Entwicklung einer CAR-T-Zelltherapie zur Behandlung von Plattenepithelkarzinomen des Kopfes und Halses (HNSCC)

Inaugural-Disseration

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
Corinna Haist
aus Köln

Düsseldorf, 31.05.2022

aus dem HNO-Forschungslabor
des Uniklinikums Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichterstatter: 1. Prof. Dr. Helmut Hanenberg
2. Prof. Dr. Matthias Kassack

Tag der mündlichen Prüfung: 12.12.2022

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Für meine Eltern

„Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.“

Albert Einstein

INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Gen- und immuntherapeutische Anwendungen rücken zunehmend in den Mittelpunkt der modernen Onkologie und werden immer häufiger zur Behandlung von Patienten eingesetzt, bei denen die Standardtherapie versagt hat. Insbesondere autologe T-Zellen, die chimäre Antigenrezeptoren (CARs) exprimieren, zeigen eine vielversprechende anti-tumorale Wirksamkeit bei Patienten mit CD19+ B-Zell-Neoplasien, weshalb es für die diese Erkrankung bereits sechs zugelassene CAR-T-Zelltherapien gibt.

Bislang konnte der erstaunliche Erfolg der CAR-T-Zelltherapie bei B-Zell-Malignomen noch nicht auf die Behandlung solider Tumore übertragen werden. Denn eine der größten Herausforderungen bei soliden Krebserkrankungen ist die Heterogenität der Zielantigenexpression auf den Tumorzellen, die häufig zum Entkommen/Überleben antigen-negativer Tumorzellen nach der Verabreichung der CAR-T-Zellen führt. Aus diesem Grund verfolgte ein Ziel dieser Dissertation, hochaffine CARs gegen Zielantigene zu entwickeln, die auf Plattenepithelkarzinomen des Kopfes und Halses (HNSCC) möglichst homogen exprimiert werden. Der epidermale Wachstumsfaktor-Rezeptor (EGFR) und die CD44-Variante 6, eine onkogene Spleißvariante von CD44, wurden als solche Zielkandidaten identifiziert, da sie auf der überwiegenden Mehrheit der getesteten HNSCC-Zelllinien exprimiert wurden. CARs gegen beide Antigene, die auf klinisch verwendeten hochaffinen einkettigen variablen Fragmenten (scFvs) der monoklonalen Antikörper Cetuximab bzw. BIWA8 basieren, wurden in lentiviralen Vektoren etabliert und ihre Funktionalität sowie ihre Spezifität an Zellen aus verschiedenen Tumorentitäten validiert.

Jedoch sind die immunsuppressiven Effekte der Tumormikroumgebung (TME) von soliden Tumoren ein weiteres großes Problem für die CAR-T-Zelltherapie, da die TME häufig die Immuneffektorzellen des Patienten inhibiert und ihre Zytotoxizität dadurch erheblich einschränkt. Deshalb wurde in dieser Arbeit versucht, maligne Zellen durch epigenetische Modulation für die CAR-T-Zelltherapie zu sensibilisieren. Als Beispiele für *Proof-of-Principle*-Studien wurden Plattenepithelkarzinom-Zelllinien der Blase mit zwei epigenetischen Inhibitoren, dem DNA-Methyltransferase-Inhibitor (DNMTi) Decitabin und dem Histon-Deacetylase-Inhibitor (HDACi) Romidepsin, vorbehandelt und anschließend mit EGFR- und CD44v6-CAR-T-Zellen koinkubiert. Diese epigenetische Vorbehandlung wirkte sich stark auf das Überleben und die Apoptose der Tumorzellen aus: Decitabin verbesserte und Romidepsin reduzierte die lytische Aktivität der CAR-T-Zellen im Vergleich zu den unbehandelten Kontrollzellen. Genexpressionsanalysen mit anschließender Validierung zeigten eine Korrelation zwischen der Zytotoxizität der CAR-T-Zellen und der Expression von zwei

Mitgliedern der BCL2-Genfamilie, wobei eine Erhöhung der BID- und ein Rückgang der BCL2L1-Expression mit einer effektiveren Lyse der Tumorzellen einherging.

Alle in dieser Arbeit genutzten CAR-Konstrukte erhielten zusätzlich eine neuartige, aus humanem CD34 abgeleitete C6-*hinge*, die von dem CD34 Antikörper Qbend-10 gebunden werden kann. Somit war es möglich CAR-T-Zellen sowohl direkt im Durchflusszytometer zu detektieren als auch mit Hilfe des magnetisch aktivierten Zellsortiersystems (MACS) von Miltenyi Biotec anzureichern; diese Verfahren können wahrscheinlich unter Einhaltung der *good manufacturing practice standards* durchgeführt werden. Zu betonen ist, dass die zusätzlichen Funktionen der C6-*hinge* keine Auswirkungen auf die Zytotoxizität der CAR-T-Zellen *in vitro* und *in vivo* in Leukämiemodellen bei Mäusen hatten.

Zusammenfassend wurden in dieser Arbeit erfolgreich hochaffine CAR-Konstrukte gegen zwei auf soliden Tumorzellen exprimierte Schlüsselantigene EGFR und CD44v6 etabliert, um CAR-positive T-Zellen, die die neuartige *hinge*-Region enthalten, effizient anzureichern und gleichzeitig solide Tumorzellen für die CAR-T-Zelltherapie durch die Vorbehandlung mit epigenetischen Inhibitoren zu sensibilisieren.

ABSTRACT

Gene and immunotherapeutic approaches are increasingly becoming the focus of modern oncology and are being applied more and more frequently in the therapy of patients failing standard treatment. In particular, autologous T-cells expressing chimeric antigen receptors (CARs) show promising anti-tumor efficacy in patients with CD19+ B-cell neoplasms. Currently, there are six approved CAR T-cell therapies, all against B-cell neoplasms.

To date, the amazing success of CAR T-cell therapy for B-cell malignancies could not be reproduced in the treatment of solid tumors. One of the major challenges in solid cancers is the heterogeneity of target antigen expression levels on tumor cells, which often leads to escape/survival of antigen-negative tumor cells after CAR T-cell administration. Therefore, one goal of this dissertation was to develop high-affinity CARs against target antigens expressed as homogeneously as possible on head and neck squamous cell carcinomas (HNSCCs). The epidermal growth factor receptor (EGFR) and the CD44 variant 6, an oncogenic splice variant of CD44, were identified as such target candidates, as they were expressed on the vast majority of the HNSCC cell lines tested. CARs against both antigens, based on clinically used high-affinity single chain variable fragments (scFvs) of the monoclonal antibodies cetuximab and BIWA8, respectively, were established in lentiviral vectors and the specificity and functionality of the CAR constructs validated on cells from different tumor entities.

The immunosuppressive effects of the tumor microenvironment (TME) are another major problem for CAR T-cell therapy of solid tumors, as the TME often inhibits the patient's immune effector cells and significantly limits their cytotoxicity. Consequently, this work attempted to sensitize malignant cells to CAR T-cell therapy by epigenetic modulation of gene expression. As examples for proof-of-principle studies, squamous cell carcinoma cell lines of the bladder were pre-treated with two epigenetic inhibitors, the DNA methyltransferase inhibitor (DNMTi) decitabine and the histone deacetylase inhibitor (HDACi) romidepsin, and then incubated with EGFR and CD44v6 CAR T-cells. This epigenetic pre-treatment strongly affected tumor cell survival and apoptosis in the co-cultures: Decitabine improved and romidepsi reduced the lytic capacity of the CAR T-cells in comparison to the untreated control cells. Gene expression analyses with subsequent validation revealed a correlation of the CAR T-cell cytotoxicity with the expression of two members of the BCL2 gene family, an increase in BID and a decrease in BCL2L1 expression with a more effective tumor cell lysis.

All CAR constructs used in this work contained a novel human CD34-derived C6-hinge, which can be bound by the CD34 antibody Qbend-10. This made it very easy to detect CAR

T-cells directly in the flow cytometer and to enrich them via the magnetic-activated cell sorting (MACS) microbeads system from Miltenyi Biotec; these procedures can probably be performed in compliance with good manufacturing practice standards. Importantly, these additional C6-hinge functions had no effect on CAR T-cell function and cytotoxicity *in vitro* and *in vivo* in leukemia models in mice.

In this thesis, high affinity CAR constructs against key antigens expressed on solid tumor cells, EGFR and CD44v6, were successfully established to efficiently enrich transduced CAR-T cells with a novel hinge region, and at the same time make solid tumor cells more sensitive towards CAR T-cell therapy by pre-treatment with epigenetic inhibitors.

1. EINLEITUNG

PLATTENEPITHERLKARZINOM DES KOPF-HALS-BEREICHS

EPIDEMIOLOGIE, ÄTIOLOGIE UND PATHOLOGIE

Plattenepithelkarzinome (SCC, *Engl.: squamous cell carcinoma*) sind maligne Tumore, die sich aus den Epithelien der Haut oder der Schleimhaut entwickeln. Mikroskopisch werden SCCs an einer polygonalen Zellstruktur und nestartigem Wachstum mit klar erkennbaren Zellgrenzen erkannt. Falls die Strukturen mikroskopisch nicht eindeutig sichtbar sind (Metastasen oder kleine Biopsien), geben immunhistologische Untersuchungen Anhaltspunkte zur Identifizierung [1].
Kopf-Hals-Plattenepithelkarzinome (HNSCCs, *Engl.: head and neck squamous cell carcinoma*) treten in den Schleimhäuten des oberen Schluck- und Atemtraktes, vornehmlich in der Mundhöhle, dem Oropharynx, dem Hypopharynx oder dem Larynx, auf. Eine Entstehung in Nase, Nasenrachen, den Nasennebenhöhlen oder auch Epithelien der Speicheldrüsen ist ebenfalls möglich. Insgesamt stellen sie den siebthäufigsten malignen Tumor weltweit dar; allein im Jahre 2019 wurden ca. 700.000 neue Fälle weltweit diagnostiziert [2], wovon 380.000 Patienten verstarben [3]. Die Mortalitätsrate liegt somit insgesamt bei 40-50 % [2] und steht hiermit an achter Stelle der tumorbedingten Todesursachen.

Tabak- und Alkoholkonsum gehören zu den Hauptrisikofaktoren für die Entstehung von HNSCCs und können in 70-80 % der Fälle mit der Erkrankung assoziiert werden [4]. Laut Hunter *et al.* (2005) führt Tabakabusus zu einer um 20-fach erhöhten Wahrscheinlichkeit für die Ausbildung von HNSCCs im Vergleich zu Nichtrauchern; bei ausgeprägtem Alkoholkonsum ist die Wahrscheinlichkeit nur 5-fach erhöht, wobei sich diese bei einem kombinierten Abusus um ca. 50-fach erhöht [1].

Seit den 80er Jahren ist bekannt, dass ein weiterer Risikofaktor für die Entstehung von HNSCCs, vor allem im Bereich des Oropharynx, eine Infektion mit onkogenen humanen Papilloma-Viren (HPV), vor allem des Subtyps 16 und 18, darstellen [5]. Obwohl über 100 verschiedene Serotypen von HPVen bekannt sind, lässt sich der HPV-Typ 16 in 90 % aller HPV-positiven HNSCCs nachweisen [6,7]. Die HPV-assoziierten Karzinome unterscheiden sich dabei auf molekularer Ebene von Tumoren ohne HPV-Befall und bilden daher in der neusten TNM-Klassifizierung eine eigene Entität [8]. Klinisch zeigen Patienten mit HPV-positiven Tumoren nicht nur ein deutlich verbessertes Ansprechen auf Radio-(Chemo)-Therapie, sondern auch ein besseres 5-Jahres-Überleben [6].

Die Metastasierung von HNSCCs erfolgt primär in die lokalen Lymphgefäße des Halses, eine hämatogene Metastasierung vor allem bei großen Tumoren oder in der Rezidiv-Situation ist ebenfalls möglich, so dass insgesamt 50 % der Patienten ein Rezidiv entwickeln [9] und somit systemische Therapieansätze benötigt werden.

THERAPIE UND PROGNOSE

Kopf-Hals Karzinome werden derzeit klinisch aufgrund Ihrer Lokalisation und der TNM-Klassifikation eingeteilt. „T“ beschreibt die Tumorgröße unter Berücksichtigung der Infiltration von Nachbarstrukturen, „N“, das Ausmaß der Lymphknotenmetastasierung und „M“ das Vorhandensein von Fernmetastasen. Aufbauend auf der TNM-Klassifikation kann zusätzlich eine Einteilung in '*union for international cancer control*' (UICC)-Stadien bei Vorliegen eines Tumorpräparates nach einem chirurgischen Eingriff durch den Pathologen vorgenommen werden. Bei Tumoren in frühen UICC-Stadien I und II können Heilungsraten von 90 % erzielt werden [10]. Jedoch werden 70 % aller Tumore meist in den höheren Stadien III oder IV diagnostiziert [10] und zeigen dann eine oft deutlich niedrigere 5-Jahres-Überlebensrate von ca. 50 %, die meistens durch fortgeschrittene Tumorstadien bei Diagnose und die häufig auftretenden lokalen Rezidive oder Metastasen verursacht werden [11].

Zusätzlich zur TNM-Klassifikation und UICC-Stadium, werden histopathologisch der Grad der Entartung „Grading“, die Infiltration von assoziierten Lymphbahnen, Nerven und Gefäßen und das Vorliegen von extrakapsulärem Wachstum bei Lymphknotenmetastasen bestimmt. Bei Oropharynxkarzinomen erfolgt weiterhin die HPV-Bestimmung mittels $p16^{Ink4a}$, dessen verminderte Expression als Surrogate-Marker für eine HPV-Infektion verwendet wird, oder den direkten Nachweis von HPV-assoziierter DNA durch PCR [12].

Diese Risikoklassifizierung schafft die Basis für Risiko-adaptierte multimodulare Therapieansätze, die als Behandlungsoptionen lokale Chirurgie, meist Platin-basierte Chemotherapie, Radiotherapie und Immuntherapie sowie Kombinationen daraus beinhalten. Das letztendliche therapeutische Vorgehen erfolgt jedoch nach interdisziplinär erarbeiteten Richtlinien (ESMO Guidelines) unter Beachtung der Patienten-individuellen Besonderheiten, der Patientenwünsche und auf Basis einer interdisziplinär getroffenen Empfehlung eines Tumorboards.

In den letzten Jahrzehnten hat die Immuntherapie die traditionelle Tumorbehandlung bei fast allen Entitäten - auch bei HNSCCs - ergänzt. Zunächst wurde 2006 der monoklonale Antikörper (mAB, *Engl.: monoclonal antibody*) Cetuximab von der US-amerikanischen *Food and Drug Administration* (FDA) zugelassen. Cetuximab blockiert die intrazelluläre Signalübertragung des epidermalen Wachstumsfaktor-Rezeptors (EGFR, *Engl.: Epidermal*

growth factor receptor) auf Tumorzellen, so dass das Wachstum des Tumors und die Invasion karzinogener Zellen in gesundes Gewebe verringert wird [13]. EGFR wird bei 80-90 % aller HNSCCs überexprimiert, so dass Cetuximab inzwischen zur Behandlung von rezidivierten und/oder metastasierten Karzinomen zusammen mit Chemotherapie als Erstlinienstandard klinisch angewendet wird [14]. In Kombination mit Bestrahlung (medianes OS 49 vs. 29 Monate im Vergleich zu Bestrahlung alleine) oder mit Chemotherapie (medianes OS 10,1 vs. 7,4 Monate im Vergleich zu Chemotherapie) kann eine Verbesserung des Gesamtüberlebens (OS, *Engl.: overall survival*) durch den Einsatz von Cetuximab erreicht werden [15]. Nichtdestotrotz sprechen nur 36 % der Patienten auf eine mit Chemotherapie kombinierte Behandlung an, während die Ansprechrate bei einer Cetuximab-Monotherapie bei nur 13 % liegt - möglicherweise bedingt durch Heterogenität des Tumors und Resistenzmechanismen [16].

