  $\mu\text{g/ml}$ ), anti-TCR (4  $\mu\text{g/ml}$ ), anti-IgG (1  $\mu\text{g/ml}$ ) antibodies, anti-idiotypic antibody (BW2064/36; 8  $\mu\text{g/ml}$ ) or recombinant HER2-Fc protein (8  $\mu\text{g/ml}$ ), respectively. After 48 h supernatants were tested for IFN- $\gamma$  and IL-2 by ELISA utilizing solid phase bound anti-IFN- $\gamma$  and anti-IL-2 capture antibodies (each 1  $\mu\text{g/ml}$ ) and biotinylated anti-IFN- $\gamma$  (0.5  $\mu\text{g/ml}$ ) and anti-IL-2 detection antibodies (1  $\mu\text{g/ml}$ ), respectively. The reaction product was visualized as described above.

## Results

Physiologically, the TCR associated CD3 $\zeta$  chain rapidly recycles on the cell membrane independently of the other TCR components (10). We asked whether a CD3 $\zeta$  chain CAR is subjected to the same rapid turn-over and tested a set of CARs with the CD3 $\zeta$  or the Fc $\epsilon$ RI  $\gamma$  signaling chain (Figure 1A); the other domains of the respective CARs were the same; the CARs were expressed by the same vector. The  $\zeta$ -chain CARs were consistently present at lower levels on the T cell surface compared to the corresponding  $\gamma$ -chain CARs. Exchange of the intracellular  $\zeta$ - and  $\gamma$ -chains reciprocally altered the CAR levels on the cell membrane, while exchange of the transmembrane domains did not, indicating that the different CAR levels on the T cell surface were due to the intracellular moiety. For comparison, the  $\gamma$ - and  $\zeta$ -chain CARs were present at equal levels in non-T cells like HEK293T cells (Figure 1A) indicating that the different CAR levels are due to their expression in T cells, most likely due to the presence of the endogenous TCR/CD3 complex.

We addressed whether the different  $\zeta$ - and  $\gamma$ -chain CAR levels go along with different protein half-life times on the T cell surface. Blocking protein synthesis and Western blot analyses revealed that the  $\zeta$ -chain CAR had a shorter half-life time than the  $\gamma$ -chain CAR in engineered MD45 T cells (Figure 1B). CAR cross-linking by an anti-IgG antibody, that binds to the common extracellular CAR domain, resulted in rapid degradation of both  $\gamma$ - and  $\zeta$ -chain CARs as expected. This goes in line with a recent study showing that antigen encounter results in rapid ubiquitination and, as a consequence of internalization and lysosomal degradation, downregulation of CARs (29). Pulse-chase analysis revealed that the  $\zeta$ -chain CAR molecules on the cell surface more rapidly declined than the  $\gamma$ -chain CARs (Figure 1C). Taken together, the  $\zeta$ -chain CAR experienced a higher turnover on the T cell membrane and a shorter half-life time than the  $\gamma$ -chain CAR.

To record CAR-driven T cell effector functions, peripheral blood T cells were engineered with  $\zeta$ - and  $\gamma$ -chain CARs with specificity for

CA19-9. Recording the cytotoxic activity against CA19-9<sup>+</sup> and CA19-9<sup>-</sup> cancer cells revealed that the  $\zeta$ -chain CAR induced higher cytolytic activity and higher IFN- $\gamma$  release than the  $\gamma$ -chain CAR indicating a higher potency of the  $\zeta$ -chain CAR in T cell activation (Figure 1D). This was the case despite a lower expression level and lower half-life time compared to the  $\gamma$ -chain CAR on the T cell surface.

Half-life time and rapid turn-over may affect T cell activation in the presence of soluble target antigen. This is a clinically relevant scenario since a substantial number of CAR-targetable surface antigens are also shed by cancer cells which may block the CAR redirected T cell activation. To address the issue, we engineered T cells with the  $\zeta$ - and  $\gamma$ -chain CAR, respectively, with the same anti-CA19-9 binding domain and co-incubated CAR T cells with CA19-9<sup>+</sup> target cells in the presence of increasing concentrations of soluble CA19-9 (Figure 1E). The induction of lytic activity triggered by the  $\gamma$ -chain CAR was blocked by soluble CA19-9 whereas the activity by the  $\zeta$ -chain CAR was less affected. We assumed that the rapid turn-over and shorter half-life of the  $\zeta$ -chain CAR goes along with a rapid replacement by antigen-free CAR chains on surface and thereby a higher resistance towards blocking by soluble antigen.

We asked whether a  $\zeta$ -chain CAR can substitute for the CD3 $\zeta$  chain in reconstituting the endogenous TCR, and whether the endogenous CD3 of the TCR affects the expression level of the CD3 $\zeta$  CAR independently of the TCR  $\alpha\beta$  chains. To address the issue, we deleted the endogenous CD3 $\zeta$  chain of Jurkat cells by CRISPR/Cas9 mediated gene editing. Flow cytometry and Western blot analysis demonstrated efficient knockout of the endogenous CD3 $\zeta$  in engineered Jurkat cells; consequently, no TCR was expressed (Figures 2A, B). For comparison, TCR deficient Jurkat76 cells express the endogenous CD3 $\zeta$  chain as reported (13).

We engineered Jurkat cells without endogenous CD3 $\zeta$  and/or CD3/TCR expression, respectively, with  $\zeta$ - and  $\gamma$ -chain CARs (Figure 2C). While the CARs were properly expressed by Jurkat cells, the CARs did not rescue TCR expression in CD3 $\zeta$  KO Jurkat cells (Figure 2D). Expression of the endogenous CD3 was not altered by the respective CARs in Jurkat cells. Same data were obtained upon engineering blood T cells (Figure 2E). More importantly,  $\zeta$ -chain CARs were expressed at lower levels in both TCR<sup>+</sup> CD3 $\zeta$ <sup>+</sup> Jurkat76 cells and in TCR<sup>+</sup> CD3 $\zeta$ <sup>+</sup> Jurkat E4 cells compared to the  $\gamma$ -chain CAR indicating that the lower levels of  $\zeta$ -CARs on T cell surface did not depend on the presence of the TCR or TCR/CD3 complex. With respect to CAR triggered functionality, the  $\zeta$ - and  $\gamma$ -chain CARs were as active in CD3 $\zeta$  KO cells as in TCR<sup>+</sup> CD3 $\zeta$ <sup>+</sup> Jurkat cells indicated by cytokine release upon CAR stimulation (Figure 2F). For comparison, engineered CD3 $\zeta$  KO Jurkat cells did not respond upon CD3 stimulation despite the presence of the  $\zeta$ -chain CAR.

Taken together we concluded that, firstly, the CAR and the CD3/TCR complex are independently regulated on the membrane surface and that the  $\zeta$ - and  $\gamma$ -chain CARs function independently of the presence of the endogenous CD3/TCR complex in T cells. Secondly, the  $\zeta$ -chain CAR did not replace CD3 $\zeta$  in rescuing TCR expression in CD3 $\zeta$  KO cells.

To address whether there is a cross-signaling at the very early stage of TCR and CAR mediated activation, we recorded by Western blot analysis the phosphorylated CD3 $\zeta$  (pCD3 $\zeta$ ) of the CAR and of

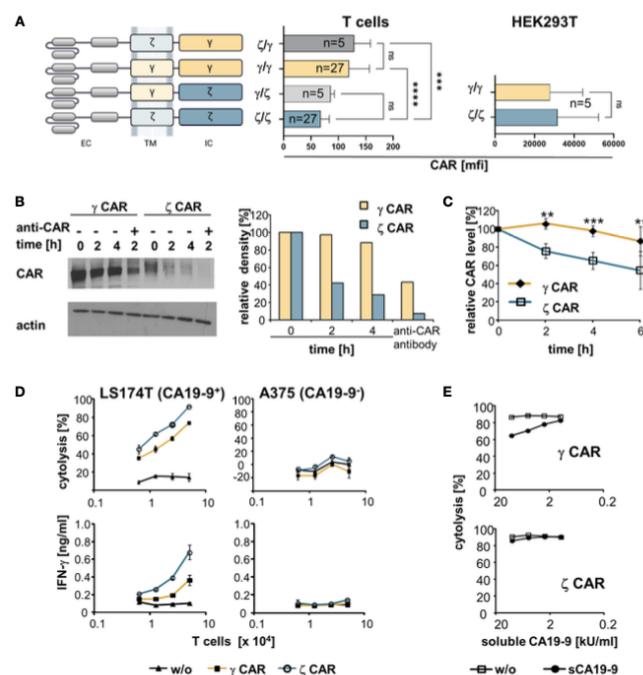
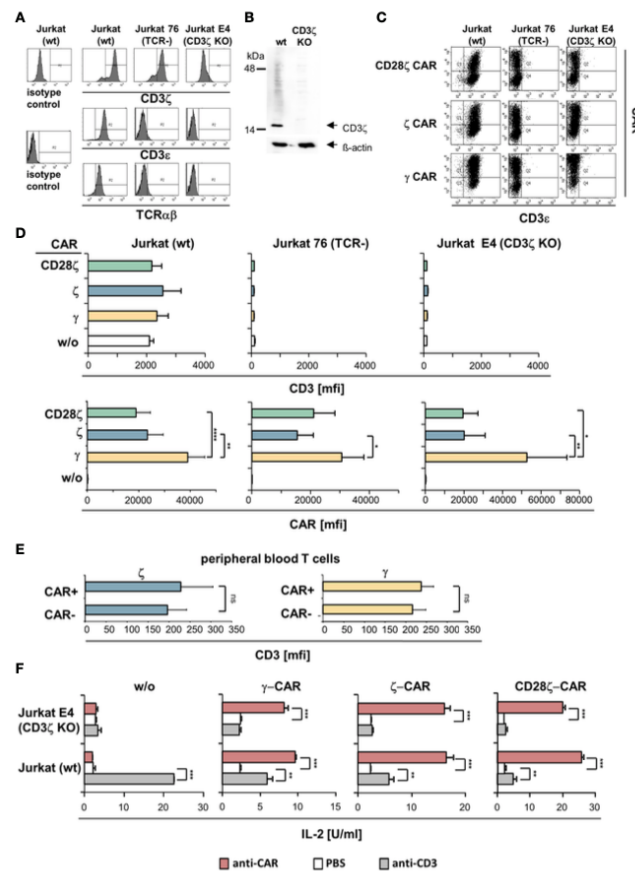


FIGURE 1

CARs with an intracellular CD3ζ chain are superior over γ-chain CARs in T cell activation despite lower cell surface expression and shorter half-life on the T cell surface. (A) Schematic representation of CARs with their respective transmembrane (TM) and intracellular (IC) signaling domains consisting of the respective CD3ζ (ζ) or the FcεR1 γ (γ) chain. All CARs harbour the same extracellular domain (EC) and were expressed by the same promoter in the same retroviral vector backbone. Cells were engineered with the respective CARs. CAR expression was monitored by an anti-IgG Fc antibody that detects the common extracellular CAR IgG1-Fc spacer domain. Background staining was determined by an isotype control antibody. Data represent the mean values of mean fluorescence intensity (mfi) ± SD. (B) MD45 cells were engineered with a ζ or a γ CAR, respectively, and incubated with cycloheximide (10 μg/ml) to block protein synthesis. For comparison, the CARs were additionally cross-linked by an anti-IgG (anti-CAR) antibody, that binds to the extracellular CAR spacer domain, for 2 h to induce CAR internalization. At different time points, 10<sup>7</sup> cells were lysed and proteins separated by SDS-PAGE on 8% (w/v) polyacrylamide gels under non-reducing conditions. CARs were detected by the anti-IgG Fc-POD antibody (1:10,000), actin was detected by the anti-actin antibody (0.5 μg/ml). Relative density of CAR bands was quantified utilizing the ImageJ software 1.48 and presented as percent of the initial amount at t=0. Data from a representative experiment out of three are shown. (C) Pulse-chase CAR labeling. CAR transfected cells were surface-labeled with biotin as described in Materials and Methods, washed and stimulated at 37°C by an anti-IgG antibody (1 μg/ml) directed against the IgG extracellular CAR domain. Aliquots of cells (5 × 10<sup>6</sup> cells) were lysed at different time points and lysates were subjected to ELISA plates coated with an anti-IgG1 mAb (1 μg/ml) to capture the CAR. Bound labeled CARs were detected by streptavidin POD and visualized with ABTS. OD at time point 0 was set at 100% and relative ODs at indicated time points were calculated. Numbers represent the mean values of three independent experiments ± SD. (D) CAR redirected T cell activation. T cells with ζ- or γ-chain anti-CA19-9 CAR were expanded in the presence of IL-2 and co-cultivated (0.625–5 × 10<sup>4</sup> cells/well) for 48 h with CA19-9<sup>+</sup> LS174T or CA19-9<sup>+</sup> A375 tumor cells (5 × 10<sup>4</sup> cells/well). Supernatants were analyzed for IFN-γ by ELISA, target cell lysis was determined by the XTT assay. Data represent mean values ± SD of two independent experiments. w/o, without CAR. (E) Activation of CAR T cells in the presence of soluble CA19-9 antigen. Anti-CA19-9 CAR T cells (5 × 10<sup>4</sup> cells/well) were co-cultivated for 48 h with CA19-9<sup>+</sup> LS174T cells (5 × 10<sup>4</sup> cells/well) in the presence of serial dilutions of supernatants of H498 tumor cells containing about 20,000 U/ml of soluble CA19-9 (sCA19-9). Target cell lysis was determined by the XTT assay. For control, cells were co-cultivated in the presence of supernatants of the CA19-9<sup>+</sup> cell line H716 lacking soluble CA19-9 (w/o). Data represent mean values ± SD of technical triplicates. For comparison of two groups, significant differences were determined by Student's T test. For comparisons of three or more groups, one-way ANOVA with Tukey's post hoc test was used. p-values < 0.05 were considered statistically significant (\*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001; ns, not significant).

TCR-associated, endogenous CD3ζ chain on stimulation. TCR stimulation increased phosphorylation of the TCR associated endogenous CD3ζ chain, but not of the CAR CD3ζ domain (Figure 3). Vice versa, stimulation of the CAR resulted in an increased phosphorylation of the CAR CD3ζ chain but not of the TCR CD3ζ chain. The same pattern was obtained with CD28-CD3ζ CAR engineered cells; phosphorylation of CAR CD3ζ increased upon CAR stimulation but not upon TCR/CD3 stimulation; pCD3ζ of the TCR, but not of the CAR, increased upon TCR/CD3 stimulation.

We also investigated whether CAR-associated CD28 signaling can induce CD3ζ signaling through the TCR. Stimulation of the CD28 CAR, that lacks the CD3ζ domain, did not produce TCR CD3ζ phosphorylation; increase in pCD3ζ occurred upon TCR/CD3 stimulation as control (Figure 3). We concluded that CD28-CD3ζ CAR stimulation did not induce phosphorylation of the endogenous TCR CD3ζ chain indicating that no substantial cross-signaling between the TCR/CD3 and the CAR at the stage of CD3ζ phosphorylation occurred.