Fast zehn Jahre nach der Zulassung von Cetuximab zeigten die KEYNOTE-12-Studie und die CheckMate-14-Studie, dass die Gabe der PD-L1-Checkpoint-Inhibitoren, Nivolumab (Zulassung für HNSCC: 2017) und Pembrolizumab (Zulassung für HNSCC: 2019), bei der Behandlung von rezidiviertem und metastasiertem HNSCC signifikante Verbesserungen für das Gesamtüberleben und die Lebensqualität herbeiführen kann [17,18]. Ähnlich gute Ergebnisse konnten auch mit den anti-PD-L1 mABs Duralumab und Atezolizumab erzielt werden [19,20]. Insgesamt profitieren jedoch nur 15-20 % der HNSCC-Patienten von einer Behandlung mit mABs gegen PD-L1 [21]. Der Checkpoint-Inhibitor Ipilimumab gegen CTLA-4 wurde bereits 2011 von der FDA zur Behandlung metastasierter Melanome zugelassen, aber erst später in Kombination mit Cetuximab und Radiotherapie in einer Phase-Ib-Studie (NCT01935921) für Stadium III HNSCCs getestet [22]. Derzeit werden weitere Studien zum Einsatz von Checkpoint-Iinhibitoren allein oder in Kombination mit Chemotherapie, Radiotherapie, Chirurgie, anderen Therapeutika (z.B. *small molecules*, andere Checkpoint-Inhibitoren, weitere immun-modulierende Substanzen) in unterschiedlichen klinischen Situationen (z.B. Adjuvanz, Neoadjuvanz, variante Studiendesigns der Checkpoint-Inhibitoren) durchgeführt.

Im Gegensatz zu Protein- oder *small molecule* Therapeutika stärkt eine adoptive Immuntherapie die Fähigkeit des Immunsystems, Tumorzellen zu erkennen und zu lysieren, indem dem Patienten spezifisch gegen die Tumorzellen gerichtete autologe oder auch allogene Immuneffektorzellen, wie z.B. T- oder NK-Zellen, verabreicht werden. Aus diesem Grund kann eine adoptive Zelltherapie mit CAR-positiven Immuneffektorzellen allein oder in Kombination mit anderen Therapeutika ein neues Feld von Therapiemöglichkeiten für HNSCCs eröffnen.

T-Zellimmuntherapie mit chimären Antigenrezeptoren

T-Zellrezeptor-vermittelte Aktivierung

T-Lymphozyten erkennen und binden mithilfe eines spezifischen Rezeptors, der im Wesentlichen aus zwei, durch eine Disulfidbrücke miteinander verknüpften Polypeptidketten, TCRα und TCRβ, besteht, fremde Antigene, wodurch eine Antigen-spezifische Immunantwort eingeleitet werden kann. Die Bindung des T-Zell-Rezeptors (TCR, *Engl.: T-cell receptor*) erfolgt jedoch nur dann, wenn ein Stück des erkannten Antigens auf Proteinen des Haupthistokompatibilitätskomplexes (MHC-Komplexes, *Engl.: major histocompatibility complex*), beim Menschen auch als humane Leukozytenantigene (HLA) bezeichnet, den T-Zellen präsentiert wird [23]. Diese HLA-Antigene sind Glykoproteine, die auf der Zelloberfläche lokalisiert sind und eine peptidbindende Furche formen, die mehrere Peptide zeitgleich binden/präsentieren kann. Die entsprechenden Peptidantigene werden intrazellulär prozessiert und als Teil des MHC-Komplexes auf dem Spalt mit hoher Affinität gebunden und an die Oberfläche gebracht [24]. Während MHC-Klasse-I-Moleküle Peptide präsentieren, die im Zytosol aus viralen oder zytosolischen Erregern prozessiert werden, können MHC-Klasse-II-Moleküle Peptide von phagozitierten Erregern oder auch malignen Zellen aus dem Endosol aufnehmen. Die Erkennung dieser Peptide ist jedoch nicht nur allein von den beiden MHC-Klassen abhängig, sondern auch von deren allelischen Varianten, was zu einer individuellen MHC-Restriktion von Peptiden führt [25].

Ein zentrales Charakteristikum für das Immunsystem des Menschen ist, dass die beiden großen MHC-Klassen von verschiedenen T-Zell-Subtypen erkannt werden. CD4-Lymphozyten nehmen MHC-Klasse-II präsentierende Zellen wahr und aktivieren unterschiedliche Effektorzellen, die die Peptid-präsentierenden Zellen letztlich eliminieren. CD8-T-Zellen hingegen beseitigen nach Erkennung des Peptids auf dem MHC-Klasse-I-Komplex jede Zelle, die ein als fremd erkanntes Peptid präsentiert [25].

Nach der Erkennung der Peptide durch den TCR erfolgt die Signalweiterleitung über den mit dem TCR verbundenen CD3-Komplex, der aus den drei Heterodimeren CD3 γε, CD3 δε und CD3 ζζ besteht. Da die zytosolischen Domänen des TCR zu kurz sind, vermitteln die Domänen von CD3 die intrazelluläre Signalübertragung über ihre insgesamt zehn Immunrezeptor-Tyrosin-basierten-Aktivierungsmotive (ITAM*, Engl.: immunoreceptor tyrosine-based activation motif*) [26]. Nach der Interaktion zwischen Rezeptor und Ligand erfolgen eine Dimerisierung der TCRs und die Phosphorylierung der ITAMs. Die dadurch aktivierte Kinasekaskade induziert verschiedene nachgeschaltete Signalwege, die unter anderem die Proliferation und Differenzierung der T-Zelle begünstigen. Für eine produktive T-Zellaktivierung benötigt die T-Zelle ein weiteres obligates Signal, das von der

antigenpräsentierenden Zelle ausgeht. Die auf der antigenpräsentierenden Zelle exprimierten B7-Moleküle, die als Kostimulator an der Aktivierung von T-Zellen beteiligt sind, werden von CD28-Rezeptoren der T-Zelle gebunden, so dass die Differenzierung und Proliferation der T-Zelle erfolgen kann [27]. Ein drittes Signal - in Form von Zytokinen - beeinflusst den Differenzierungsgrad der T-Zelle und stimuliert ihre Entwicklung zur T-Responderzelle.

Ist die T-Zelle zu einer T-Effektorzelle ausdifferenziert, kann sie nach ihrer Aktivierung zytotoxische Proteine sezernieren. Programmierter Zelltod wird z.B. durch eine Apoptoseinduktion über zellulären Kontakt oder auch durch die Ausschüttung von Perforin und Granzym B ausgelöst: Perforin perforiert die Membran, so dass das Granzym B einfacher in die Zelle eindringen kann [28].

Allerdings können Tumorzellen sich vor dem induzierten Zelltod schützen, indem sie z.B. die MHC-Klasse-I-Moleküle auf ihrer Oberfläche herunterregulieren. Dadurch werden die Erkennung des Zielantigens bzw. der Zielzellen und damit die Aktivierung der T-Zelle vermieden. Damit autologe oder auch allogene Immuneffektorzellen trotzdem zur Behandlung von Krebserkrankungen effektiv eingesetzt werden können, werden zurzeit zahlreiche Ansätze zur adoptive Zellimmuntherapie entwickelt.

ADOPTIVE T-ZELLIMMUNTHERAPIE

Die adoptive zelluläre Immuntherapie ergänzt die konventionelle Behandlung von malignen Erkrankungen, indem *ex vivo* expandierte immunologische Effektorzellen, wie im Falle der adoptiven T-Zellimmuntherapie autologe T-Lymphozyten, nach Stimulation mit Interleukinen (IL) in den Patienten infundiert werden, so dass eine spezifisch, gegen den Tumor gerichtete Immunantwort, im Patienten induziert wird. Die dazu genutzten Immunzellen können entweder von Spendern (z.B. allogene NK-Zellen oder auch TCR$\gamma\delta$ T-Zellen) oder auch vom Patienten selber stammen [29].

Das Konzept, Immunzellen therapeutisch für die Krebsbehandlung zu nutzen, wurde erstmals in den 80er Jahren klinisch eingesetzt. Um die Anti-Tumor-Reaktion des Immunsystems zu verstärken, wurde zunächst versucht die Anzahl der tumorspezifischen T-Zellen im Blutkreislauf und im malignen Gewebe zu erhöhen. Die Gruppe von Rosenberg zeigte 1988 erstmalig, dass die *ex vivo* Expansion von Melanom-Metastasen infiltrierten T-Lymphozyten (TILs, Tumor-infiltrierende-Lymphozyten) mittels IL-2 möglich ist und diese TILs nach autologer Reinfusion bei Patienten mit metastasierenden Melanomen eine Tumorregression bewirken können [30]. Die dennoch schlechten Ansprechraten der Patienten konnten mithilfe einer nicht-myeloablativen Lymphodepletion vor dem TIL-Transfer - wahrscheinlich aufgrund der Eliminierung immunregulatorischer Zellen,

verringerter Konkurrenz um Wachstumsfaktoren und Erhöhung der Interleukinkonzentration im Serum - verbessert werden. Letztendlich führte die *in vitro* Inkubation der TILs mit IL-2 in Kombination mit einer Lymphodepletion vor der Gabe an den Patienten bei der Behandlung des metastasierendem Melanoms zu einer klinischen Ansprechrate von 40-50 % [31–34]. Die hohen Kosten und die begrenzte klinische Wirksamkeit limitierte jedoch diese zeitaufwendige Therapie, so dass es notwendig wurde, die adoptive Immuntherapie weiterzuentwickeln.

Um die Tumorspezifität von TILs auf Lymphozyten aus dem peripheren Blut zu übertragen, wurden T-Zellen durch Transfervektoren mit einem TCR, der spezifisch gegen ein definiertes Antigen auf der Tumorzelloberfläche gerichtet war, ausgestattet [35]. So konnte die Affinität der TCR-modifizierten T-Zellen gegenüber des Tumors wesentlich gesteigert werden [35]. Durch die Expression von TCRs auf T-Zellen wird auch der physiologische Weg der T-Zell-Erkennung beibehalten, weshalb neben Oberflächenantigenen auch prozessierte intrazelluläre Antigene erkannt werden können [35].

Die TCR-basierte Therapie zeigt jedoch auch Nebenwirkungen in Form von Autoimmunreaktionen, denn Fehlpaarungen zwischen den endogenen und rekombinanten TCR-Ketten zu Heterodimeren kann zu neuen unerwünschte Rezeptorspezifitäten gegen körpereigene MHC-Peptidkomplexe führen [36]. *In vivo*-Studien demonstrierten ebenfalls, dass TCR-modifizierte T-Zellen nicht in jedem Fall eine autoimmune Gewebeinfiltration oder –schädigung vermitteln [37]. Ein weiterer Nachteil der Therapie besteht darin, dass Tumorzellen der T-Zellerkennung durch Reduktion der MHC-Moleküle entgehen können [31].

Um auch eine MHC-unabhängige Erkennung durch nicht-MHC-restringierte Immuneffektorzellen beim Menschen einsetzen zu können, wurden chimärer Antigen-Rezeptoren (CARs) entwickelt, bei denen die auf der Tumorzelle exprimierten Zielantigene direkt erkannt werden. Dieser Ansatz ist im Folgenden beschrieben und ein zentrales Thema meiner Arbeit.

AUFBAU UND FUNKTION CHIMÄRER ANTIGENREZEPTOREN

Im Vergleich zur TCR-basierten Erkennung von Antigenen durch prozessierte und im MHC-Kontext präsentierte Peptide erkennen CAR-T-Zellen spezifische Epitope in Oberflächenantigenen über eine von einem Antikörper abgeleitete Bindedomäne, so dass die Elimination der Zielzellen in MHC-unabhängiger Weise funktioniert [38]. Bei einem TCR wird nach erfolgreicher MHC-Peptidbindung ein Multiproteinkomplex, bestehend aus mehreren Ketten, aufgebaut, wohingegen die Antigenbindung und Signalweiterleitung eines CARs durch funktionelle Einheiten in einem einzigen Polypeptid miteinander verbunden sind [39].

CARs sind artifizielle Konstrukte, die prinzipiell aus einer extrazellulären antigenbindenden Domäne, einer *hinge* (oder auch *spacer* genannt), einer Transmembrandomäne und mindestens einer intrazellulären Signalkette (Abbildung 1A) bestehen.

Die extrazelluläre **Antigenbindedomäne** setzt sich aus einer schweren (V_H, *Engl.: variable heavy*) und einer leichten (V_L, *Engl.: variable light*) Kette eines murinen oder humanen mAB, die durch einen flexiblen Linker zu einem Einzelkettenfragment (scFv) fusioniert sind, zusammen und ist für die spezifische Bindung des CARs an das idealerweise tumorspezifische Zielantigen, welches meist extrazellulär auf der Tumorzelle vorliegt, zuständig [40]. Neben der Spezifität des CARs zum Zielantigen beeinflusst die Wahl der Antigenbindedomäne auch die Affinität des CARs. Bei der Wahl der scFv muss beachtet werden, dass eine sehr hohe Affinität mit Dauerstimulation (häufig) zu einem aktivierungsbedingten Zelltod der CAR-T-Zelle oder zu einer *on-target/off-tumor*-Toxizität führen kann. Bei der *on-target/off-tumor* Toxizität bindet der CAR sein Antigen außerhalb des Tumors, wenn dieses - in der Regel in geringerer Dichte - auf anderen körpereigenen Zellen ebenfalls vorkommt. Eine zu geringe Affinität der scFv begünstigt eine ineffektive Bindung an das Zielantigen, eine unzureichende Aktivierung der Zytotoxizität der CAR-T-Zelle und somit einen mangelnden therapeutischen Effekt [41–43].

Die ***hinge*** und die **Transmembrandomäne** (TM) verknüpfen die Antikörperbindedomäne mit den intrazellulären Signaldomänen. Die Charakteristika der *hinge* müssen dabei so an das Zielantigen angepasst werden, dass dieses ohne sterische Einschränkungen gebunden werden kann. Für eine optimale Antigenbindung und Signalweiterleitung gehören hierzu sowohl eine ausreichende Flexibilität als auch eine adäquate Länge der *hinge*, wobei letztere ganz wesentlich von der Entfernung der Binderegion des Antigens zur Oberfläche der Zielzelle abhängig ist [42,44]. In der Vergangenheit wurden Fragmente von menschlichem Immunglobulin G1 (IgG1) oder Immunglobulin G4 (IgG4) als *hinge* genutzt, die jedoch durch Kreuzreaktionen mit Fc-Rezeptoren auf myeloischen Zellen eine unspezifische Aktivierung der T-Zellen auslösten, wodurch *in vitro* und auch *in vivo off-target*-Toxizität entstand [45,46]. Die **TM-Domäne** verankert den CAR in der Zellmembran der Effektorzelle und trägt wesentlich zur Stabilität des CARs bei. Dotti *et al*. konnten z.B. zeigen, dass der Einbau einer CD28 TM, im Vergleich zur Nutzung einer CD3ζ TM, zu einer besseren und stabileren CAR-Expression führte [47,48].

Zu Beginn der CAR-Entwicklung beinhaltete der modular aufgebaute CAR lediglich eine intrazelluläre **Signaldomäne** (Abbildung 1D), die CD3ζ-Kette. Diese stammt aus einem TCR-Komplex und enthält drei ITAMs, die durch ihre Phosphorylierung die

Aktivierung der CAR-T-Zelle induzieren. Da dieser CAR der ersten Generation keine kostimulatorische Domäne enthielt und somit die Aktivierung der CAR-T-Zellen nicht ausreichte, um eine Expansion der CAR-T-Zellen und eine kontinuierliche Antitumor-Aktivität *in vivo* zu erzeugen [49], wurden in der zweiten Generation der CARs zusätzliche intrazelluläre Signaldomänen, meist CD28 oder CD137 (4-1BB), eingebaut, die die Aktivierung, Proliferation und Persistenz der CAR-T-Zellen verbesserten [50].

Durch die Nutzung zweier intrazellulärer Signaldomänen, CD3ζ und CD137, konnte erstmals *in vivo* die vollständige Aktivierung der CAR-T-Zellen und ihr zytotoxisches Potential dokumentiert und später klinisch mithilfe von gegen CD19 gerichtete CARs der zweiter Generation Patienten mit B-Zell-Malignomen erfolgreich behandelt werden [51–54]. Mittlerweile sind CARs mit CD28 oder CD137 als zweite Kostimulationsdomänen aufgrund der hohen Ansprechraten bei Patienten mit B-Zellneoplasien von der FDA und der EMA zugelassen [39].