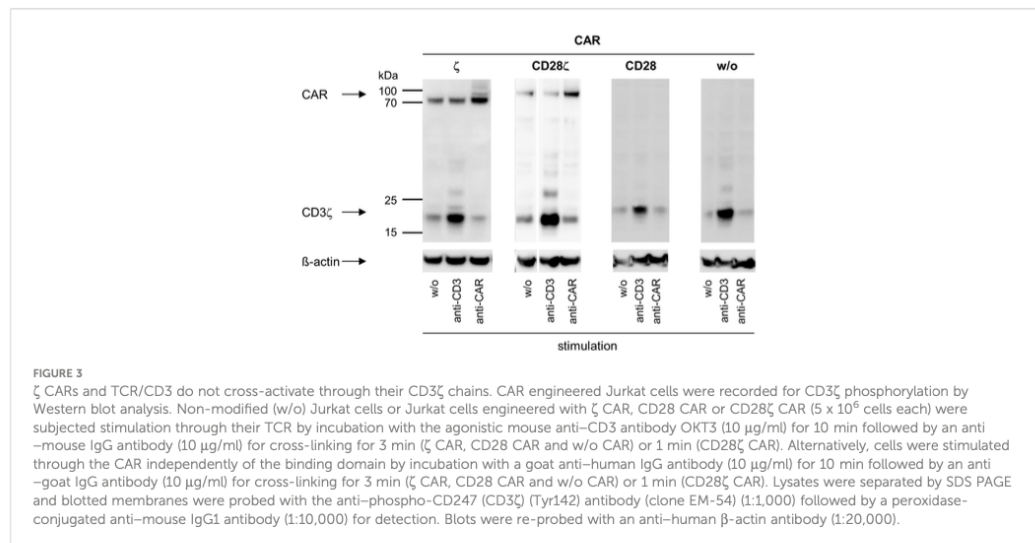


**FIGURE 2**  
 ζ CARs did not rescue the CD3/TCR complex in CD3ζ KO Jurkat cells. (A) The CD3ζ locus in Jurkat cells was deleted by CRISPR/Cas9 engineering as described in Materials and Methods. Non-modified Jurkat cells (wt), Jurkat76 cells lacking TCR (TCR<sup>-</sup>) and Jurkat E4 CD3ζ knock-out (KO) cells were tested by flow cytometry for intracellular CD3ζ expression and for surface expression of CD3ε and TCR, respectively. Histograms of a representative analysis are shown. (B) Western blots of genome edited Jurkat cells. Lysates of non-modified (wt) and CD3ζ KO Jurkat cells (5 μg protein lysate/lane) were separated by SDS PAGE, blotted, probed with a mouse anti-human CD3ζ antibody (1:500) and detected by a HRP-conjugated anti-mouse antibody (1:5,000). Blots were re-probed with an anti-β-actin antibody (1:20,000). (C) TCR<sup>+</sup> Jurkat (wt), Jurkat76 (TCR<sup>-</sup>) and Jurkat E4 (CD3ζ KO) cells were engineered with the CD28ζ, ζ or γ CAR, respectively. Expression of CARs and surface expression of CD3 was recorded by flow cytometry and mean fluorescence intensity (mfi) was determined. Dot plots of a typical experiment and mean values of 5 independent experiments ± SD (D) are shown. Significant differences were determined by Student's T test. (E) Peripheral blood T cells engineered with ζ-chain and γ-chain CAR, respectively, were stained for CAR and CD3 expression and analyzed by flow cytometry. CAR<sup>+</sup> and CAR<sup>-</sup> T cells were gated and mean fluorescence intensity (mfi) of CD3 was determined. Data represent mean values of 4 healthy donors ± SD. Statistical differences were determined by Student's T test. (F) Jurkat (wt) and Jurkat E4 (CD3ζ KO) cells with and without CAR, respectively, were stimulated through the CAR and CD3 by incubation on 96-well plates (4 × 10<sup>4</sup> cells/well) coated with the agonistic anti-CD3 antibody OKT3 or anti-IgG Fc antibody (5 μg/ml each) that binds to the CAR extracellular domain. After 48 h supernatants were tested for IL-2 by ELISA. Values represent the means of technical triplicates ± SD. Significant differences were determined by Student's T test. A representative experiment out of two is shown. p-values <0.05 were considered statistically significant (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001; ns, not significant).

To investigate whether CAR and TCR are recruited into similar regions during immunological synapse formation, we engineered peripheral blood T cells with Her2-specific CD28-CD3ζ CARs linked to GFP (Figure 4A). The distribution of CAR and TCR in the contact region between CAR T cell and immobilized Her2 molecules was recorded via TIRF microscopy. The CAR was localized by its linked GFP and verified by staining with an

AF647-conjugated anti-CAR antibody; the TCR was localized by an anti-TCRαβ AF647-conjugated antibody; the transferrin receptor (TfR) was localized by an anti-TfR AF647-conjugated antibody (Figure 4B). There was no difference in size of the contact regions formed by the T cell on surfaces coated either with Her2 as CAR target or with the anti-CD3 antibody OKT3 as TCR target (Figure 4C). While there was no indication for synapse formation of anti





–Her2 CARs on the OKT3 antibody coated surface, recognition of the cognate antigen Her2 led to an accumulation of anti-Her2 CARs in the contact region, but not of the TCR (Figure 4D). Notably, the Pearson's correlation coefficient (PCC) between CAR and TCR distribution was not different compared to the negative distribution (Figure 4E, median PCC = 0.384). As negative control, the Tfr, that is distributed on the cell surface independently of the CAR and TCR, showed no substantial correlation with the CAR distribution (median PCC = 0.306). As positive control, the GFP-CAR signal strongly correlated with the signal of anti-CAR antibody (median PCC = 0.949).

The distribution of CAR and TCR in the contact region between anti-Her2 CAR T cell and Her2<sup>+</sup> tumor cell was studied by 3D fast AiryScan microscopy (Figure 4F; Supplementary Figure 1A; Supplementary Video 1, 2, 3). The analysis confirmed accumulation of the anti-Her2 CAR, but not of the TCR, in the synaptic region (Figure 4G). In fact, TCR was present at lower mean intensities in the synaptic than in the extra-synaptic regions (Supplementary Figure 1B), making up a significant difference after normalization to the entire membrane intensity. In anti-Her2 CAR T cells engaging Her2<sup>+</sup> tumor cells, PCC between CAR and TCR in the synapse was not different from the Tfr negative control (mean  $PCC_{CAR\_TCR} = 0.149$ ;  $PCC_{CAR\_TFR} = 0.040$ ), while the GFP-CAR signal showed a strong correlation with the anti-CAR antibody as positive control signal (mean  $PCC_{CAR\_CAR} = 0.628$ ) (Figure 4H). No co-distribution of the CAR with the TCR or the Tfr as control occurred when the synaptic region, the extra-synaptic region, and the unstimulated CAR T cell membranes were compared (Figure 4H). Taken together, data indicate that the CAR synapse formed upon engagement of cognate antigen did not recruit the TCR into the same region.

As cross-signaling between the CAR and TCR/CD3 can occur at more downstream steps in the activation pathway at the level of effector functions, we recorded cytokine production as a near final step in the activation of effector functions. CAR engineered T cells

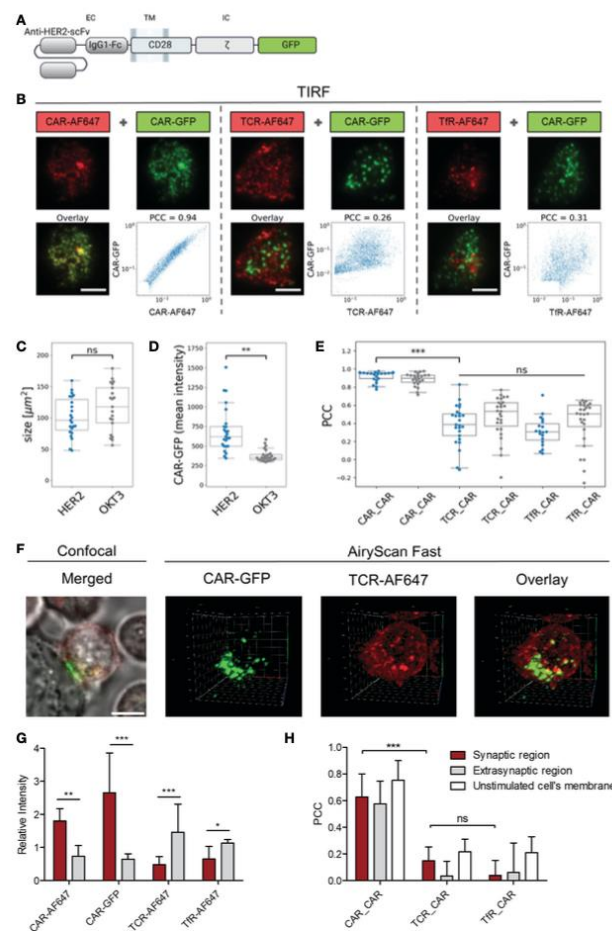
were stimulated through the CAR, CD3 and TCR, respectively, and IFN-γ and IL-2 release was recorded. The threshold for IFN-γ release by TCR and CD3 activation, respectively, was not altered by the presence of a CD3ζ or CD28-CD3ζ CAR compared to unmodified T cells (Figure 5A). While CD3ζ-chain signaling by the CAR was sufficient for IFN-γ secretion, IL-2 release required additional CD28 co-stimulation as provided through the CD28-CD3ζ CAR as expected. No IL-2 release occurred upon TCR or CD3 stimulation in the presence of the CD28-CD3ζ CAR indicating that the co-expressed CD28 CAR domain was not cross-activated by TCR stimulation to complement for IL-2 release.

While TCR and CAR did not cross-activate upon engagement of either cognate antigen, we assessed whether CAR and TCR can complement in T cell activation when both are engaging their respective target. T cells were engineered with a CD28 CAR lacking the CD3ζ domain and recognizing CEA or HER2, respectively. CAR T cells were stimulated through the CAR by binding to their cognate antigen or through their TCR/CD3 (Figure 5B). Simultaneous binding to the respective CAR ligand and to an agonistic anti-CD3 antibody induced IL-2 release indicating successful complementation of the TCR/CD3 signaling with CAR CD28 signaling; IL-2 release was not obtained upon TCR/CD3 or CAR stimulation alone. For control, the CD28-CD3ζ CAR induced IL-2 upon binding to the CAR ligand without additional TCR stimulation; stimulation of CD3 plus CD28 independently of the CAR also induced IL-2 release. Data indicate that CAR and TCR/CD3 could complement in the downstream T cell activation pathway when engaging their respective cognate ligand.

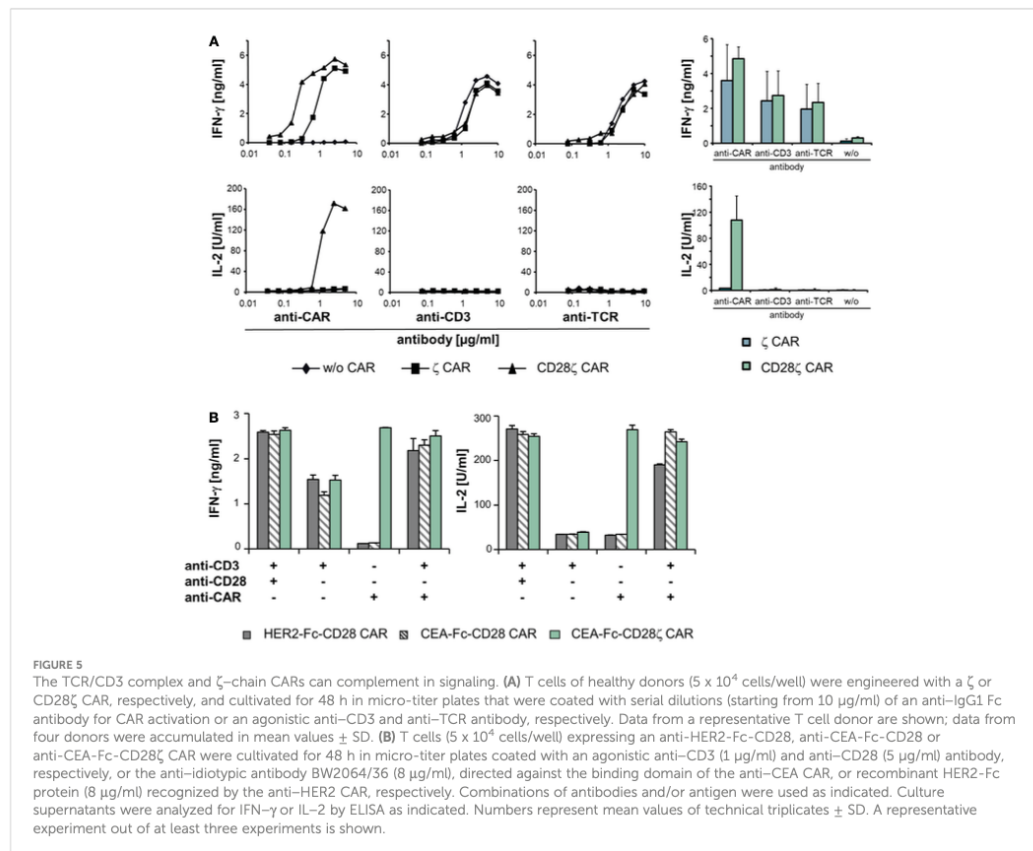
## Discussion

Nearly all CARs used in clinical trials signal through the CD3ζ chain by engaging downstream signaling proteins associated with the





**FIGURE 4**  
 $\zeta$  CAR engagement of antigen induced clustering without TCR integration. Anti-HER2 CAR T cells were labeled with either AF647-conjugated F(ab')<sub>2</sub> goat anti-human IgG antibody that recognizes the CAR, anti-TCR $\alpha/\beta$  AF647-conjugated antibody, or AF647-conjugated anti-transferrin receptor (TfR) antibody. (**A**) Schematic representation of the used anti-HER2 CD28 $\zeta$  CAR linked to GFP at the intracellular site (IC). (**B**) Cells were seeded onto surfaces coated with HER2 protein or anti-CD3 antibody OKT3 and contacts were formed for 20 minutes. Cells were fixed and TIRF microscopy was used to image the localization of CAR-GFP (green) and either CAR-AF647, TCR-AF647, or TfR-AF647 (red). Scale bar represents 5  $\mu$ m. For each cell, the pixel-wise correlation of the brightness values recorded in the red and in the green channel were plotted along the x- and the y-axis, respectively. Pearson's correlation coefficient (PCC) for each cell was calculated. Contact size (**C**) and Mean intensity (**D**) of anti-HER2 CAR-GFP plated on HER2 (blue) and OKT3 coated slides (grey) are displayed for  $n \geq 16$  cells per group in a Whisker box plot. Statistical differences were determined by Wilcoxon-Mann-Whitney test with Python code (\*\* $p < 0.01$ ). (**E**) PCC for CAR-GFP and TCR-AF647 was calculated for anti-HER2 CAR T cells plated on HER2 (blue) and OKT3 (grey) coated slides and displayed for  $n \geq 24$  cells per group in a Whisker box plot. Statistical differences were determined by Kruskal-Wallis test with Dunn's post-hoc test performed with Python code (\*\*\* $p < 0.001$ ). (**F**) Labelled anti-HER2 CAR T cells were seeded on HER2 expressing N87 target cells plated on chambered coverslips. 3D fluorescence images of live anti-HER2 CAR T cells forming contacts with the tumor target were recorded in AiryScan Fast mode. Confocal images of each analyzed cell were recorded as controls. (**G**) Differential 3D distribution of CAR-GFP and either CAR-AF647, TCR-AF647, or TfR-AF647 in the synaptic contact region and the extrasynaptic membrane was normalized to total intensity ( $n_{\text{CAR-GFP}}=11$ ,  $n_{\text{TCR-AF647}}=11$ ,  $n_{\text{CAR-AF647}}=10$ ,  $n_{\text{TIR-AF647}}=6$ ; 3D images contained approximately 50-80 slices). (**H**) PCC for CAR-GFP and either CAR-AF647, TCR-AF647, or TfR-AF647 in the synaptic contact region and the extrasynaptic membrane was quantified separately for each slice and averaged for each individual cell and presented as the mean of multiple cells across 3 independent experiments. 2D AiryScan Fast images of unstimulated cells were used as control ( $n_{\text{CAR-CAR}}=15$ ,  $n_{\text{TCR-CAR}}=7$ ,  $n_{\text{TIR-CAR}}=14$ ). Data are presented as mean  $\pm$  SD (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; ns, not significant).



endogenous TCR/CD3 complex (6, 7). The impact of TCR/CD3 on the CAR redirected T cell activation and vice versa was so far not addressed. A mutual functional interaction is a relevant issue since CAR engineered T cells harbor in addition a functionally active TCR that may interfere with or add to CAR-mediated signaling.