Die dritte Generation der CARs enthält neben CD3ζ, zwei weitere intrazelluläre Domäne, z.B. CD28 und CD137. Alternative kostimulatorische Domänen, wie z.B. CD134 [55], CD27 [56] oder ICOS [57], funktionieren ebenfalls, wurden aber bislang kaum in Phase I/II klinischen Studien an Patienten getestet, da schwere Zytokine-Release-Syndromen schon bei den zweite Generationen CARs auftreten und Befürchtungen bestehen, dass die niedrige Signalschwelle durch die drei Signaldomänen zur Aktivierung der CAR-T-Zellen auch ohne vorherige Antigenerkennung ausgelöst werden könnte.

Als zusätzliche Steigerung wurden die sogenannten TRUCK´s (*Engl.: T-cells redirected for universal cytokine-mediated killing*) speziell für die Eliminierung von soliden Tumorzellen entwickelt; diese Trucks werden auch als vierte Generation CARs bezeichnet. In den TRUCKS kann die bislang unzureichende Produktion von pro-inflammatorischen Zytokinen der zweiten und dritten Generation CAR-T-Zellen durch die zusätzliche induzierbare Freisetzung von transgenen Immunmodifikatoren wie z.B. IL-12 zu einer lokaleren Produktion von Zytokinen im Tumormikromilieu (TME) führen und die Proliferation und das Überleben der CAR-T-Zellen während der Ansammlung im soliden Tumor begünstigen [58]. Da die konstitutive Expression von z.B. IL-12 zum Absterben der T-Zellen führt, wird in den TRUCKS prinzipiell ein zusammengesetzter Promotor aus mehreren *Nuclear Factor of Activated T-Cells* (NFAT)–Reaktionselementen in Kombination mit einem minimalen Interleukin-2 Promoter in den CAR-Vektor integriert, so dass die Zytokinfreisetzung erst nach der Erkennung des TAAs (tumorassoziierte Antigene) und dem *Signaling* über den CAR induziert wird [59].

Eine fünfte Generation CAR-T-Zellen enthält zusätzlich eine intrazelluläre Domäne eines Zytokinrezeptors (z.B. IL-2R) im CAR-Konstrukt, die die Signaltransduktion über den

STAT/JAK-Weg induzieren. Dadurch kann die CAR-T-Zelle nach Antigenstimulation zusätzlich die Produktion von entzündlichen Zytokinen induzieren, so dass die Aktivierung verbessert und die Proliferation erhöht wird [60].

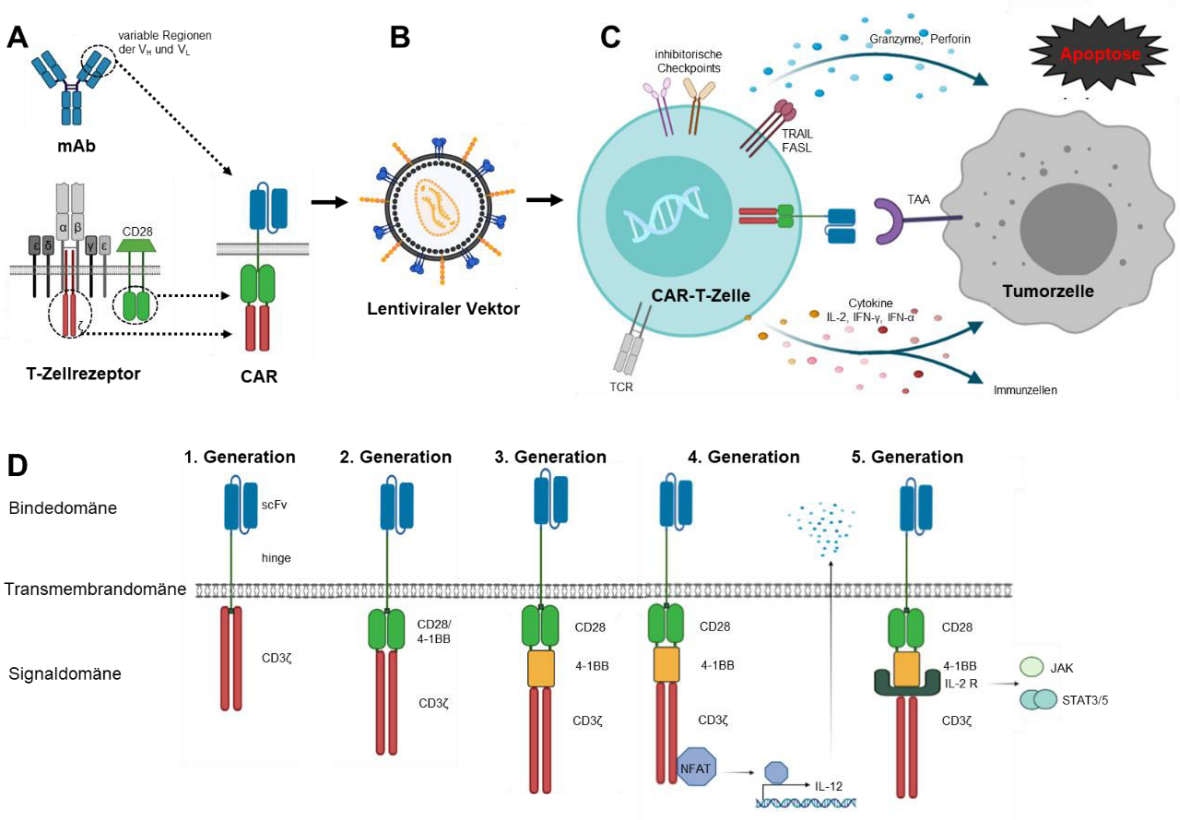

Abbildung 1: Aufbau und Funktion von CAR-T-Zellen. Ein zweite Generation CAR besteht prinzipiell aus einem scFv eines mABs und zwei intrazellulärer Signaldomänen (CD28 und CD3ζ) eines T-Zellrezeptors, die über eine *hinge* und eine TM-Domäne miteinander verbunden sind (A). Nach Integration des CAR-Vektors (z.B. durch lentiviralen Gentransfer) und Expression auf primären T-Zellen (B) kann die gebildete CAR-T-Zelle das Tumorantigen in einer MHC-unabhängigen Weise binden, wodurch die T-Zelle zur Proliferation und Zytotoxizität aktiviert wird (C). Insgesamt wurden bisher fünf Generationen von CARs bislang entwickelt, um die Proliferation, die Zytotoxizität, die Beständigkeit und die Aktivierung der CAR-T-Zellen durch die Integration verschiedener intrazellulärer Signaldomänen zu verbessern (D). Diese Abbildung wurde modifiziert nach [61,62] und mit Biorender.com erstellt.

Für die klinische Anwendung der CAR-T-Zelltherapie werden autologe T-Zellen aus dem Blut des Patienten isoliert und *ex vivo* mit dem CAR-Konstrukt ausgestattet. Die CAR-Sequenz wird mithilfe eines Vektors, meist durch lentiviralen/retroviralen Gentransfer oder häufiger auch durch Transposons (*PiggyBac*/*Sleeping Beauty*) in die *in vitro* expandierten T-Zellen des Patienten eingebracht (Abbildung 1B), so dass diese schließlich den CAR exprimieren [63–65]. Die Expansion der transduzierten Zellen erfolgt entweder durch Aktivierung des TCRs und/oder durch Präkonditionierung mit stimulierenden Zytokinen wie IL-2. Nachdem schließlich die CAR-T-Zellen dem Patienten nach Vorbehandlung mit

Chemotherapie in die peripheren Venen reinfundiert wurden, werden sie durch die Bindung des scFv an das Antigen auf der Zielzelle durch die intrazytoplasmatischen Signaldomänen aktiviert und üben ihre zytotoxischen Funktionen aus [62]. Die Übertragung der Aktivierungssignale der CARs in den Zellkern der T-Zelle führt dann u.a. zu einer Ausschüttung von Perforin, das die Zelloberfläche der Tumorzelle perforiert, so dass Granzym B in die Tumorzelle eindringen und Apoptose auszulösen kann (Abbildung 1C). Zusätzlich werden auch pro-inflammatorische Zytokine wie IL-2, Interferon-gamma (IFNγ) oder Tumornekrosefaktor-alpha (TNFα) freigesetzt, um weitere Immunzellen zu aktivieren [66,67].

Die Ansprechraten der Neoplasien auf eine CAR-T-Zelltherapie sind, vor allem im Hinblick auf die Behandlung solider Tumore, von einer Vielzahl unterschiedlicher Faktoren abhängig: Dazu gehören die Expression der tumorassoziierten (TAAs) wie auch -spezifischen (TSAs) Antigene, die Immuncheckpoint-Expression und die HLA-Expression auf den Zielzellen, der Apoptose-Bereitschaft der malignen Zellen, dem immunsuppressiven *Signaling* im TME und dem Vorhandensein von TILs und von Zellen im TME (Tregs, TAMs). Hofft man auf einen zusätzlichen unterstützenden Effekt des Immunsystems auf die adoptive zelluläre Immuntherapie, so können folgende Faktoren wichtig sein: das TCR-Repertoire der TILs, die Mutationslast in den malignen Zellen, die durch unzureichende DNA-Reparatur zur Expression von Neoantigenen führt, die häufig durch punktuelle genetische Veränderungen entstehen.

Viele dieser Mechanismen stehen unter epigenetischer Kontrolle [71]. Deshalb untersuchen viele Ansätze und erste klinische Studien zusätzliche Manipulationen der Tumorzellen mit epigenetischen Substanzen wie Histon-Deacetylase-Inhibitoren (HDACi) oder auch DNA-Methyltransferase-Inhibitoren (DNMTi), um die malignen Zellen gegenüber den Immuneffektorzellen zu sensibilisieren [68–71].

EPIGENETISCHE THERAPIE

Unter dem Begriff Epigenetik versteht man die reversible Modifikation von Genexpressionsmustern auf mRNA-Ebene, ohne eine Änderung der zugrundeliegenden DNA-Sequenz herbeizuführen, um die Differenzierung und Physiologie verschiedener Zelltypen zu beeinflussen. Diese Regulation der Genexpression erfolgt durch Konformationsänderungen des Chromatins und der damit verbundenen Zugänglichkeit bestimmter Gene für die Transkriptionsmaschinerie. Genexpressionsveränderungen werden mithilfe unterschiedlicher epigenetischer Prozesse wie DNA-Methylierung, Auswirkungen von nicht-kodierenden RNAs, posttranslationalen kovalenten Modifikationen von Histonen und Veränderungen der Chromatinstruktur und erreicht [72].

POSTTRANSLATIONALE MODIFIKATION VON HISTONEN

Der Mensch besitzt ungefähr 20.000 bis 25.000 Gene, die auf der chromosomalen Desoxyribonukleinsäure (DNA) codiert sind. Die Gesamtlänge der DNA wird im haploiden Chromosomensatz auf einen Meter geschätzt, unterläuft während der Metaphase eine strukturelle Reorganisation und wird schließlich um das 10.000-fache verkürzt. Die Verpackung der DNA erfolgt in Nukleosomen, die aus Histon-Proteinen (Histone) und der außen herumlaufenden DNA-Helix bestehen [73]. Histone, die zusammengelagert in Form eines rundlichen Oktamers auftreten (Abfolge: H2A, H2B, H4, H3, H3, H4, H2B, H2A), bestehen zu einem großen Teil aus positiv geladenen Aminosäuren (Arginin, Lysin), wodurch die negativ geladene DNA diese mit einer Sequenzlänge von ca. 146 Basenpaaren 1,65-Mal umwinden kann [74]. Die vier basischen Kernhistone binden mithilfe ihrer globulären Struktur das Phosphatdiester-Band der DNA, wodurch das Nukleosom stabilisiert wird. Die flexiblen Histonschwänze ragen jedoch aus dem Komplex heraus und können Interaktionen mit benachbarten Nukleosomen, DNA und Proteinen im Nukleus eingehen oder z.B. mithilfe von epigenetischen Enzymen auf unterschiedliche Weise modifiziert werden; dadurch werden die Bindungseigenschaften und Anordnung der Nukleosomen im Chromatin beeinflusst [73]. Durch die offenen bzw. geschlossenen Zustände des Chromatins kann die Transkription der gebundenen DNA entweder erleichtert oder verhindert werden, wodurch der Informationsgehalt des genetischen Codes erhöht wird (Abbildung 2) [73].

Die genomische Prägung (*Engl.: Imprinting*) stellt wohl die bekannteste Form der epigenetischen DNA-Modifikation dar und aktiviert entweder das väterliche oder mütterliche Allel eines Gens oder vermittelt die X-chromosomale Inaktivierung durch DNA-Methylierung [75]. Fehler während der Prägung beeinflussen die epigenetischen Muster und die Expression der Gene innerhalb eines geprägten Genclusters und führen zu *Imprinting*-Krankheiten, wie

z.B. Prader-Willi-Syndrom, Angelman-Syndrom, Beckwith-Wiedemann-Syndrom oder Silver-Russell-Syndrom [75].

Weitere zelluläre Prozesse, wie die Entwicklung des Immunsystems [76], die Regulation von Gehirnfunktionen [77] oder die Aufrechterhaltung der genomischen Integrität [78] werden ebenfalls durch epigenetische Mechanismen beeinflusst, wodurch z.B. unterschiedliche Verhaltensweisen bei eineiigen Zwillingen erklärt werden können [79]. Mithilfe dieser reversiblen Modifikationen können Organismen also temporär auf interne oder äußere Reize mit einer Änderung des Phänotyps reagieren und sich anpassen, ohne dass es zu Veränderungen in der DNA kommen muss [80].

Neben genetischen Mutationen spielen aberrante epigenetische Veränderungen auch eine wichtige pathophysiologische Rolle bei der Tumorentstehung [74]. So ist für viele Krebsarten, inklusive HNSCCs, gezeigt, dass die Krebsentstehung, -erhaltung und -progression durch Veränderungen der DNA-Methylierungs- und Histonmodifikationsmuster sowie durch veränderte Expressionsprofile chromatin-modifizierender Enzyme ausgelöst werden [72,81]. In den meisten Fällen resultieren die epigenetischen Modifikationen in einer Dysfunktion der Nukleosomdynamik oder Transkription, wodurch abnorme Zellproliferation, Invasion und Metastasierung sowie Chemoresistenz entstehen können [82]. Wenn die Missregulation der epigenetischen Signalwege zu einem Selektionsvorteil der malignen Zelle führt, kann dieser Phänotyp sogar stabil vererbt werden.

Zu den am häufigsten beschriebenen epigenetischen Mechanismen für die Entwicklung von HNSCC gehören die Histonacetylierung und DNA-Methylierung [83], deren Reaktionen durch verschiedene Klassen DNA- und Histon-modifizierender Enzyme, wie Methyltransferasen (DNMT) und Histondeacetylasen (HDACs), katalysiert werden.

Da im Gegensatz zu genetischen Mutationen epigenetische Regulation reversibel ist, werden immer häufiger Wirkstoffe, die die Aktivität epigenetischer Enzyme hemmen, in klinischen Studien eingesetzt, um z.B. epigenetisch stillgelegte Tumorsuppressorgene zu reaktivieren [84]. Demethylierende oder deacetylierende Inhibitoren hemmen dabei Enzyme, die Methylgruppen an die DNA anfügen bzw. Enzyme, die Acetylgruppen von den Histonen entfernen, so dass die epigenetischen Modifikationen entfernt wird, das Chromatin seinen Ausgangszustand annimmt und stillgelegte Gene erneut transkribiert werden können [85,86].

HDAC-Inhibition als therapeutischer Ansatz

Unter Histonacetylierung versteht man die Übertragung einer Acetylgruppe von Acetyl-Coenzym A auf die ε-Aminogruppe der Lysinseitenketten am aminoterminalen Ende der Histonproteine, wodurch die positive Ladung der Lysine neutralisiert wird [87]. Die Affinität

zwischen den Histonenden und der negativ geladenen DNA nimmt ab, das Chromatin lockert sich, so dass die Nukleosomstruktur destabilisiert wird; diese leichtere Zugänglichkeit von z.B. Transkriptionsfaktoren an die DNA führt dazu, dass dort lokalisierte Gene effizienter abgelesen werden können [87]. Die zügige Umkehrung dieses Prozesses ist jedoch für die epigenetische Kontrolle der eukaryotischen Gentranskription essentiell und wird von HDACs ausgeführt, indem durch die erneute Deacetylierung der Histone die Zugänglichkeit zur DNA für Transkriptionsfaktoren wieder verringert wird [72].