Physiologically, the endogenous CD3 $\zeta$  stabilizes the CD3/TCR complex; in the absence of CD3 $\zeta$ , the levels of TCR $\alpha\beta$  chains are substantially reduced (30, 31). Moreover, CD3 $\zeta$  has a rapid turnover on the cell membrane independently of the other TCR components (10). Here we revealed that CD3 $\zeta$ -chain CARs likewise have a shorter half-life and are expressed at lower levels on the T cell membrane than the Fc $\epsilon$ RI  $\gamma$ -chain CARs. The low expression levels are mediated by the CAR intracellular CD3 $\zeta$  and not by the transmembrane domain; the effect holds also true for the second generation CD28-CD3 $\zeta$  CAR. In contrast to the situation in T cells,  $\gamma$ -chain CARs are less expressed in Fc receptor expressing cells compared with the  $\zeta$ -chain CARs, like macrophages and neutrophils (32–36); in non-lymphoid cells both  $\gamma$ - and  $\zeta$ -chain CARs are expressed at similar levels. In CD3 $\zeta$  KO and in TCR<sup>−</sup> Jurkat cells the  $\zeta$ -chain CARs were also less expressed than the  $\gamma$ -chain CARs indicating that the levels of  $\zeta$ -chain CARs on the T cell surface are affected by downstream elements of the TCR/CD3

complex and not by the presence of the TCR and CD3 $\zeta$  themselves. Notably, the expression level does not correlate with the activation capacity since CD3 $\zeta$  CARs require less amounts of antigen than  $\gamma$ -chain CARs to activate engineered T cells.

At the membrane receptor level, the CAR does not co-recruit the TCR into its synapse as revealed by TIRF and fast AiryScan microscopy; vice versa, the TCR does not recruit the CAR into its synapse (37). Consequently, CAR and TCR do not cross-signal with respect to CD3 $\zeta$  phosphorylation; TCR/CD3 stimulation did not result in increase in CAR CD3 $\zeta$  phosphorylation and, conversely, CAR stimulation did not increase TCR/CD3 phosphorylation. The conclusion holds true for both the CD3 $\zeta$  and the CD28-CD3 $\zeta$  CAR. In line with this finding, costimulatory CD28 signaling through the CD28 CAR did not increase TCR/CD3 $\zeta$  phosphorylation. However, there is a convergence in TCR and CAR downstream signaling, since the adaptor protein LAT, which is a linker between proximal and distal signaling events, becomes phosphorylated by each TCR and CAR activation, although at different levels (38).

We asked whether lack of cross-signaling at the early step was associated with lack of cross-activation of downstream pathways like the release of effector molecules including cytokines. To address this

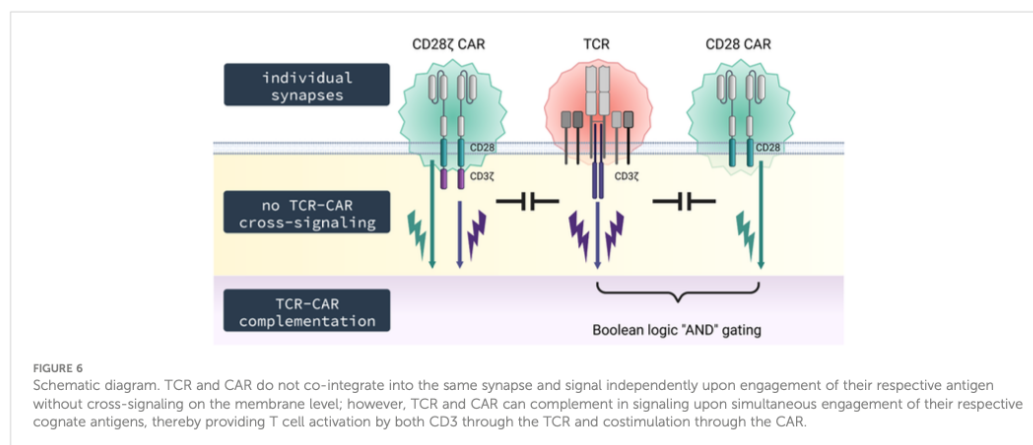
scenario in a well-defined antigen stimulation assay, we took advantage of the different signaling requirements for IFN- $\gamma$  and IL-2 release; IFN- $\gamma$  release indicates CD3 $\zeta$  signaling while IL-2 release depends on combined CD3 $\zeta$  and CD28 signaling in T cells. Using these cytokines as indicators, we revealed that signaling through TCR/CD3 did not activate CAR-associated CD28 and vice versa (Figure 6). However, TCR/CD3 stimulation can complement with CAR-provided CD28 co-stimulation when both TCR and CAR are engaging their respective cognate antigen; signaling through only the TCR or the CD28 CAR was not sufficient. Taken together data indicate lack of cross-signaling between CAR and TCR not only on the level of the cell membrane associated kinases but also in the downstream pathway of effector molecules. In addition to our findings, potential physical interaction between CAR and endogenous signaling molecules can occur. Muller et al. showed that CAR T cells harboring a CD28-derived transmembrane domain form heterodimers with the endogenous CD28; such CAR-CD28 heterodimers can activate CAR T cells (39). The number of molecules captured in heterodimers may differ and the functional consequences still need thorough investigation.

Our conclusions are of relevance for clinical applications in various aspects. Firstly, T cells will undergo terminal differentiation towards hypo-responsive cells with terminally differentiated KLRG-1<sup>+</sup> CD57<sup>+</sup> CD7<sup>-</sup> phenotype once extensively stimulated through their TCR. In a previous study we revealed that hypo-responsiveness of CMV-specific late-stage CD8<sup>+</sup> T cells is due to reduced TCR synapse formation compared to younger cells which is the result of galectin-mediated membrane-anchoring of TCR components (40). However, transgenic CAR expression and CAR triggering produced full effector functions in TCR hypo-responsive T cells indicating that the defect is restricted to TCR membrane components while synapse formation of the transgenic CAR was not blocked. CAR engineered late-stage T cells released cytokines and mediated redirected cytotoxicity as efficiently as younger effector T cells. Together with our recent analysis, data presented here sustain the model that CAR mediated activation occurs TCR-independently and can by-pass hypo-responsiveness of late-stage T cells upon repetitive TCR encounter.

Secondly, we do not expect an increase in signaling through the endogenous TCR in presence of a CAR, for instance, when EBV-specific T cells are used for a CAR redirected anti-tumor attack (41). Clinical observation indicates that both CAR and TCR can trigger T cells as TCR stimulation of virus-specific T cells in addition to CAR engagement of antigen enhances expansion of CAR T cells and finally their anti-leukemic function (42). Moreover, TCR and CAR can complement in signaling when simultaneously engaging their respective cognate antigen. This is of benefit when achieving complementation in target recognition; one target is recognized by the TCR, the other by the CD28 CAR as shown in our model system. Complementing in activation while lacking cross-signaling is the basis for creating Boolean logic “AND” gating by co-signaling through a CD28 CAR without primary signal while the latter is provided by signaling through the TCR upon engagement of its respective antigen. In this situation, only engagement of both targets will be capable to sustain a lasting T cell activation. The combination may also be used for specific T cell inhibition using an inhibitory CAR that dampens TCR driven activation upon CAR antigen recognition.

The concept of combinatorial antigen recognition was primarily introduced by Kloss et al. (43) aiming at complementing signals between two CARs, one CAR harboring a suboptimal activation signal and the other CAR harboring a costimulatory signal. So-called RevCARs are a further improvement as they represent an artificial receptor platform for controllable T cell activation (44). Herein, universal receptors are redirected by adaptor molecules to the respective targets allowing dosing of the adaptor molecules, flexible targeting and, notably in this context, combinatorial antigen recognition. Again, prerequisite for successful “AND” gating is lack of dimerization and cross-talk between the signaling receptors.

Thirdly, we do not expect altered CAR signaling under conditions where the endogenous CD3 $\zeta$  chain is down-regulated as it occurs under chronic inflammatory conditions (45). CAR redirected T cell activation does not depend on primary TCR signals as it is mediated through the CAR intrinsic CD3 $\zeta$  and costimulatory domain. However, the presence of the endogenous TCR/CD3 substantially prolongs the persistence of CAR T cells in a mouse model compared to TCR  $\beta$ -chain KO cells (11). This is the case despite similar CAR



expression in both cells indicating the impact of the endogenous TCR/CD3 on sustaining CAR T cell function. The “tonic” activation through the TCR and thereby an active downstream signaling cascade, although at low levels, seems to be crucial for the overall therapeutic success given the less persistence of CAR redirected TCR KO T cells and the pivotal impact of CAR T cell persistence on their efficacy in controlling leukemia/lymphoma in the long-term. Along with this hypothesis, in patients treated with CAR engineered allogeneic TCR KO T cells only contaminating TCR<sup>+</sup> CAR T cells, but not TCR<sup>-</sup> CAR T cells, persisted while producing TCR signaling and finally graft-versus-host disease (9).

Taken together, CD3ζ CARs are similarly regulated as the CD3ζ chain of the TCR. However, the CAR cannot substitute for CD3ζ within the TCR complex underlining the concept that CAR and TCR form individual synapses in structure as verified by microscopic analyses and in function as shown by phospho-CD3ζ analyses. This specific situation allows logic “AND” gating by combinatorial target recognition through TCR and CAR. On the other hand, CAR engineered TCR KO T cells, designed for allogeneic “off-the-shelf” therapy, lack TCR support through “tonic” signaling and likely may lose functional capacities in the long-term.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

MB, AH, LV and MM-C conducted, designed, and analyzed experiments. AS, GV, AAH, GS, and HA interpreted the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1110482/full#supplementary-material>

**SUPPLEMENTARY FIGURE 1**  
Distribution of CAR, TCR and TIR in unstimulated and synapse forming cells. (A) Confocal and AiryScan Fast 2D fluorescence images of live anti-HER2 CAR T cells forming contacts with the tumor target. (B) Mean intensity of CAR-AF647, CAR-GFP, TCR-AF647, and TIR-AF647 in the synaptic region, extrasynaptic region, and both regions.

**SUPPLEMENTARY VIDEO 1**  
3D distribution of CAR-GFP and CAR-AF647 in the synaptic contact region of one exemplary cell. 3D fluorescence images of live anti-HER2 CAR T cells forming contacts with the tumor target were recorded in AiryScan Fast mode.

**SUPPLEMENTARY VIDEO 2**  
3D distribution of CAR-GFP and TCR-AF647 in the synaptic contact region and the extrasynaptic membrane of one exemplary cell. 3D fluorescence images of live anti-HER2 CAR T cells forming contacts with the tumor target were recorded in AiryScan Fast mode.

**SUPPLEMENTARY VIDEO 3**  
3D distribution of CAR-GFP and TIR-AF647 in the synaptic contact region and the extrasynaptic membrane of one exemplary cell. 3D fluorescence images of live anti-HER2 CAR T cells forming contacts with the tumor target were recorded in AiryScan Fast mode.



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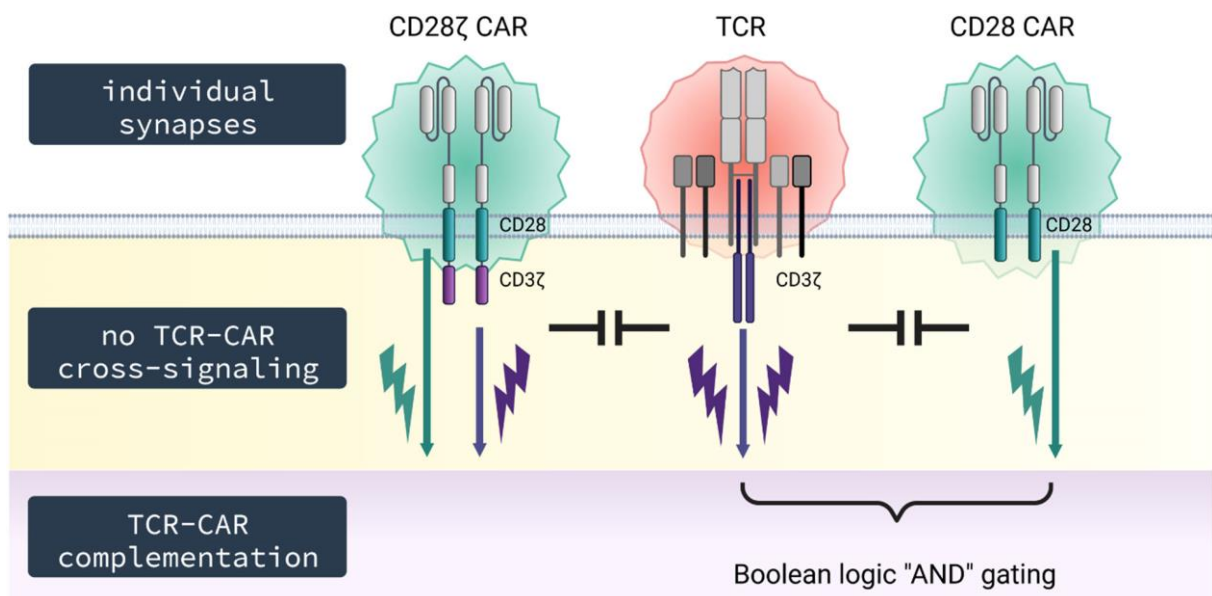
## 4. Diskussion

Der physiologische TCR ist ein Multi-Protein-Komplex, der präzise reguliert ist <sup>33</sup>. Der synthetische CAR kombiniert die Signaldomänen des TCRs in einer einzigen Rezeptorkette <sup>34</sup>. Fast alle klinisch eingesetzten CAR T-Zell-Produkte exprimieren zusätzlich zum CAR den endogenen TCR/CD3-Komplex. Der CAR verwendet Signalmoleküle des TCRs <sup>35</sup>. Eine mögliche funktionale Interaktion von CAR und TCR ist deswegen sehr wahrscheinlich auf der untergeordneten Ebene der Signalkette und von großer Relevanz, da dadurch die CAR-vermittelte T-Zell-Aktivierung erheblich beeinflusst werden könnte. Salter et al. haben gezeigt, dass die Zusammensetzung der CAR-Signaldomänen die Stärke der CAR-vermittelten T-Zell-Aktivierung beeinflusst, jedoch keine klaren Rückschlüsse auf die vom CAR verwendeten Signalwege aus dem TCR Komplex zulässt <sup>35,36</sup>. Eine von Ramello et al. durchgeführte systematische Untersuchung der CAR-Signalwege mittels Massenspektrometrie deutet darauf hin, dass die Signaldomänen des CARs ein komplexes Netzwerk von Proteinen ansteuern, welches über den klassischen TCR/CD3 $\zeta$ -Signalweg hinausgeht <sup>36</sup>.