Bis heute wurden 18 verschiedene HDACs identifiziert, die aufgrund ihrer Sequenzähnlichkeit zu Hefe-HDACs in vier Klassen unterteilt werden [88]. Klasse I (HDAC1, 2, 3, 8), Klasse II (HDAC4, 5, 6, 7, 9,10) und Klasse IV (HDAC11) benötigen Zink für die Deacetylierung, wohingegen Klasse III-HDACs Nicotinamid-Adenin-Dinukleotid als Cofaktor für ihre katalytische Funktion nutzen [87]. Zur Klasse I gehören die HDACs 1-3 und 8, welche alle im Nucleus und HDAC 8 zusätzlich im Cytoplasma lokalisiert sind. Enzyme dieser Klasse zeigen erhebliche Ähnlichkeit zu dem Transkriptionsfaktor Rpd-3 der Hefe und deacetylieren unter anderem p53 oder STAT-3 [88]. Klasse II HDACs (HDACs 4-7, 9, 10) weisen Homologien zur HDAC1-Deacetylase der Hefe auf. Besonders HDAC 6 ist aufgrund der Beteiligung an der Deacetylierung von α-Tubulin, Cortactin, Chaperonen und der Regulierung der Autophagie und des Leberstoffwechsels von besonderer Wichtigkeit in der Zelle [87]. Klasse III-HDACs sind ebenfalls an der Deacetylierung von nicht-Histonen beteiligt. Zu Klasse IV-HDAC gehört zurzeit nur ein Vertreter (HDAC 11), der noch nicht im Detail untersucht wurde.

Ein exaktes Zusammenspiel zwischen Histonacetyltransferasen (HATs) und HDACs ist entscheidend für die exakt regulierte Expression von Genen, die mit der Signalübertragung, dem Zellwachstum und dem Zelltod in Verbindung stehen [87]. Ein Ungleichgewicht dieser Enzyme kann mit der Proliferation maligner entarteter Zellen in Verbindung gebracht werden. Bei HNSCCs werden immer häufiger Überexpressionen von HDACs beschrieben, die zu einer schlechten Prognose des Patienten führen [89]. Eine Überexpression von HDAC 6 wird hauptsächlich in fortgeschrittenen HNSCCs gefunden und somit mit einer aggressiveren Erkrankung in Zusammenhang gebracht. Eine HDAC 2 Überexpression führt zu einer Stabilisierung des Proteins Hypoxia-induzierbarer Faktor Alpha (HIF-1α), wodurch die Migration und Invasion von Tumorzellen begünstigt wird [89]. Aufgrund dieser Zusammenhänge wird die Nutzung von HDAC-Inhibitoren immer häufiger zur Behandlung von malignen Erkrankungen genutzt [90].

Ein in vielen klinischen Studien verwendeter Kandidat stellt Romidepsin (ROM) dar. ROM (Istodax ®, auch bekannt als Depsipeptid, NSC 630176, FR 901228, FK228) ist ein zyklisches Tetrapeptid, das aus dem Fermentationsprodukt des Bakteriums

Chromobacterium violaceum isoliert wurde und als Prodrug verabreicht wird. Nach Reduktion seiner Disulfitbindung durch Glutathion ist es schließlich in der Lage, HDACs zu binden [86]. Im niedrigen nM-Bereich werden vorrangig HDACs der Klasse I, bei höheren Konzentrationen jedoch auch Klasse II HDACs mithilfe einer besonders starken und langanhaltenden Bindung im Vergleich zu anderen HDAC-Inhibitoren inhibiert [91].

Der Einsatz vom ROM bei Patienten mit T-Zell-Lymphomen zeigte vielversprechende initiale Ergebnisse [92], so dass die FDA dieses Medikament im Jahr 2009 zur Behandlung von kutanen T-Zell-Lymphomen (nach Behandlung mit mindestens einer systemische Behandlung im Vorfeld) und im Jahr 2011 zur Behandlung von peripheren T-Zell-Lymphomen zuließ [86].

INHIBITION VON DNMTS ALS THERAPEUTISCHER ANSATZ

Neben der Histonmethylierung stellt eine Form der Methylierung die durch DNA-Methyltransferasen (DNMT) vermittelte Addition von Methyl-Gruppen (CH3) an Cytosinnukleotiden der DNA dar. Dabei findet die Methylierung des Cytosins durch Übertragung des S-Adenylmethionin auf den fünften Kohlenstoff eines Cytosinrestes am Häufigsten in C-G reichen Stellen (CpG Inseln) statt [93]. Diese CpG Inseln befinden sich primär an Stellen im Genom, die für die Regulation der Genexpression wichtig sind. Ca. 70 % der humanen Promotoren - vor allem diejenigen, die für stabile und ubiquitär exprimierte Gene (*housekeeping* Gene) verantwortlich sind - sind in bzw. nahe von CpG Inseln lokalisiert und liegen in Mensch (und Maus) konserviert vor [93–95]. Die Methylierung an diesen Bereichen verhindert entweder die Bindung von Transkriptionsfaktoren an die DNA oder reguliert die Genexpression mittels Rekrutierung verschiedener Proteine (z.B. HDACs) [94].

Drei Mitglieder der DNMT-Familie spielen bei der DNA-Methylierung eine entscheidende Rolle und katalysieren diese Reaktion. Die DNMT1 zeigt eine hohe Effizienz bei der Methylierung hemimethylierter DNA, da diese für die Erhaltung der Methylierung während der DNA-Replikation verantwortlich ist [93]. Im Gegensatz dazu methylieren DNMT3a und DNMT3b, die sich in Struktur und Funktion sehr ähneln, DNA unabhängig vom Methylierungsstatus und werden deshalb auch als *de novo*-Methyltransferasen bezeichnet [93].

In malignen Zellen findet man häufig Abweichungen von den standardmäßigen Methylierungsmustern, die jedoch nicht immer die Ursache der Tumorentstehung sein müssen. Diese Methylierungsveränderungen können dabei entweder zu einem späten Zeitpunkt der Tumorbildung auftreten oder als frühes Ereignis, meist durch Hypo- oder Hypermethylierung, zur Bildung der malignen Zellen beitragen.

Hypomethylierung bezeichnet die verringerte Gesamt-DNA-Methylierung, die um bis zu 70 % im Vergleich zum normalen Zelltyp vermindert sein kann, und betrifft hauptsächlich intergene DNA-Regionen, wie Wiederholungssequenzen oder transponierbare Elemente. Durch Hypomethylierung wird die konstitutive Transkription von Genen bedingt und die Genexpression im gesamten Genom erhöht, so dass daraus eine chromosomale Instabilität und erhöhte Mutationsereignisse resultieren.

Wenn Promotor-assoziierte CpG-Inseln, die meist während ihrer gesamten Entwicklung in nahezu allen Geweben unmethyliert bleiben, hypermethyliert werden, wird die transkriptionelle Stilllegung von z.B. Tumorsuppressorgenen gefördert, die ebenfalls bei HNSCCs (z.B. CDK-Inhibitor 2A*, Tumor Protein 53, Phosphatase and tensin homolog, association domain-containing protein 1*, methylierte-DNA-Cystein-S-methyltransferase) eine Rolle spielen [72,96]. Die Stilllegung der Expression von Tumorsuppressorgenen führt, genauso wie genetische Mutationen, zum Verlust ihrer normalen Funktion [72]. Da die Hypermethylierung jedoch nach jeder Zellteilung von den DNMTs erneuert werden muss, kann durch Inhibition der DNMTs das ursprüngliche Methylierungsmuster wiederhergestellt werden [72]. Dieser Ansatz wird immer häufiger durch die Gabe von DNMT-Inhibitoren wie Decitabin (DEC) verfolgt [85].

Bei DEC (Handelsname Dacogen) handelt es sich um ein Cytosin-Analogon, bei dem das Kohlenstoffatom an Position 5 im Pyrimidinring durch ein Stickstoffatom ersetzt wurde [97]. Nach Verabreichung wird es von den Zellen aufgenommen, in das entsprechende Deoxinukleotid-Triphosphat umgewandelt und während des Zellzyklus an Stelle des Cytosins in die DNA eingebaut, so dass die DNMTs das fehlende Cytosin nicht mehr methylieren können. Ursprünglich wurde eine zytotoxische Wirkung bei Gabe hoher Dosen beschrieben, jedoch stellte sich anschließend heraus, dass durch eine niedrige Dosierung die aberranten Methylierungsmuster durch die Inaktivierung der Methyltransferase-Aktivität nicht mehr auf die Tochterstränge übertragen werden können [97]. Denn nach dem Einbau in die DNA werden DNMTs kovalent an die DNA gebunden, so dass die DNMTs ihre Funktion nicht mehr ausführen können und die genomweite Abnahme der DNA-Methylierung zur Reaktivierung der inaktivierten Gene führt.

Auf der Basis von positiven Erkenntnissen in klinischen Studien erhielt DEC im Jahr 2010 eine FDA-Zulassung zur Behandlung von myelodysplastischen Syndromen und wird als Standardbehandlung für Patienten mit einem myelodysplastischen Syndrom mit höherem Risiko eingesetzt. 2020 folgte die Zulassung zur Behandlung von chronischen myelomonozytären Leukämien; derzeit werden viele klinische Studien zur Behandlung von akuter myeloischer Leukämie, Gliomen und soliden Tumoren durchgeführt [98].

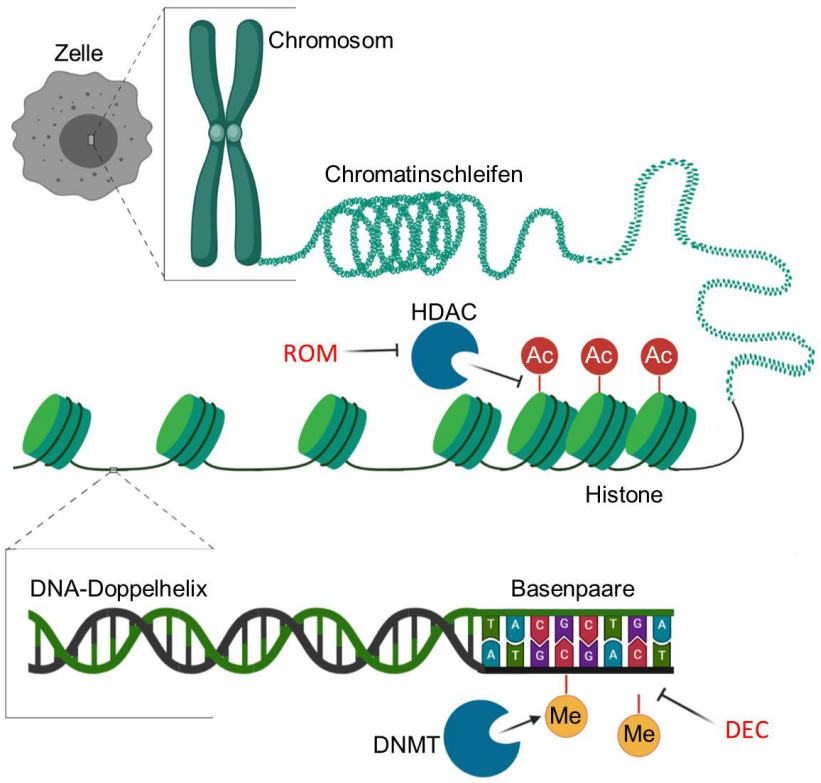

Abbildung 2: Modifikation des Epigenoms mittels Romidepsin (ROM) und Decitabin (DEC). Die DNA wird im Zellkern mithilfe von Histonen verpackt. Die positiv geladenen Histone binden die negativ geladene DNA und falten sich zu einer 30 nm großen Chromatinfaser, die Schleifen bildet. Epigenetische Modifikationen modulieren die Zugänglichkeit des Chromatins für Transkriptionsfaktoren. Dabei führt die Histonacetylierung (Ac) zu einer aufgelockerten Verpackungsweise der DNA, wodurch die Genexpression gefördert wird. HDACs entfernen diese Acetylierung und unterdrücken dadurch die Expression bestimmter Gene. Die DNA-Methylierung (Me) wird mit Hilfe von DNMTs katalysiert und unterdrückt die Transkription, indem sie die Bindung von Transkriptionskomplexen an die Promotoren der Gene blockiert. Eine Dysregulierung dieser epigenetischen Modifikationen kann zu einer abnormen Genexpression führen, die das Auftreten von malignen Zellen begünstigt bzw. verursacht. DNA/Histon-modifizierende Enzyme stellen die Ziele epigenetischer Substanzen, wie ROM und DEC, dar. Diese Abbildung wurde modifiziert nach [99] und mit Biorender.com erstellt.

ZIELSETZUNG

Das Ziel dieser Arbeit war es, innovative und effiziente immunologische Therapieansätze für Kopf-Hals-Karzinome mit CAR-T-Zellen zu etablieren. Zur Steigerung der Spezifität und Effizienz der CAR-T-Zelltherapie sollten drei kritische Teilbereiche untersucht werden. Im **ersten Teil** sollten verschiedene CARs unter Berücksichtigung folgender Punkte etabliert werden:

- Identifizierung und Darstellung von spezifischen Zielantigenen für die CAR-T-Zelltherapie bei HNSCC und Verifikation ihrer Anwendbarkeit anhand von Expressionsstudien.
- Etablierung von hoch-affinen CARs der zweiten Generation gegen primäre HNSCC-Zelllinien sowie Charakterisierung ihrer Spezifität und Funktionalität anhand von HNSCC-Zelllinien mit unterschiedlicher Zielantigendichte

Der therapeutische Nutzen von CAR-T-Zellen zur Behandlung von soliden Tumoren konnte bisher, im Vergleich zur Behandlung von hämatologischen Erkrankungen, keine Durchbrüche in der Prognose von Patienten mit fortgeschrittenen Malignomen erzielen, was u.a. auf das Vorhandensein eines immunsuppressiven TME im soliden Tumorgewebe zurückzuführen ist. Deshalb sollte im **zweiten Teil** der Arbeit die zusätzliche Behandlung der Tumorzellen mit epigenetischen Substanzen untersucht werden, um diese für die Zytotoxizität der CAR-T-Zellen zu sensibilisieren. Durch die Vorbehandlung der Tumorzellen mit Vertretern von DNMTi (Decitabin) und HDACi (Romidepsin) sollten Gene, die durch die epigenetische Behandlung variant exprimiert vorliegen und zu einer Verbesserung der Lyse beitragen identifiziert werden.

Der **dritte Teil** dieser Arbeit sollte sich mit dem Design der CARs beschäftigen. Mithilfe verschiedener *in vitro* und *in vivo* Untersuchungen sollte die Nutzbarkeit universeller *hinge*-Regionen menschlichen Ursprungs, deren Expression sich spezifisch durch mABs nachweisen lässt und die gleichzeitig Selektion von CAR-exprimierenden T-Zellen *in vitro* unter GMP (*Engl.: Good Manufacturing Practice*) -konformen Bedingungen ermöglichen, mit der von anderen, klinisch bereits in CAR-Konstrukten eingesetzten *hinges* verglichen und ihr Nutzbarkeit für eine Vielzahl von scFvs gegen membran-nahe und -ferne Epitope auf den Zielzellen genauer charakterisiert werden.

2. ERGEBNISSE

Der folgende Ergebnisteil dieser Arbeit umfasst fünf Manuskripte, die in enger Zusammenarbeit mit dem Forschungslabor der Urologie der medizinischen Fakultät der Heinrich-Heine-Universität, dem Institut für pharmazeutische und medizinische Chemie der Heinrich-Heine-Universität, dem Institut der experimentellen medizinischen Physik der Heinrich-Heine-Universität sowie der Klinik für Kinderheilkunde III des Universitätsklinikums Essen entstanden sind. Die eigene Leistung wurde unter Berücksichtigung der Folgenden Aspekte für jedes Manuskript separat geschätzt: Versuchsplanung / Durchführung der Experimente / Datenanalyse / Verfassen des Manuskripts. Des Weiteren wird der aktuelle Stand eines jeden vorgestellten Manuskripts und des Journals zum Zeitpunkt der Abgabe dieser Arbeit angegeben.