Endogenes CD3 $\zeta$  stabilisiert den TCR/CD3-Komplex auf der Zellmembran. Das Fehlen von CD3 $\zeta$  bei einem Patienten mit primärer T-Zell-Immundefizienz führte zu substantieller Reduktion der Expressionsdichte der TCR $\alpha\beta$ -Ketten <sup>37</sup>. Eine spontane Re-expression von CD3 $\zeta$  in einem initial CD3 $\zeta$ -defizienten Patienten bewirkte die Wiederherstellung des TCR Komplexes und dessen Funktion <sup>38</sup>. CD3 $\zeta$  wird unabhängig vom TCR-Komplex exprimiert und unterliegt einem raschen Austausch auf der Zelloberfläche durch neusynthetisiertes CD3 $\zeta$  <sup>39</sup>. In dieser Arbeit wurde gezeigt, dass CD3 $\zeta$ -CARs ebenfalls eine kurze Halbwertszeit auf der Zellmembran haben und nur für kurze Zeit auf der Zelloberfläche verweilen. Die Expressionsdichte der CD3 $\zeta$ -CARs ist geringer als von vergleichbaren CARs mit Fc $\epsilon$ RI  $\gamma$ -Signaldomäne. Die  $\gamma$ -Signaldomäne ähnelt der CD $\zeta$  Signaldomäne dahingehend, dass ein ITAM gleichen Typs verliert, jedoch nur ein ITAM statt 3 ITAMs bei der CD $\zeta$ -Signalkette. Die geringe Expressionsdichte des CD3 $\zeta$ -CARs ist dabei vom intrazellulären CD3 $\zeta$  abhängig und wird nicht von der Transmembrandomäne beeinflusst. Der Effekt ist auf CD28-CD3 $\zeta$ -CARs der zweiten Generation übertragbar. Im Gegensatz zur Situation in T-Zellen ist die Expressionsdichte von Fc $\epsilon$ RI  $\gamma$ -CARs in Fc-Rezeptor exprimierenden Zellen wie Makrophagen und Neutrophilen geringer als die Expressionsdichte von CD3 $\zeta$ -CARs <sup>40</sup>. Der Fc $\epsilon$ RI  $\gamma$ -CAR kompetitiert mit endogenen Fc-Rezeptoren um eine limitierte Anzahl von Fc-Rezeptor- $\beta$ - und  $\gamma$ -Ketten <sup>41–44</sup>. In nicht-lymphatischen Zellen ist die Expressionsdichte von CD3 $\zeta$ - und Fc $\epsilon$ RI  $\gamma$ -CARs ähnlich hoch, was anzeigt, dass die jeweiligen

Expressionsdichten in den verschiedenen Zellen von der gleichzeitigen Anwesenheit des CD3 $\zeta$ - oder des Fc  $\gamma$ -Rezeptors abhängt. In CD3 $\zeta$  KO oder TCR<sup>-</sup> Jurkat-Zellen ist die Expressionsdichte von CD3 $\zeta$ -CARs geringer als von Fc $\epsilon$ RI  $\gamma$ -CARs. Die Expressionsdichte von CD3 $\zeta$ -CARs wird folglich nicht vom Vorhandensein der CD3 $\zeta$ -Kette oder des TCRs auf der Zelloberfläche, sondern von deren Signalelementen beeinflusst. Die Expressionsdichte des CARs auf der Zelloberfläche korreliert nicht mit der CAR-vermittelten Aktivierungsstärke. CD3 $\zeta$  CARs benötigen zur T-Zell-Aktivierung eine geringere Antigenexpressionsdichte als Fc $\epsilon$ RI  $\gamma$ -CARs.

CAR T-Zellen bilden ähnlich wie unmodifizierte T-Zellen einen Kontakt mit ihrer Zielzelle, um ein Aktivierungssignal in der T-Zelle zu generieren und schließlich die Zielzelle mit Hilfe von Perforin und Granzym zu eliminieren. Die Bildung dieses Kontakts läuft beim CAR scheinbar ungeordneter und deutlich schneller ab als die Bildung der TCR-Immunsynapse<sup>30</sup>. In dieser Arbeit haben wir mittels TIRF- und AiryScan-Mikroskopie nachgewiesen, dass sich die Kontaktregionen von CAR und TCR auf Membranebene separat ausbilden und sich nicht überschneiden. Der CAR rekrutiert den TCR nach Antigenerkennung nicht in seine Kontaktregion. Dieses steht in Übereinstimmung mit der bestehenden Beschreibung, dass vice versa der TCR den CAR nicht in seine Immunsynapse integriert<sup>45</sup>.



**Abbildung 5: Schematische Darstellung der Aktivierungsebenen von CAR und TCR**

TCR und CAR werden nicht in dieselbe Kontaktregion rekrutiert und leiten ihre Aktivierungssignale nach Antigenerkennung unabhängig voneinander weiter. CAR und TCR können dennoch in der Signalgebung komplementieren (Abbildung mit Genehmigung der Co-Autoren aus der dieser Arbeit zugrundeliegenden Publikation übernommen<sup>46</sup>).

Auch auf Ebene der zellmembranassoziierten Kinasen interferieren die Signale von CAR und TCR im Hinblick auf die CD3 $\zeta$  Phosphorylierung nicht. Eine Stimulation des CD28 CARs, des CD3 $\zeta$  CARs oder des CD28-CD3 $\zeta$  CARs resultierte nicht in einer Phosphorylierung des TCR/CD3-Komplexes. Andererseits wurden der CD3 $\zeta$  CAR und der CD28-CD3 $\zeta$  CAR nicht durch eine Stimulation des TCR/CD3-Komplexes phosphoryliert.

CAR- und TCR-Aktivierung münden jeweils in der Phosphorylierung des Adapterproteins LAT, jedoch in unterschiedlich starker Ausprägung<sup>47</sup>. Die Konvergenz der Signalwege auf der Ebene der LAT-Phosphorylierung könnte eine Kreuzaktivierung von spezifischen Effektorfunktionen der T-Zelle bewirken, die das distale Ende der Signalketten darstellen. Um dies zu überprüfen, haben wir uns die unterschiedlichen Voraussetzungen für eine Sekretion der Zytokine IFN- $\gamma$  und IL-2 zu Nutze gemacht. IFN- $\gamma$  wird bereits bei alleinigem CD3 $\zeta$ -Signal sezerniert, wohingegen die IL-2-Sekretion eine Kombination aus CD3 $\zeta$ - und CD28-Signalen voraussetzt. Die TCR/CD3 oder CD28 CAR-Stimulation alleine induzierten keine IL-2 Sekretion. Eine gleichzeitige Stimulation von TCR/CD3 und CD28 CAR resultierte hingegen in einer IL-2 Sekretion.

Wir schließen daraus, dass die Signale von CAR und TCR trotz der Verwendung identischer Signalmoleküle unabhängig voneinander übermittelt werden und sich nicht gegenseitig in der Ansteuerung von Effektorfunktionen der T-Zelle beeinflussen. Allerdings können das Primärsignal aus dem TCR/CD3-Komplex und das CD28 Signal des CAR komplementieren, sofern beide ihr Zielantigen erkennen. Muller et al. haben gezeigt, dass CAR T-Zellen mit einer CD28-Transmembrandomäne Heterodimere mit endogenem CD28 bilden und auf diese Weise eine T-Zell-Aktivierung bewirken können<sup>22</sup>. Unklar ist, auf welche Weise der CD28 CAR durch die Heterodimerisierung mit dem TCR kommuniziert.

Die Erkenntnisse dieser Arbeit sind für verschiedene Aspekte der klinischen Anwendung von CAR T-Zellen relevant. Die Aufrechterhaltung einer T-Zell-Population mit funktionalem Phänotyp ist Voraussetzung für den langfristigen Therapieerfolg eines applizierten T-Zell-Produkts. Anhaltende Stimulation durch den TCR führt zur terminalen Differenzierung von T-Zellen mit Ausbildung eines KLRG-1<sup>+</sup> CD57<sup>+</sup> CD7<sup>-</sup> Phänotyps<sup>48</sup>. Die CMV-spezifischen CD8<sup>+</sup> T-Zellen altern nach wiederholter Stimulierung mit Antigen und erleiden eine Hyposensitivität, d.h., die Aktivierungsschwelle steigt mit zunehmenden Runden der Aktivierung, was sich in einer im Vergleich zu jüngeren T-Zellen reduzierten TCR-Synapsenbildung widerspiegelt<sup>48</sup>. Die CAR-vermittelte Zytokinfreisetzung und Effektorfunktion sind ebenfalls unabhängig von der T-Zell-Alterung<sup>48</sup>. Die Rekrutierung von TCR-Komponenten in die Kontaktregion wird in gealterten T-Zellen durch eine Galektin-3



vermittelte Membranverankerung gestört <sup>48</sup>. Die Ausbildung der CAR-Kontaktregion funktioniert in gealterten T-Zellen hingegen ungehindert; Galectin-3 in diesen Zellen stört nicht die CAR Synapsenbildung, während die TCR Synapse blockiert ist. CARs können die durch anhaltende TCR-Stimulation verursachte Hyporesponsivität gealterter T-Zellen überwinden. Zusammen mit in dieser Arbeit generierten Daten spricht dies für ein Modell der TCR-unabhängigen CAR-vermittelten T-Zell-Aktivierung.

Umgekehrt erwarten wir keine Veränderung der Aktivierungskapazität des endogenen TCRs bei gleichzeitigem Vorhandensein des CARs. Epstein-Barr Virus spezifische T-Zellen konnten durch die Expression von anti-CD30-CARs für eine gezielte Tumorzelleliminierung genutzt werden; die Funktionalität ihres endogenen TCRs blieb erhalten <sup>49</sup>. CAR und TCR können parallel zur Stimulation von T-Zellen beitragen. Klinische Daten zeigen, dass eine Aktivierung von anti-CD19-CAR T-Zellen über ihren endogenen TCR eine Expansion und funktionelle Persistenz der CAR T-Zellen bewirkt <sup>50</sup>. Selbst die Infusion einer niedrigen CAR T-Zell-Dosis ohne vorherige zytoreduktive Chemotherapie führte bei viralem Stimulus durch die Stimulation des endogenen TCR zu einer potenten Depletion von CD19<sup>+</sup> Leukämiezellen <sup>50</sup>.

Die fehlende Kreuzaktivierung zwischen CAR und TCR ermöglicht eine kombinatorische Antigenerkennung im Sinne Boolesche Logik als UND-Funktion. Unsere Modellsysteme zeigen, dass ein rein kostimulatorisches Signal des CD28-CARs durch ein primäres Aktivierungssignal des TCRs komplementiert werden kann; der CD28 CAR allein erzeugt keine primäre Aktivierung. Allein die Erkennung der Zielstrukturen beider Rezeptoren ermöglicht eine anhaltende T-Zell-Aktivierung. Diese Kombinatorik bietet gleichfalls die Möglichkeit zur spezifischen Inhibition der T-Zell-Antwort. Ein inhibitorischer CAR kann die TCR-vermittelte T-Zell-Aktivierung nach Erkennung des CAR-spezifischen Antigens bremsen.

Das Konzept der kombinatorischen Antigenerkennung wurde von Kloss et al. eingeführt mit dem Ziel, Signale von zwei CARs zu integrieren <sup>51</sup>. Ein CAR mit suboptimaler Aktivierungsstärke komplementiert nach Erkennung seines Antigens mit einem kostimulatorischen CAR, welcher ein zweites Antigen erkennt <sup>51</sup>. Diese Strategie ermöglicht die tumorselektive T-Zell-Aktivierung in Abwesenheit eines einzelnen tumorspezifischen Antigens und erweitert somit das Anwendungsgebiet adoptiver Zelltherapien.

Die Kombinatorik von zwei CARs entfaltet seine Variabilität erst dann, wenn die Erkennungsdomänen beliebig ausgetauscht werden können. Dieses wurde durch die Entwicklung der Rev-CARs erzielt. Diese sind eine artifizielle Rezeptorplattform zur kontrollierbaren T-Zell-Aktivierung und stellen eine weitere Evolutionsstufe der CARs dar.

Anstelle einer scFv-Bindedomäne besitzen Rev-CARs lediglich ein kleines Peptidepitop als Extrazellulärdomäne <sup>52</sup>. In Anwesenheit eines passenden Adaptermoleküls, welches auf einem bispezifischen Antikörper basiert, kommt es zur spezifischen T-Zell-Aktivierung <sup>52</sup>. Auswahl des Adaptermoleküls sowie Dosierbarkeit erlauben eine flexible Steuerung der T-Zell-Antwort. Zur kombinatorischen Antigenerkennung können zwei RevCARs mit unterschiedlichen extrazellulären Peptidepitopen auf derselben T-Zelle eingesetzt werden <sup>52</sup>. Erneut ist eine fehlende Dimerisierung und Kreuzaktivierung Grundvoraussetzung für die erfolgreiche Anwendung einer UND-Funktion.

Letztlich erwarten wir ebenfalls kein verändertes CAR-Signal in Situationen reduzierter Expression der endogenen CD3 $\zeta$ -Kette, wie zum Beispiel bei chronischer Inflammation. Aus der Synovialflüssigkeit von Patienten mit rheumatoider Arthritis isolierte T-Zellen wiesen eine im Vergleich zu T-Zellen aus dem peripheren Blut des selben Patienten niedrigere CD3 $\zeta$ -Expressionsdichte und eine damit einhergehende reduzierte proliferative Kapazität auf <sup>53</sup>. Die CAR-vermittelte T-Zell-Aktivierung ist unabhängig vom endogenen CD3 $\zeta$  und weiteren primären TCR-Signalen und basiert auf CAR-intrinsischen CD3 $\zeta$ - und kostimulatorischen Domänen. Nichtsdestotrotz verbessert das Vorhandensein des endogenen TCR/CD3-Komplexes die Persistenz von CAR T-Zellen im Vergleich zu TCR-defizienten CAR T-Zellen maßgeblich <sup>32</sup>. CAR T-Zellen, in welchen mit Hilfe von CRISPR/Cas9 eine Defizienz der TCR-  $\beta$ -Kette erzeugt wurde, besaßen eine mit konventionellen CAR T-Zellen vergleichbare Expressionsdichte des CARs <sup>32</sup>. In vitro zeigten sich keine funktionellen Unterschiede zwischen den TCR-defizienten und konventionellen CARs <sup>32</sup>. Im Leukämie-Mausmodell war die Persistenz der CAR T-Zellen mit endogenem TCR verglichen mit den TCR-defizienten CAR T-Zellen jedoch signifikant höher <sup>32</sup>. Die "tonische" Aktivierung durch den TCR und damit einhergehende niederschwellige intrazelluläre Signalfortleitung scheint für den langfristigen Erfolg der CAR T-Zell-Therapie höchstgradig relevant zu sein. Langanhaltende CAR T-Zell-Persistenz ist notwendig, um Leukämien und Lymphome therapeutisch zu kontrollieren. Die Berichte von zwei Patienten mit akuter lymphatischer Leukämie, welche mit allogenen und zu 99% TCR-defizienten CAR T-Zellen behandelt wurden, unterstützen unsere Hypothese. Im Verlauf ihrer Therapie persistierten lediglich TCR-exprimierende CAR T-Zellen, die das ursprüngliche CAR T-Zell Produkt kontaminierten, und bewirkten eine Graft-versus-Host Reaktion <sup>5</sup>.

Zusammengefasst unterliegen CD3 $\zeta$ -CARs vergleichbaren Regulationsmechanismen wie die CD3 $\zeta$ -Kette des TCRs. Dennoch kann der CAR das endogene CD3 $\zeta$  des TCRs nicht ersetzen. Das Konzept der Bildung unabhängiger Synapsen von CAR und TCR wird durch strukturelle

Nachweise anhand mikroskopiebasierter Verfahren und funktionelle Analyse der membranständigen Signalgenerierung, z.B. von phospho-CD3 $\zeta$ , unterstrichen.

Über den Rahmen dieser Arbeit hinausgehend werden aktuell Versuche unternommen, den CAR in die Maschinerie des TCRs zur Regulation der T-Zell-Aktivierung einzubetten. Unter anderem konnte vor Kurzem durch Kreuzvernetzung des CARs über PDZ-Bindemotive, die eine Kopplung des CARs and des Zytoskeletts wie beim TCR vermitteln, eine verstärkte Synapsenbildung mit verbesserter Effektorfunktion nachgewiesen werden <sup>54</sup>. Ein tiefergehendes Verständnis der Interaktion von CAR und TCR mit nachfolgender Feinjustierung der CAR vermittelten T-Zell-Aktivierung würde die Kontrolle über die CAR T-Zell-Therapie als „Living Drug“ erhöhen und damit grundlegend zu ihrer Effizienz und Sicherheit beitragen.

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## 6. Anhang

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