CD44v6 CAR-T-ZELLEN ELIMINIEREN SPEZIFISCH CD44 ISOFORM 6 EXPRIMIERENDE HNSCC ZELLEN

Titel:	CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells
Autoren:	**Corinna Haist**, Elena Schulte, Nina Bartels, Arthur Bister, Zoe Poschinski, Tabea C. Ibach, Katja Geipel, Constanze Wiek, Martin Wagenmann, Cornelia Monzel, Kathrin Scheckenbach*, Helmut Hanenberg* * Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen
Eigenleistung:	50 % • Planung, Durchführung und Auswertung der Experimente, Schreiben und Korrektur des Manuskripts
Veröffentlicht in:	Oral Oncology
Impact Factor:	5,337
Status:	veröffentlicht
DOI:	10.1016/j.oraloncology.2021.105259

Contents lists available at ScienceDirect

Oral Oncology

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

CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells

Corinna Haist [a,b], Elena Schulte [a], Nina Bartels [c], Arthur Bister [a,b], Zoe Poschinski [a], Tabea C. Ibach [a], Katja Geipel [a], Constanze Wiek [a], Martin Wagenmann [a], Cornelia Monzel [c], Kathrin Scheckenbach [a,1,*], Helmut Hanenberg [a,b,1,*]

[a] Department of Otorhinolaryngology, Head & Neck Surgery, Heinrich Heine University, Düsseldorf, Germany
[b] Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Essen, Germany
[c] Department of Experimental Medical Physics, Heinrich Heine University, Düsseldorf, Germany

ARTICLE INFO

Keywords:
Immunotherapy
Solid tumor
Chimeric antigen receptor
T-cell
CD44v6
HNSCC

ABSTRACT

Immune checkpoint blockade can cause regression of recurrent and/or refractory head and neck squamous cell carcinoma (HNSCC). As a second type of immunotherapy, adoptive cellular therapy with genetically modified patient's T-cells redirected against the autologous malignant cells by expressing chimeric antigen receptors (CARs) recognizing tumor-associated antigens has been established as highly efficient personalized treatment for hematological malignancies. In solid cancers however, the application of these genetically modified immune effector cells still lacks equal response rates. CD44v6 is an isoform of the hyaluronic receptor CD44 that is almost exclusively expressed at high levels on solid cancers and has been associated with tumorigenesis, tumor cell invasion and metastasis. Here, we established a highly specific CAR against CD44v6 on HNSCC cells that can be expressed on normal T-cells with lentiviral vectors. Using primary human HNSCC cells in combination with CRISPR/Cas9 and overexpression approaches allowed us to confirm the high specificity of our CAR construct for the tumor-associated CD44v6 as target antigen and to demonstrate a direct correlation between CD44v6 expression levels and cytotoxicity of the CAR T-cells. Importantly, the design of our clinically applicable lentiviral vector facilitates to co-express a second transgene for *in vivo* control of CAR T-cells, if undesired side-effects or toxicities occur.

Introduction

More than 600,000 patients are annually diagnosed with head and neck squamous cell carcinoma (HNSCC), making HNSCC the 6th most frequent cancer worldwide [1,2]. Multimodal treatment approaches for HNSCC include surgery, platinum-based chemotherapy and radiation [3], however only result in five-year survival rates of approximately 50%, due to high metastasis and recurrence rates [4]. In 2006, the EGF receptor antibody Cetuximab was approved as the first targeted immunotherapy for HNSCC, either as single agent after cisplatin-based therapy or in combination with radiation [5]. While no other specific targeting reagent proved to be clinically effective for HNSCC patients, the antibodies Nivolumab [6,7] and Pembrolizumab [8] as general immune checkpoint inhibitors directed against PD-1 were recently introduced for the treatment of HNSCC for 1st and 2nd line treatment in metastatic and/or recurrent disease.

Chimeric antigen receptors (CARs) are another option to direct autologous immune effector cells against antigens expressed on the surface of malignant cells. CARs are synthetic molecules that combine a single chain variable fragment (scFv), derived from a monoclonal antibody, with cytoplasmatic T-cell activation motifs from the zeta-chain of CD3 and co-stimulatory domains in a single molecule [9]. Expression of CARs on immune cells results in highly efficient recognition and killing of any antigen positive cell in an HLA independent manner [10]. While major clinical breakthroughs have been achieved in recent years for refractory or relapsed acute lymphoblastic leukemia (ALL) and diffuse

* Corresponding authors at: Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Hufelandstraße 55, 45122 Essen, Germany (H. Hanenberg). Department of Otorhinolaryngology, Head and Neck Surgery, UKD, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany (K. Scheckenbach).
E-mail addresses: scheckenbach@med.uni-duesseldorf.de (K. Scheckenbach), Helmut.Hanenberg@uk-essen.de (H. Hanenberg).
[1] contributed equally

https://doi.org/10.1016/j.oraloncology.2021.105259
Received 5 December 2020; Received in revised form 4 February 2021; Accepted 28 February 2021

large B-cell lymphoma using the patient's autologous T-cells equipped with CARs against CD19 or CD20 [11,12], the establishment of CAR T-cell therapy for solid tumors is more challenging [13]. For HNSCCs, only a handful of target antigens have been described as potential CAR candidates, including EGFR, HER2neu/ERBB2 and a specific variant of CD44 [11,12,14].

CD44 is a cell surface glycoprotein, that serves as hyaluronic acid receptor and is involved in tumor growth, differentiation and metastasis [15]. Alternative splicing of the *CD44* gene leads to nineteen CD44 isoforms with different variant exons between the constant upstream (exons 1–5) and down-stream (exons 16–20) regions [16]. The smallest isoform is CD44s, which lacks all variant exons. CD44v6 additionally translates exon 11 [15] and is abundantly expressed on various malignancies including squamous cell carcinomas of the head and neck, lung, skin, cervix and esophagus as well as breast, gastrointestinal, hepatocellular and colorectal cancers and some subtypes of acute myeloid leukemias (AML) [11,15,16]. In normal tissues, CD44v6 is expressed on keratinocytes in the skin and oral mucosa and on cells of the monocytic lineage [17,18]. Experimental studies revealed that expression of CD44v6 on tumors cells is directly associated with tumor progression and metastatic potential [19] and that administration of CD44v6 monoclonal antibodies inhibits the formation of metastases [20]. Clinically, patients with CD44v6 positive tumors experienced more aggressive tumors with reduced overall survival [21,22].

To immunologically target CD44v6 in humans, murine monoclonal antibodies were used that bind to overlapping epitopes encoded by exon 11 of CD44 [16,17]. In clinical phase I/II studies, administration of these antibodies labeled with radionucleotides demonstrated highly tumor-specific uptake of the antibodies but only limited tumor responses were achieved [23,24]. As the murine antibodies led to allergic reactions in patients, humanized derivatives were developed, among them BIWA4 (bivatuzumab) with medium affinity and BIWA8 with high affinity for CD44v6 [25]. To date, numerous studies have been conducted with bivatuzumab conjugated to radionuclides [26] or to mertansine, an anti-microtubule agent [27,28]. While the combination with radionuclides showed no severe side effects and at best stable disease, the administration of bivatuzumab-mertansine led to specific fatal toxicity and the closure of all bivatuzumab studies [28]. Recently however, the use of a CD44v6 CAR became the subject of clinical phase I/II studies for treatment of AML and multiple myeloma as well as solid malignancies such as breast cancer and HNSCCs.[2]

In this study, we investigated the specificity and efficiency of a BIWA8-derived high affinity CD44v6 second generation CAR against HNSCC cell lines *in vitro* as proof-of-principle for *in vivo* mouse models and ultimately for human clinical studies.

Material and methods

Cell culture

HEK293T cells were obtained from DSMZ (Braunschweig, Germany), human oral keratinocytes (HOK) from ScienCell Research Laboratories (Carlsbad, USA), dysplastic oral keratinocytes (DOK) from Merck KGaA (Darmstadt, Germany) and primary human HNSCC cell lines (UM-10B, UM-11B, UM-14C, UM-17A, UT-24A) from the University of Michigan (UM), USA, or the University of Turku (UT), Norway, respectively. All adherent cells were cultured in DMEM GlutaMAX with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Thermo Fisher Scientific, Schwerte, Germany).

Lentiviral constructs and transduction

Standard lentiviral vectors were used for overexpression of CD44s and CD44v6 cDNAs [29]. The latter one was synthesized by BioCat GmbH (Heidelberg, Germany) and the CD44s spliceform generated by removing exon 11 via overlap PCR. The CD44v6 single-chain fragment variable (scFv) sequences were derived from the BIWA8 sequence [25], synthesized after optimization for human codon usage by GeneArt (Regensburg, Germany) and cloned into our CD19 CAR lentiviral vector [29]. For the CD44s and CD44v6 knockout constructs, DNA oligonucleotides for six different gRNAs were designed, synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and cloned into the lentiviral CRISPR/Cas9 vector LentiCRISPRv2 [30].

VSV-G-pseudotyped replication-deficient infectious lentiviral particles were produced in HEK293T cells [31]. Primary human T-cells were obtained from healthy volunteers, as approved by the local institutional review board/ethics committee (study #4687). After density purification, human primary T-cells were prestimulated on CD3/CD28-coated plates (OKT3, Ortho Biotech, Neuss, Germany/CD28, BD Biosciences, Heidelberg, Germany) for two days and then transduced on the fibronectin fragment CH296 (Retronectin®, Takara Bio Inc., Japan) in the presence of 100 IU/ml IL-2 [29,31]. 72–96 h after transduction, CAR T-cells were enriched by the CD34 MACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. For overexpression of CD44s or CD44v6, $1x10^6$ cells were transduced with limiting dilutions of supernatants with lentiviral CD44s or CD44v6 expression plasmids and selected with 1 μg/ml puromycin 48 h post transduction. Lentiviral CRISPR/Cas9 vectors were used in limiting dilutions followed by puromycin selection.

Flow cytometry analysis

CD44v6 and CD44 expression was assessed on a MACSQuantX after staining $1x10^6$ HNSCC cells with the monoclonal antibodies CD44v6-PE-Vio770 antibody or CD44-APC-Vio770 (Miltenyi Biotec). To analyze the expression of the CARs on T-cells, staining was performed with a CD34-PE monoclonal antibody (QBEND/10, Thermo Fisher Scientific) and analyzed on a FACSCalibur (BD Biosciences).

Cytotoxicity assay

The cytotoxicity of CD44v6 CAR T-cells against HNSCC cells was determined by the CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega, Walldorf, Germany). To this end, $2x10^4$ cells were seeded in 96-well plates and cultured overnight at 37 °C. The next day, MACS-selected CAR T-cells were added at different effector to target cell ratios. After 16 h at 37 °C, plates were washed three times to remove non-adherent T-cells and dead tumor cells, incubated with the CellTiter96® substrate according to manufacturer specifications and the number of viable cells was determined with a TECAN sunrise analyzer (Tecan Group AG, Männedorf, Switzerland). The percent lysis was calculated as

100% - (absorption of target cells incubated with T-cells/ absorption of target cellsx100).

Time lapse live video microscopy

For visualization of the interactions between CD44v6 CAR T-cells and HNSCC cells, 10^5 UM-17A cells were seeded per well of an 8-well glass bottom chamber slide (ibidi, Gräfelfing, Germany). The next day, the cells were cultured with 0.5 μl CellEvent Caspase-3/7 Green Detection Reagent in 200 μl of Leibovitz's L-15 imaging medium (both Thermo Fisher Scientific) and incubated with CD19 or CD44v6 CAR T-cells in 100 μl Leibovitz's L-15 medium supplemented with 100 IU/ml IL-2 on a temperature-controlled stage at 37 °C. Time-lapse videos were acquired with a 60x (NA 0.65–1.25) oil objective and the CellSense Dimensions Software (Olympus, Hamburg, Germany) by sequential imaging of the CellEvent marker (excitation 470/40 nm), the T-cells' mCherry red fluorescence (excitation 545/30 nm) and phase-contrast

[2] https://clinicaltrials.gov/ct2/show/NCT04097301

mode on an IX83 microscope system (Olympus). Frames were acquired every 5 min over 16 h at multiple positions. Image analysis was performed with the ImageJ open source software, using an intensity-based threshold to the green channel in order to detect the time of the emerging CellEvent apoptosis signals in the nuclei.

Results

CD44s and CD44v6 expression profiles of primary HNSCC cell lines

We initially employed flow cytometry to analyze the expression profiles of CD44 and its splice variant CD44v6 in 31 primary HNSCC cell lines, which were established from primary tumor lesions or from metastatic sites of human patients (Table 1). All cell lines and dysplastic human oral keratinocytes (DOK) expressed CD44 and CD44v6, while normal human oral keratinocytes (HOK) and HeLa.P3 were only positive for CD44 (Figure 1) [32]. 26 out of the 31 HNSCC lines expressed CD44 on more than 80% of cells, while only 12 cell lines showed equally high CD44v6 expression. We were unable to detect any correlation between these expression patterns and the tumor and patient characteristics (Table 1), similarly to others [33,34].

A CD44v6 CAR for efficient targeting of HNSCC cells

Our CD44v6 CAR construct with the BIWA8 scFv sequences [25], the CD28 transmembrane and cytoplasmic regions and the CD3 zeta (ς) -chain was cloned behind the T2A site into our standard lentiviral vector [29] shown in Fig. 2A. We also included a 99 amino acid stretch from the

Table 1
Main characteristics of the cell lines used in this study.

Cell line	Sex	Type of lesion	HPV	P53	Literature
UD-01	M	Primary	–	c.96+1G>A, splice	[58–60]
UD-02	M	Primary	+	WT	[58–60]
UD-03	M	Metastasis	–	p.Q224X	[58–60]
UD-04	M	Primary	–	NT 664 del13	[58–60]
UD-05	M	Primary	–	p.H179Y	[59,60]
UD-06	M	Primary	–	p.Y220C	[59,60]
UT-02	M	Primary	–	p.C275F	[60,61]
UT-04	F	Metastasis	–	p.R248-P250del	[60–62]
UT-05	M	Primary	–	p.P151H	[60,63]
UT-06B	F	Metastasis	–	WT	[60,63,64]
UT-07	M	Metastasis	–	p.G266E	[60–62,65]
UT-09	M	Metastasis	–	deletion exon 2-9	[60,62,63]
UT-14	M	Primary	–	c.919+1G>T, splice	[60,63,65]
UT-15	M		–	c.560-1G>T, splice	[60,65–67]
UT-24A	M	Primary	–	c.673-2A>T, splice	[60,63,65–67]
UT-24B	M	Metastasis	–	c.673-2A>T, splice	[60,63,65–67]
UT-33	F		–	p.R282W	[60,65,68]
UT-34	M		–	no transcript	[60,65,68]
UT-50	M		–	c.919+59del46bp	[60,65]
UM-10A	M	Primary	–	p.G245C	[59,65,67,69,70]
UM-10B	M	Metastasis	–	p.G245C	[59,60,65,67,69,71]
UM-11B	M	Primary	–	p.C242S	[59,60,65,67,69,71]
UM-14A	F	Recurrent	–	p.R280S	[59,60,65,67,69,71]
UM-14B	F	Recurrent	–	p.R280S	[59,60,65,67,69,71]
UM-14C	F	Recurrent	–	p.R280S	[59,60,65,67,69,71]
UM-17A	F	Primary	–	WT	[59,60,65,67,69,71]
UM-17B	F	Metastasis	–	WT	[59,60,65,67,69,71]
UM-22B	F	Metastasis	–	p.Y220C	[59,60,67,69,71]
UM-74A	M	Primary	–	WT	[60,65,69]
UM-74B	M	Primary	–	WT	[60,65,69]
UM-104	/	Recurrent	+	WT	[72]

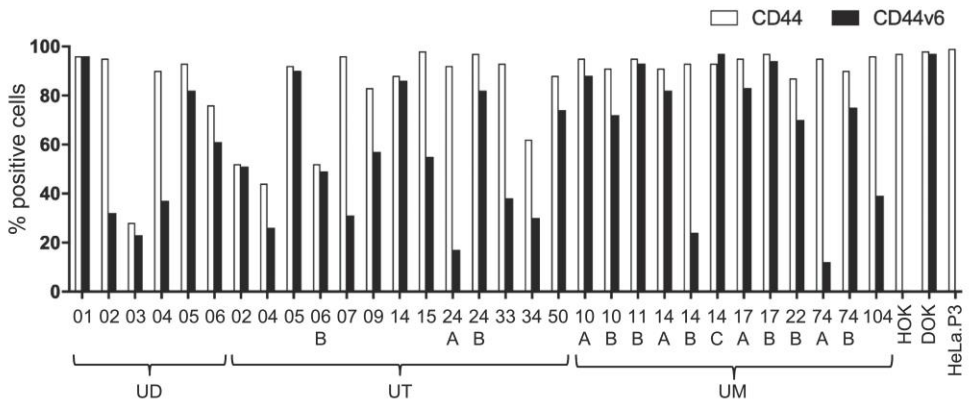

Fig. 1. Screening of CD44 and CD44v6 gene expression in primary HNSCC cell lines. Cells were stained with CD44-APC Vio 770 or CD44v6-PE Vio 770 antibodies according to manufacturer's specifications and analysed by flow cytometry. Percent positive cells (y-axis) are indicated for HNSCC cell lines and reference cells (HOK, DOK, HeLa.P3).

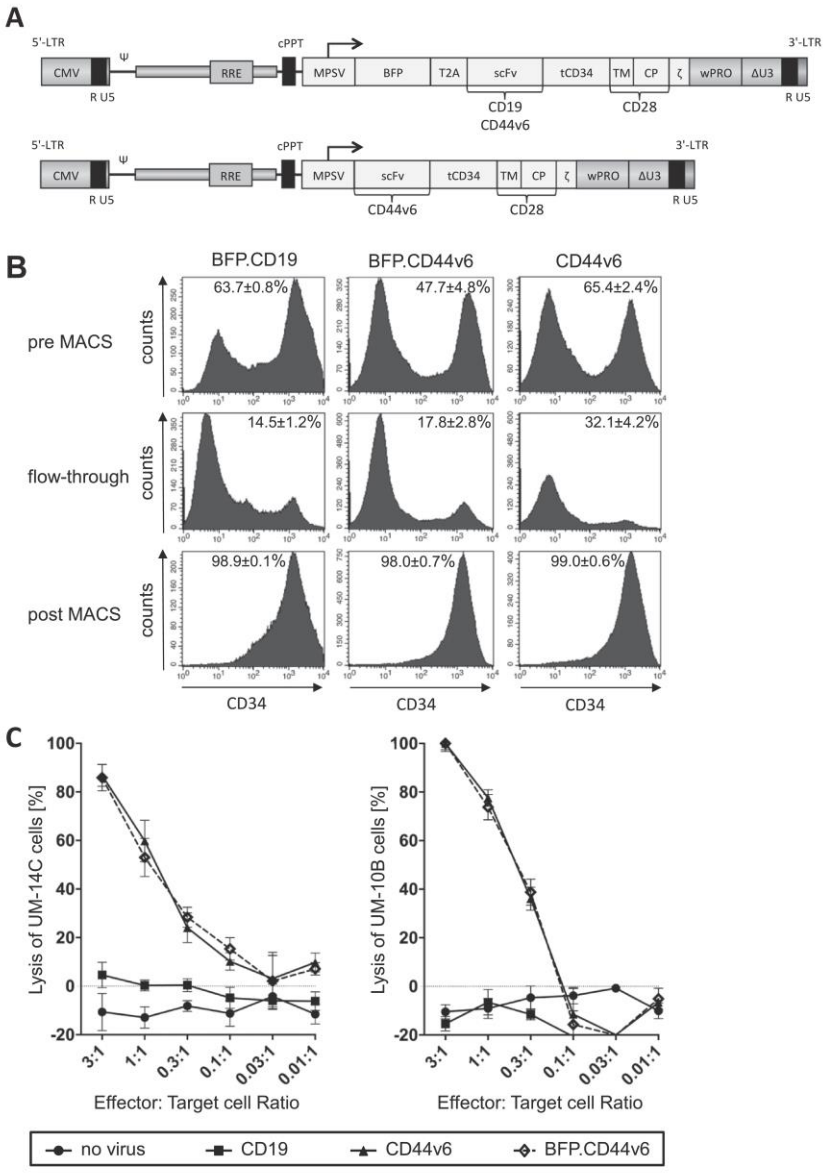

Fig. 2. Design and enrichment of CAR positive T-cells. A) Lentiviral vectors for expression of CARs. The constructs contained CD19 or CD44v6 scFvs, the CD34 hinge domain, the CD28 transmembrane and intracellular domains, and the CD3ζ cytosolic domain. BFP and T2A site were used optionally. B) Flow cytometry analyses of purification and enrichment steps via MACS system. Transduced primary T-cells were sampled for flow cytometry analysis prior to MACS separation and after MACS from the eluate as well as from the flow-through fractions. All constructs were separated with CD34 microbeads. CAR expression (x-axis) was plotted against the relative cell count (y-axis). The histograms also showed the percentage of positive cells as mean ± SEM for three different experiments. C) Identical killing of CD44v6 positive cell lines UM-14C and UM-10B by CD44v6 CAR with or without second transgene. Primary human T-cells were transduced with a CD44v6 and CD19 CAR. After three days, the genetically modified primary human T-cells were selected by MACS, cocultred with target cells for 16 h and finally lysis of target cells was determined via CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay. Data were depicted as mean ± SEM of three independent experiments.

human CD34 antigen in the CAR as a hinge region (Bister *et al.* in preparation, patent EP3293199[3]). This region of CD34 is recognized by the QBEND10 monoclonal antibody and therefore can be used to detect the expression of the CAR with directly conjugated QBEND10 by flow cytometry or to enrich CAR expressing T-cells by the MACS technology (Miltenyi Biotech). As a control, we also generated a second lentiviral vector, where the CD44v6 CAR was expressed as the sole transgene off the MPSV promoter (Fig. 2A). For the bi-cistronic CD44v6 and CD19 CAR vectors, the TagBFP fluorescent protein was included as a marker gene for the detection of transduced cells in front of the T2A site [29].

Lentiviral particles were produced for the three vectors (BFP-T2A-CD19, BFP-T2A-CD44v6 and CD44v6) and used to transduce peripheral blood-derived T-cells [29,35]. Three days after transduction, 63.7 ± 0.8%, 47.7 ± 4.8% and 65.4 ± 2.4% of the T-cells expressed the three CARs, respectively (condition: pre MACS, Fig. 2B). After enriching the transduced T-cells once on standard MS columns with CD34 magnetic microbeads, the purity of strongly CAR positive T-cells in the eluates post MACS consistently reached ≥ 98%, while only 14.5 ± 1.2%, 17.8 ± 2.8% and 32.1 ± 4.2% CAR T-cells were present in the flow-through for the three lentiviral constructs BFP-T2A-CD19, BFP-T2A-CD44v6 and CD44v6, respectively (Fig. 2B).

To analyze whether the cytotoxicity of the CD44v6 CAR constructs is affected, if a second transgene is expressed via a T2A site, we incubated cells of the strongly CD44v6 expressing cell lines UM-14C and UM-10B with MACS-enriched CD44v6 CAR T-cells with or without BFP as second transgene. Untransduced or MACS-enriched CD19-CAR transduced T-cells served as negative controls. As shown in Fig. 2C, the specific killing of HNSCC cells after 16 h of co-incubation was identical for the two CD44v6 constructs at all target to effector cell ratios, while both the CD19 CAR and the untransduced T-cells showed no lysis of the HNSCC

[3] https://data.epo.org/publication-server/rest/v1.0/publication-dates/20180314/patents/EP3293199NWA1/document.pdf

cells. These experiments confirmed that the CD44v6 CAR facilitated efficient recognition and killing of cells from both cell lines and that the cytotoxicity of our CD44v6 CAR was not affected by the presence of a second transgene in the lentiviral vector.

Killing of HNSCC cells largely depends on the CD44v6 expression level

To determine the correlation between the CD44v6 expression and the cytotoxicity of CD44v6 CAR T-cells, three HNSCC cell lines with distinct CD44 and CD44v6 expression patterns were chosen (Fig. 3A-C): UT-24A cells had low (MFI: 6.5 ± 0.3), UM-11B cells intermediate (MFI: 11.2 ± 1.2) and UM-14C high (MFI: 14.2 ± 3.2) CD44v6 expression. Analyzing the cytotoxicity of CD44v6 CAR T-cells revealed a clear correlation between the CD44v6 expression levels and the killing of the target cells (Fig. 3A-C): Less than 40% of UT-24A cells, 60–80% of the UM-11B and almost 100% of UM-14C cells were killed at effector to target cell ratios of 3:1 and 1:1, respectively. In contrast, the killing efficiencies did not correlate with the CD44 expression levels detected on the surface of the target cells with an antibody binding to CD44.

In order to assess the specificity of the CAR, we overexpressed CD44v6 on the low expressing UT-24A cells with a lentiviral vector [36]. Compared to the UT-24A cells that were transduced with the control vector and selected in parallel with puromycin (MFI for CD44v6: 4.7 ± 0.6, Fig. 3D), the strongly overexpressing UT-24A + CD44v6 cells (MFI 648.2 ± 28.7) were killed much more efficiently (Fig. 3E).

CD44v6 CAR T-cells specifically eliminate CD44v6 expressing cells

The variable exon 11 in the *CD44* gene is only 204 base pairs long and encodes, when included as variant 6 in the CD44v6 transcript, as little as 68 amino acids. We therefore wanted to prove that the CD44v6 CAR solely and specifically recognized these amino acids.

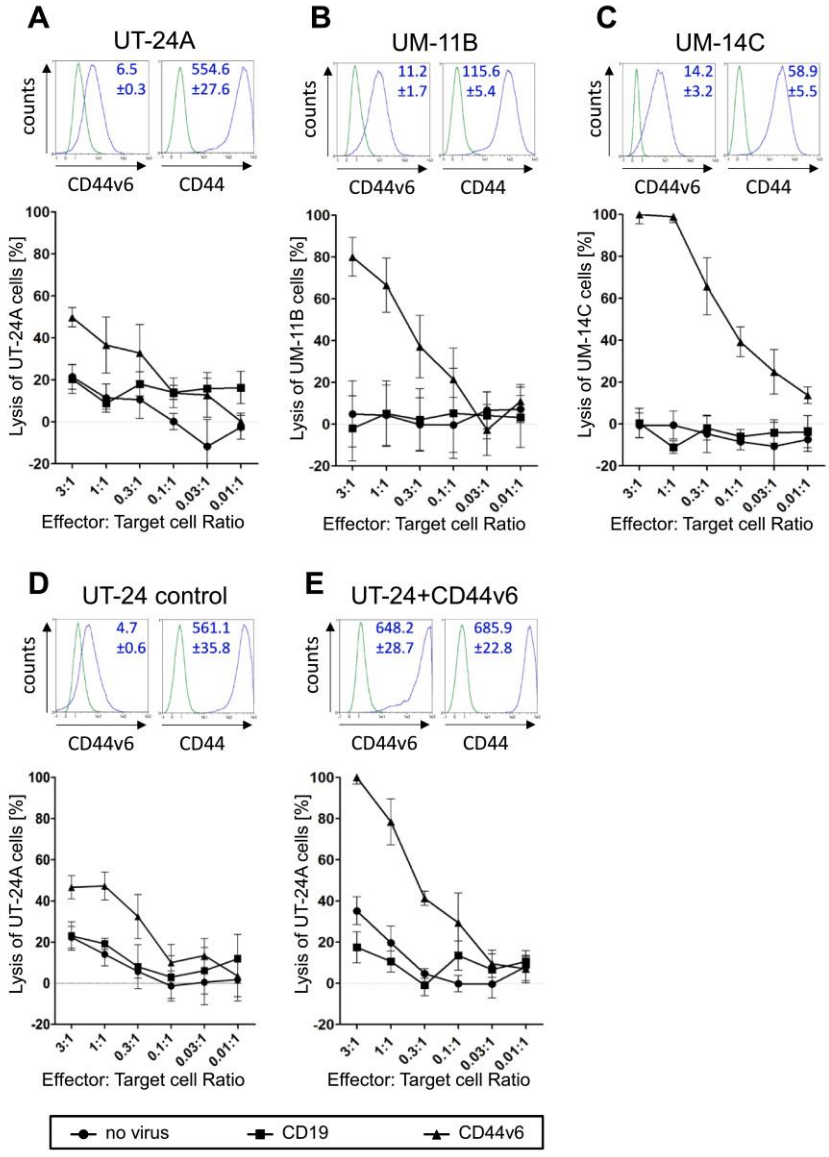

Fig. 3. Correlation between antigen density and killing efficiency. CD44 and CD44v6 expression on the cell surface of primary HNSCC cell lines UT-24A (A), UM-11B (B), UM-14C (C), UT-24A transduced with an empty control vector (D) and UT-24A transduced with an CD44v6 overexpression plasmid (E) were determined by binding of CD44-APC-Vio770 or CD44v6-PE-Vio770 antibodies. Antigen expression in flow cytometry (x-axis) was plotted against the absolute cell count (y-axis) for unstained control cells (green) and stained cells (blue). The mean fluorescence intensities (MFIs) of stained cells were shown from three experiments as mean ± SEM. Primary human T-cells were transduced with a CD44v6 or CD19 CAR. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with target cells. After 16 h, lysis of the target cells was determined via CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). Data were depicted as mean ± SEM for three different experiments.

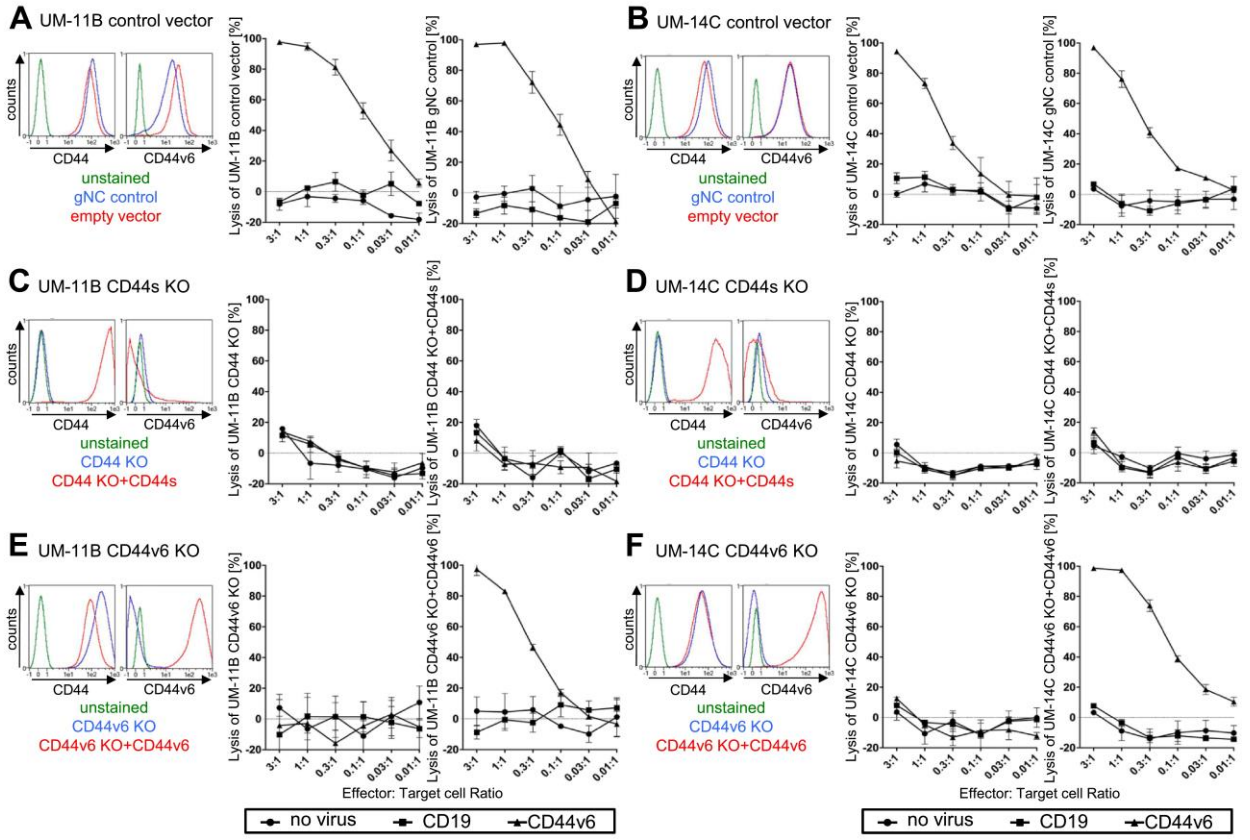

Fig. 4. CD44v6 CAR T-cells specifically recognized CD44v6 on UM-11B and UM-14C cells. Both cell lines were transduced with the CRISPR/Cas9 control vectors (A, B) or contain either CD44s (C,D) or CD44v6 guides (E,F). CD44 and CD44v6 expression on the cell surface of HNSCC cell lines was determined by flo cytometry after staining with CD44-APCVio770 or CD44v6-PE-Vio770 antibodies. Antigen expression (x-axis) was plotted against the absolute cell count (y-axis) for each sample. Primary human T-cells were transduced with a CD44v6 or CD19 CAR. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with target cells. After 16 h, lysis of the target cells was determined via CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). Data were depicted as mean ± SEM for three different experiments.

For targeting of all CD44 transcripts, we generated three 20-nucleotide guide RNAs (gRNAs) against a common region in *CD44* exon 2 using the chopchop.cbu.uib.no website [37] and then cloned the gRNAs into the LentiCRISPR V2 vector. To accurately knockout CD44v6, we designed three different gRNAs that specifically target the variable exon 11. We used the empty LentiCRISPRv2 (empty vector) and additionally cloned a gRNA targeting the chicken actin promoter as an off-target control (gNC) [38]. Lentiviral vector particles were used to transduce the robustly CD44v6 expressing cells of the HNSCC cell lines UM-11B and UM-14C at MOIs of less than 0.1. Flow cytometry analysis of the CD44 isoform expression patterns of the CRISPR/Cas9 expressing cells revealed that, depending on the lentiviral construct used, >80% (~89 to 96%) of cells had clearly diminished CD44 and CD44v6 expression levels (data not shown).

We subsequently generated single cell clones with either complete CD44 or CD44v6 knockout and then re-introduced either CD44s or CD44v6 cDNAs with lentiviral expression vectors [36]. Importantly, the CD44 and CD44v6 expression levels were largely unaffected after transduction of the UM-11B and UM-14C cells with the two CRISPR/Cas9 control vectors. Alsothe cytotoxicity of the CD44v6 CAR T-cells remained the same (Fig. 4A/B, left/right panels). Transduction with the CD44 exon 2 CRISPR/Cas9 vector eliminated all CD44 expression; however, it was specifically restored by re-introducing the CD44s isoform (Fig. 4C/D left panels, red curves). Neither the cells without CD44 expression (KO) nor the knockout cells with CD44s overexpression were killed by the CD44v6 CAR T-cells (Fig. 4C/D right panels). Only when CD44v6 was re-introduced into CD44v6 KO cells, both the expression of CD44v6 (Fig. 4E/F left panels, red curves) and the killing of the HNSCC cells by the CD44v6 CAR T-cells were restored (Fig. 4E/F right panels), thus proving the high specificity of our CD44v6 CAR construct.

Killing dynamics of CD44v6 CAR T-cells against HNSCC cells

Finally, in order to understand the killing dynamics of the CD44v6 CAR T-cells, we co-incubated UM-17A cells, characterized by high CD44v6 and CD44 expression (Figure 1), at ratios of 3:1 (red) and 1:1 (yellow) with CD44v6 CAR T-cells and at a 1:1 (blue) ratio with CD19 CAR T-cells for 16 h. The CAR T-cells additionally expressed the red fluorescent protein mCherry (Fig. 5A/B). The HNSCC cells actively undergoing apoptosis showed a green fluorescence by using the CellEvent™ Caspase-3/7 Green Detection Reagent (Fig. 5B). Interestingly, although CAR T-cells were quickly in contact with the HNSCC cells, apoptosis of HNSCC cells was not detected in the first two hours of co-culture (Fig. 5B/C). At an effector:target cell ratio of 3:1, the apoptosis became clearly visible after 3–5 h of co-culture; within 8 h, the T-cells had induced apoptosis in ≥ 80% of HNSCC cells (Fig. 5C). Using an effector:target cell ratio of 1:1 did not delay the induction of apoptosis, but slowed down the killing. These different apoptosis kinetics for the two 3:1 and 1:1 cultures were also visualized in Fig. 5D, demonstrating that the killing of HNSCC cells extends over longer time periods (ratio

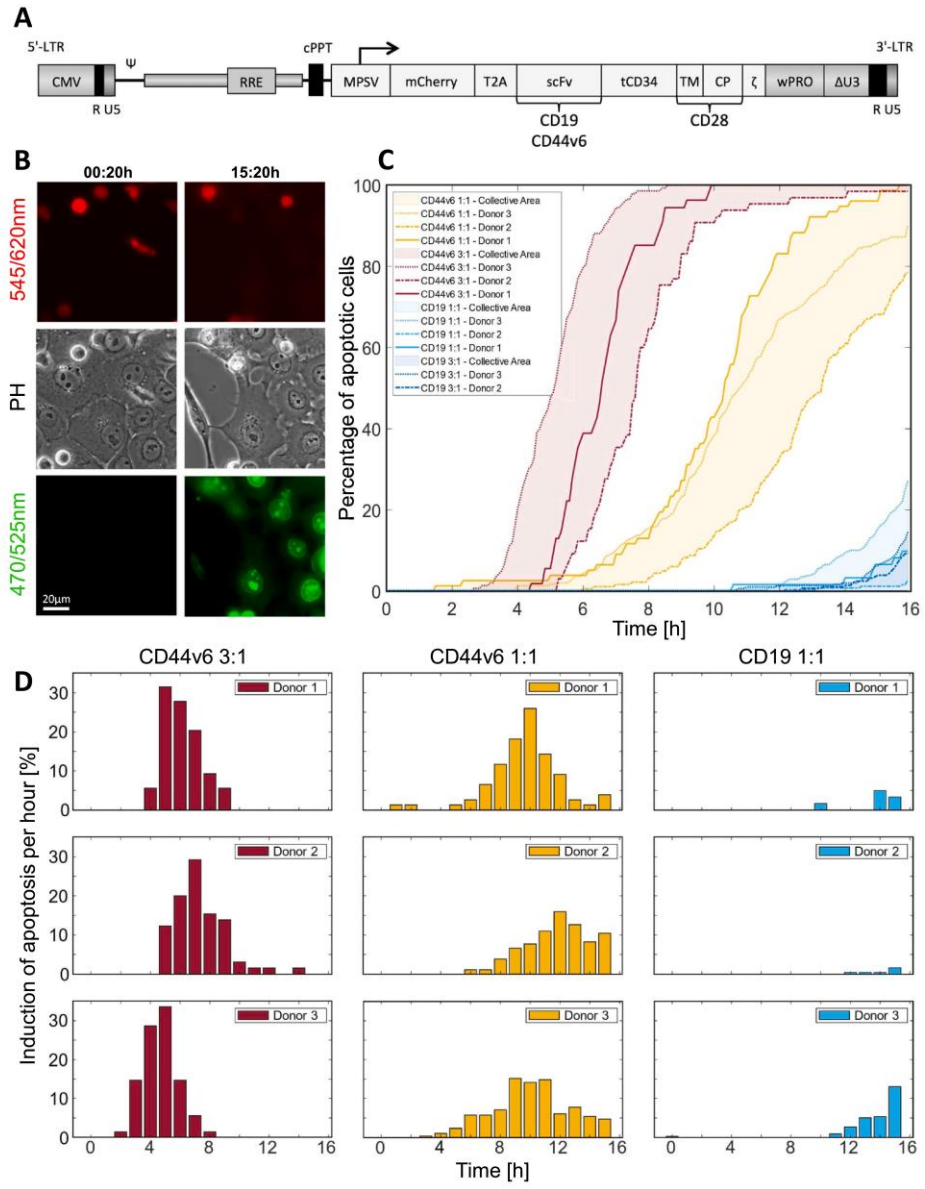

Fig. 5. Killing dynamic of CD44v6 CAR T-cells against CD19^{neg} CD44v6^{pos} UM17-A cells. Primary human T-cells from three healthy 3 donors) were transduced with lentiviral CD44v6 or CD19 CAR vectors. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with UM-17A cells in two effector:target cell ratios (3:1, 1:1) for 16 h while performing live cell imaging to detect apoptotic cells (CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen™, 470/525 nm channel). (A) Lentiviral vector with mCherry as a second transgene for detection of the transduced T-cells (B) Co-culture of UM-17A (apoptotic cells shown in green) with CD44v6-CAR-T-cells (red) in the beginning (t = 0:20 h) and at the end (15:20 h) of the measurement. (C) Apoptosis of UM-17A cells lysed by CD44v6 or CD19 CAR T-cells. (D) Apoptosis of UM-17A cells per hour.

3:1 Ø7.6 h, ratio 1:1 Ø12.6 h), if less effector cells are present. Importantly, CD19 CAR T-cells did not exert relevant killing activity during co-cultivation with the UM-17A cells, demonstrating that activated T-cells did not express any receptors that recognized the malignant HNSCC cells.

Discussion

The key for selective targeting of HNSCCs with immune effector cells requires immunologically recognisable differences between the malignant cells and their normal counterparts [39]. Among the different classes of target antigens that have been considered for immunotherapy of HNSCCs are tumor neoantigens, which are derived from somatic mutations in the malignant cells. However, the two most frequently mutated gene in HNSCCs, *TP53* and *RAS*, are both not expressed on the cell surface and can therefore not be targeted by either antibodies or CARs [39]. Although engineering T-cells to express mutation-specific T-cell receptors for altered peptides expressed on HLA class I molecules of the malignant cells is possible [40], this approach is not widely applicable. Another class of target antigens in HNSCCs are cancer testis antigens, that are only present during embryogenesis and in normal germ cells in the testis of adults [41,42]. Peptides of these intracellular antigens are presented on the surface of certain HLA class I molecules and are currently targeted by several immunotherapeutic approaches [39], including TCR mimics [42,43]. However, data on the clinical outcome in larger patient cohorts are still missing [42–44]. A number of antigens, generally not mutated, are overexpressed on HNSSCs. Among those are EGFR, which is the target for Cetuximab that is not well suited for CAR therapies, and the human epidermal growth factor receptor 2 (ErbB2), which has been frequently used in preclinical and clinical trials albeit not really successful yet [45–47]. Finally, tumor–associated antigens, e. g. the alternatively spliced surface protein EGFRvIII, or abnormal glycoforms, e.g. αvβ6 or MUC1TN, are currently evaluated as targets for CAR T–cell therapy [48–51].

We decided to focus here on CD44v6, an alternatively spliced surface protein that is strongly overexpressed on HNSCCs [24], and where autologous CAR T-cells can be tested in preclinical studies in monkeys [14]. For early human trials, two different types of therapeutic reagents were coupled to CD44v6 antibodies, radionuclides and the highly toxic antimicrotubuli agent mertansine [14]. Phase I clinical studies with radioimmunotherapeutic CD44v6 antibodies were associated with stable disease at higher radioactivity dose levels and also with myelotoxicity and mild oral mucositis [52]. However, the combination of BIWA4/bivatuzumab with mertansine in a phase I dose escalation study in seven patients with advanced untreatable HNSCCs led to fatal grade 4 toxicity in the 7th patient, who experienced loss of the epidermis and defoliation of the whole skin and died three days after the second infusion [28]. Although similarly severe toxicities were not observed with bivatuzumab in animal studies or in the clinical trial, skin toxicities such as depigmentation and desquamation were still present in other patients and in the monkey studies; therefore further clinical testing of CD44v6 antibodies in humans was stopped [52].

For CD44v6 as targeting moiety for autologous CAR T-cells, we chose the single chain variable fragment (scFv) sequences of the humanized high-affinity BIWA8 antibody for our construct [29]. To confirm the specificity of the CAR for HNSCC cells, we assessed the CD44 and CD44v6 expression profiles for 31 HNSCC lines and then chose four cell lines with low to high CD44v6 expression levels as targets in our cytotoxicity assays. The cytotoxicity of our CAR was comparable when lentivirally expressed either as the single transgene or as the second transgene off the MPSV promoter and the killing efficiency directly correlated with the CD44v6 expression levels on the target cells. Using the CRISPR/Cas9-generated HNSCC knock-out cells with overexpression of CD44s or CD44v6 and a CD19 CAR as control proved the high specificity of our CD44v6 CAR constructs for the amino acid sequence encoded by exon 11 of the *CD44* gene. Finally, the time course studies revealed that the apoptosis in the target cells started approximately 2–3 h after contact with the immune effector cells and was clearly influenced by the target to effector cell ratio.

Based on these results, we expect that expression of our CD44v6 CAR on T-cells will effectively kill CD44v6 positive HNSCC cells in patients. However, as T-cells can readily leave the blood stream and recognize antigens present on cells in tissues, the expression of CD44v6 on normal cells can be a problem. According to the literature, CD44v6 is robustly expressed on keratinocytes in the skin as well as on epithelium in the cervix, in the cornea and in tonsils [53], however the expression levels were lower compared to those on malignant cells [18]. It will be very interesting to see if the expression of CD44v6 on normal tissues will be a major problem in the clinical phase I/II CD44v6 CAR T-cell study that has been opened for AML and multiple myeloma by scientists from Milano and Rome in 2019[2]. To stop any unwanted T-cell activation in off-target organs, they also included a modified thymidine kinase (TK) cDNA as suicide gene in their retroviral vector [54]. A second phase I trial for CD44v6 positive cancers treated with autologous CAR T-cells transduced with a lentiviral vector opened in 2020 in China[4]. The information, whether a suicide gene is also included in the lentiviral vector, has not been disclosed[4].

An attractive possibility to avoid cytotoxicity for normal tissues is to inject multiple doses of CAR T-cells directly into the tumor, similarly as it was already performed in the T4 clinical trial for HNSCC targeting the ErbB receptor family[5]. Although the final results from this phase I trial have not been published, preliminary results demonstrated that the injected T-cells hardly ever left the tumor tissues and never caused off-target effects, even when injecting escalating T-cell doses [55]. Using CARs with lower affinities for the target antigen is another attractive possibility that has been explored for EGFR and ErbB2 overexpressing tumors [46,56]. The Italian CD44v6 study also used the BIWA8-derived scFv for their CAR T-cell trials. Therefore, in case of severe toxicity, we can readily engineer a reduced-affinity CD44v6 CAR by changing two amino acids in the light chain of BIWA8 to residues present in the middle-affinity CD44v6 scFv of BIWA4 at the corresponding positions [25] and then test the two constructs in comparison. Other possibilities to change the activation level of our construct are to include a different co-stimulatory domain (e.g. 4-1BB or 2H4) or to pursue additional strategies for reducing off-target effects of CAR T-cells in solid tumors [57]. While we will proceed with *in vivo* xenograft studies in immunodeficient mice for testing the efficacy of intratumor versus intravenous application of the CD44v6 CAR T-cells, the clinical results including the toxicity from the two open CD44v6 CAR T-cell phase I/II studies in humans will finally guide us which strategy is most likely be successful for our HNSCC patients.

[4] https://clinicaltrials.gov/ct2/show/NCT04427449

[5] https://clinicaltrials.gov/ct2/show/NCT01818323

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that have influenced the work reported in this paper.

Acknowledgements

We gratefully acknowledge the healthy donors that provided peripheral blood for the cytotoxicity studies. We would like to thank Jörg Schipper, MD, director of ENT clinic, for his support of this research project. We are also in debt to Wolfgang Schulz, PhD, and Michèle Hoffmann, PhD, both in the department of Urology, Heinrich Heine University, for the use of the MACSQuant-10.

Funding

This work was supported, in part, by funding from the Medical Research School Düsseldorf, DSO, Heinrich Heine University, Düsseldorf, and within the framework of the iCAN33 project, funded by the European Regional 470 Development Fund NRW (ERDF, German EFRE) 2014-2020. CM acknowledges the support from VolkswagenFoundation (Freigeist fellowship, project ID 94195). NB and CM acknowledge the support within the DFG Collaborative Research Center 1208 'Identity and dynamics of biological membranes' (project ID 267205415).

References

[1] Morris LGT, Chandramohan R, West L, Zehir A, Chakravarty D, Pfister DG, et al. The molecular landscape of recurrent and metastatic head and neck cancers. JAMA Oncol 2016;3:244. https://doi.org/10.1001/jamaoncol.2016.1790.

[2] Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol 2010;11:781–9. https://doi.org/10.1016/S1470-2045(10)70017-6.

[3] Mehra R, Ang KK, Burtness B. Management of human papillomavirus-positive and human papillomavirus-negative head and neck cancer. Semin Radiat Oncol 2012;22:194–7. https://doi.org/10.1016/J.SEMRADONC.2012.03.003.

[4] Hartmann J, Schüßler-Lenz M, Bondanza A, Buchholz CJ. Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts. EMBO Mol Med 2017;9:1183–97. https://doi.org/10.15252/emmm.201607485.

[5] Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006;354:567–78. https://doi.org/10.1056/NEJMoa053422.

[6] Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med 2016;375:1856–67. https://doi.org/10.1056/nejmoa1602252.

[7] Harrington KJ, Ferris RL, Blumenschein G, Colevas AD, Fayette J, Licitra L, et al. Nivolumab versus standard, single-agent therapy of investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck (CheckMate 141): health-related quality-of-life results from a randomised, phase 3 trial. Lancet Oncol 2017;18:1104–15. https://doi.org/10.1016/S1470-2045(17)30421-7.

[8] Rischin D, Harrington KJ, Greil R, Soulieres D, Tahara M, de Castro G, et al. Protocol-specified final analysis of the phase 3 KEYNOTE-048 trial of pembrolizumab (pembro) as first-line therapy for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). J Clin Oncol 2019;37. https://doi.org/10.1200/jco.2019.37.15_suppl.6000. 6000–6000.

[9] Stoiber S, Cadilha BL, Benmebarek M-R, Lesch S, Endres S, Kobold S. Limitations in the design of chimeric antigen receptors for cancer therapy. Cells 2019;8:472. https://doi.org/10.3390/cells8050472.

[10] Chmielewski M, Hombach AA, Abken H. Antigen-specific T-cell activation independently of the MHC: Chimeric antigen receptor-redirected T cells. Front Immunol 2013;4.. https://doi.org/10.3389/fimmu.2013.00371.

[11] Ling DC, Bakkenist CJ, Ferris RL, Clump DA. Role of immunotherapy in head and neck cancer. Semin Radiat Oncol 2018;28:12–6. https://doi.org/10.1016/j.semradonc.2017.08.009.

[12] Davies DM, Foster J, Van Der Stegen SJC, Parente-Pereira AC, Chiapero-Stanke L, Delinassios GJ, et al. Flexible targeting of ErbB dimers that drive tumorigenesis by using genetically engineered T cells. Mol Med 2012;18:565–76. https://doi.org/10.2119/molmed.2011.00493.

[13] Mirzaei HR, Rodriguez A, Shepphird J, Brown CE, Badie B. Chimeric antigen receptors T cell therapy in solid tumor: Challenges and clinical applications. Front Immunol 2017;8.. https://doi.org/10.3389/fimmu.2017.01850.

[14] Spiegelberg D, Nilvebrant J. CD44v6-targeted imaging of head and neck squamous cell carcinoma: antibody-based approaches. Contrast Media Mol Imaging 2017;2017:2709547. https://doi.org/10.1155/2017/2709547.

[15] Martegani MP, Del Prete F, Gasbarri A, Natali PG, Bartolazzi A. Structural variability of CD44v molecules and reliability of immunodetection of CD44 isoforms using mAbs specific for CD44 variant exon products. Am J Pathol 1999;154:291–300. https://doi.org/10.1016/S0002-9440(10)65275-3.

[16] Cortes-Dericks L, Schmid RA. CD44 and its ligand hyaluronan as potential biomarkers in malignant pleural mesothelioma: Evidence and perspectives. Respir Res 2017;18:1–12. https://doi.org/10.1186/s12931-017-0546-5.

[17] Casucci M, Di Robilant BN, Falcone L, Camisa B, Norelli M, Genovese P, et al. CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. Blood 2013;122:3461–72. https://doi.org/10.1182/blood-2013-04-493361.

[18] Xu YY, Guo M, Yang LQ, Zhou F, Yu C, Wang A, et al. Regulation of CD44v6 expression in gastric carcinoma by the IL-6/STAT3 signaling pathway and its clinical significance. Oncotarget 2017;8:45848–61. https://doi.org/10.18632/oncotarget.17435.

[19] Wang Z, Zhao K, Hackert T, Zöller M. CD44/CD44v6 a reliable companion in cancer-initiating cell maintenance and tumor progression. Front Cell Dev Biol 2018;6:97. https://doi.org/10.3389/fcell.2018.00097.

[20] Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, et al. Prevention of tumor metastasis formation by anti-variant CD44. J Exp Med 1993;177:443–55. https://doi.org/10.1084/jem.177.2.443.

[21] Mulder JWR, Sewnath M, Offerhaus G, Pals S, Oosting J, Kruyt P, et al. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. Lancet 1994;344:1470–2. https://doi.org/10.1016/S0140-6736(94)90290-9.

[22] Kaufmann M, von Minckwitz G, Heider KH, Ponta H, Herrlich P, Sinn HP. CD44 variant exon epitopes in primary breast cancer and length of survival. Lancet 1995;345:615–9. https://doi.org/10.1016/S0140-6736(95)90521-9.

[23] Stroomer J, Roos J, Sproll M, Quak J, Heider KH, Wilhelm BJ, et al. Safety and biodistribution of 99mTechnetium-labeled anti-CD44v6 monoclonal antibody BIWA 1 in head and neck cancer patients. Clin Cancer Res 2000;6:3046–55.

[24] de Bree R, Roos JC, Quak JJ, den Hollander W, Snow GB, van Dongen GA. Radioimmunoscintigraphy and biodistribution of technetium-99m-labeled monoclonal antibody U36 in patients with head and neck cancer. Clin Cancer Res 1995;1.

[25] Verel I, Heider K-H, Siegmund M, Ostermann E, Patzelt E, Sproll M, et al. Tumor targeting properties of monoclonal antibodies with different affinity for target antigen CD44V6 in nude mice bearing head-and-neck cancer xenografts. Int J Cancer 2002;99:396–402. https://doi.org/10.1002/ijc.10369.

[26] Börjesson PK, Postema EJ, Roos JC, Colnot DR, Marres HA, van Schie MH, et al. Oyen WJ van DG. Phase I therapy study with 186Re-labeled humanized monoclonal antibody BIWA 4 (Bivatuzumab) in patients with head and neck squamous cell carcinoma. Clin Cancer Res 2003;9:3961–72.

[27] Sauter A, Kloft C, Gronau S, Bogeschdorfer F, Erhardt T, Golze W, et al. Pharmacokinetics, immunogenicity and safety of bivatuzumab mertansine, a novel CD44v6-targeting immunoconjugate, in patients with squamous cell carcinoma of the head and neck. Int J Oncol 2007;30:927–35. https://doi.org/10.3892/ijo.30.4.927.

[28] Tijink BM, Buter J, De Bree R, Giaccone G, Lang MS, Staab A, et al. A phase I dose escalation study with anti-CD44v6 bivatuzumab mertansine in patients with incurable squamous cell carcinoma of the head and neck or esophagus. Clin Cancer Res 2006;12:6064–72. https://doi.org/10.1158/1078-0432.CCR-06-0910.

[29] Roellecke K, Virts EL, Einholz R, Edson KZ, Altvater B, Rossig C, et al. Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies. Gene Ther 2016;23:615–26. https://doi.org/10.1038/gt.2016.38.

[30] Virts EL, Jankowska A, Mackay C, Glaas MF, Wiek C, Kelich SL, et al. AluY-mediated germline deletion, duplication and somatic stem cell reversion in UBE2T defines a new subtype of Fanconi anemia. Hum Mol Genet 2015;24:5093–108. https://doi.org/10.1093/hmg/ddv227.

[31] Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 1996;2:876–82. https://doi.org/10.1038/nm0896-876.

[32] Mulhall HJ, Labeed FH, Kazmi B, Costea DE, Hughes MP, Lewis MP. Cancer, pre-cancer and normal oral cells distinguished by dielectrophoresis. Anal Bioanal Chem 2011;401:2455–63. https://doi.org/10.1007/s00216-011-5337-0.

[33] van Hal NL, van Dongen GA, Stigter-van Walsum M, Snow GB, Brakenhoff RH. Characterization of CD44v6 isoforms in head-and-neck squamous-cell carcinoma. Int J Cancer 1999;82:837–45. https://doi.org/10.1002/(SICI)1097-0215(19990909)82:6<837::AID-IJC12>3.0.CO;2-H.

[34] Soukka T, Salmi M, Joensuu H, Häkkinen L, Sointu P, Koulu L, et al. Regulation of CD44v6-containing isoforms during proliferation of normal and malignant epithelial cells. Cancer Res 1997;57:2281–9.

[35] Pollok KE, Hanenberg H, Noblitt TW, Schroeder WL, Kato I, Emanuel D, et al. High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. J Virol 1998;72:4882–92. https://doi.org/10.1128/jvi.72.6.4882-4892.1998.

[36] Wiek C, Schmidt EM, Roellecke K, Freund M, Nakano M, Kelly EJ, et al. Identification of amino acid determinants in CYP4B1 for optimal catalytic processing of 4-ipomeanol. Biochem J 2015;465:103–14. https://doi.org/10.1042/BJ20140813.

[37] Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H, Valen E. CHOPCHOP v3: Expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Res 2019;47:W171–4. https://doi.org/10.1093/nar/gkz365.

[38] Kearns NA, Genga RMJ, Enuameh MS, Garber M, Wolfe SA, Maehr R. Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. Development 2014;141:219–23. https://doi.org/10.1242/dev.103341.

[39] Von Witzleben A, Wang C, Laban S, Savelyeva N, Ottensmeier CH. cells HNSCC: tumour antigens and their targeting by immunotherapy. Cells 2020;9.. https://doi.org/10.3390/cells9092103.

[40] Theoret MR, Cohen CJ, Nahvi AV, Ngo LT, Suri KB, Powell DJ, et al. Relationship of p53 overexpression on cancers and recognition by anti-p53 T cell receptor-transduced T cells. Hum Gene Ther 2008;19:1219–31. https://doi.org/10.1089/hum.2008.083.

[41] Figueiredo DLA, Mamede RCM, Proto-Siqueira R, Neder L, Silva WA, Zago MA. Expression of cancer testis antigens in head and neck squamous cell carcinomas. Head Neck 2006;28:614–9. https://doi.org/10.1002/hed.20380.

[42] Jakobsen MK, Gjerstorff MF. CAR T-cell cancer therapy targeting surface cancer/testis antigens. Front Immunol 2020;11:1568. https://doi.org/10.3389/fimmu.2020.01568.

[43] Al-Khadairi G, Decock J. Cancer testis antigens and immunotherapy: Where do we stand in the targeting of PRAME? Cancers (Basel) 2019;11.. https://doi.org/10.3390/cancers11070984.

[44] von Witzleben A, Wang C, Laban S, Savelyeva N, Ottensmeier CH. HNSCC: tumour antigens and their targeting by immunotherapy. Cells 2020;9:2103. https://doi.org/10.3390/cells9092103.

[45] Ferris RL. Immunology and immunotherapy of head and neck cancer. J Clin Oncol 2015;33:3293–304. https://doi.org/10.1200/JCO.2015.61.1509.

[46] Caruso HG, Hurton LV, Najjar A, Rushworth D, Ang S, Olivares S, et al. Tuning sensitivity of CAR to EGFR density limits recognition of normal tissue while maintaining potent antitumor activity. Cancer Res 2015;75:3505–18. https://doi.org/10.1158/0008-5472.CAN-15-0139.

[47] Van Schalkwyk MCI, Papa SE, Jeannon JP, Urbano TG, Spicer JF, Maher J. Design of a phase i clinical trial to evaluate intratumoral delivery of ErbB-targeted chimeric antigen receptor T-cells in locally advanced or recurrent head and neck cancer. Hum Gene Ther Clin Dev 2013;24:134–42. https://doi.org/10.1089/humc.2013.144.

[48] Jiang H, Gao H, Kong J, Song B, Wang P, Shi B, et al. Selective targeting of glioblastoma with EGFRvIII/EGFR bitargeted chimeric antigen receptor T cell. Cancer Immunol Res 2018;6:1314–26. https://doi.org/10.1158/2326-6066.CIR-18-0044.

[49] Whilding LM, Parente-Pereira AC, Zabinski T, Davies DM, Petrovic RMG, Kao YV, et al. Targeting of aberrant αvβ6 integrin expression in solid tumors using chimeric antigen receptor-engineered T cells. Mol Ther 2017;25:259–73. https://doi.org/10.1016/j.ymthe.2016.10.012.

[50] Maher J, Wilkie S, Davies DM, Arif S, Picco G, Julien S, et al. Targeting of tumor-associated glycoforms of MUC1 with CAR T cells. Immunity 2016;45:945–6. https://doi.org/10.1016/j.immuni.2016.10.014.

[51] Wang Z, Cao YJ. Adoptive cell therapy targeting neoantigens: a frontier for cancer research. Front Immunol 2020;11:176. https://doi.org/10.3389/fimmu.2020.00176.

[52] Börjesson PKE, Postema EJ, De Bree R, Roos JC, Leemans CR, Kairemo KJA, et al. Radioimmunodetection and radioimmunotherapy of head and neck cancer. Oral Oncol 2004;40:761–72. https://doi.org/10.1016/j.oraloncology.2003.11.009.

[53] Mack B, Gires O. CD44s and CD44v6 expression in head and neck epithelia. PLoS ONE 2008;3:e3360. https://doi.org/10.1371/journal.pone.0003360.

[54] Porcellini S, Asperti C, Corna S, Cicoria E, Valtolina V, Stornaiuolo A, et al. CAR T cells redirected to CD44v6 control tumor growth in lung and ovary adenocarcinoma bearing mice. Front Immunol 2020;11:99. https://doi.org/10.3389/fimmu.2020.00099.

[55] Larcombe-Young D, Papa S, Maher J. PanErbB-targeted CAR T-cell immunotherapy of head and neck cancer. Expert Opin Biol Ther 2020;20:965–70. https://doi.org/10.1080/14712598.2020.1786531.

[56] Liu X, Jiang S, Fang C, Yang S, Olalere D, Pequignot EC, et al. Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. Cancer Res 2015;75:3596–607. https://doi.org/10.1158/0008-5472.CAN-15-0159.

[57] Newick K, O'Brien S, Moon E, Albelda SM. CAR T cell therapy for solid tumors. Annu Rev Med 2017;68:139–52. https://doi.org/10.1146/annurev-med-062315-120245.

[58] Balló H, Koldovsky P, Hoffmann T, Balz V, Hildebrandt B, Gerharz CD, et al. Establishment and characterization of four cell lines derived from human head and

neck squamous cell carcinomas for an autologous tumor-fibroblast in vitro model. Anticancer Res 1999;19:3827–36.
[59] Hauser U, Balz V, Carey TE, Grénman R, van Lierop A, Scheckenbach K, et al. Reliable detection of p53 aberrations in squamous cell carcinomas of the head and neck requires transcript analysis of the entire coding region. Head Neck 2002;24:868–73.
[60] Lansford CD, Grenman R, Bier H, Somers KD, Kim SY, Whiteside TL, et al. Head and Neck Cancers. In: Masters JRW, Palssons B, editors. Human Cell Culture, Vol. 2. Kluwer Academic Publishers: Dordrecht; 1999. p. 185–255.
[61] Soukka T, Salmi M, Joensuu H, Häkkinen L, Sointu P, Koulu L, et al. Regulation of CD44v6-containing isoforms during proliferation of normal and malignant epithelial cells. Cancer Res 1997;57:2281–9.
[62] Pekkola-Heino K, Kulmala J, Grenman R. Sublethal damage repair in squamous cell carcinoma cell lines. Head Neck 1992;14:196–9.
[63] Johansson N, Airola K, Grenman R, Kariniemi A-L, Saarialho-Kere U, Kahari V-M. Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. Am J Pathol 1997;151:499–508.
[64] Servomaa K, Kiuru A, Grénman R, Pekkola-Heino K, Pulkkinen JO, Rytömaa T. p53 mutations associated with increased sensitivity to ionizing radiation in human head and neck cancer cell lines. Cell Prolif 1996;29:219–30.
[65] Lin CJ, Grandis JR, Carey TE, Gollin SM, Whiteside TL, Koch WM, et al. Head and neck squamous cell carcinoma cell lines: established models and rationale for selection. Head Neck 2007;29:163–88.
[66] Pekkola-Heino K, Servomaa K, Kiuru A, Grenman R. Increased radiosensitivity is associated with p53 mutations in cell lines derived from oral cavity carcinoma. Acta Otolaryngol 1996;116:341–4.
[67] Grénman R, Carey TE, McClatchey KD, Wagner JG, Pekkola-Heino K, Schwartz DR, et al. In vitro radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. Cancer 1991;67:2741–7.
[68] Kiuru A, Servomaa K, Grénman R, Pulkkinen J, Rytömaa T. p53 mutations in human head and neck cancer cell lines. Acta Oto-Laryngologica, Suppl., Taylor and Francis A.S. 1997:237–40.
[69] Bradford CR, Zacks SE, Androphy EJ, Gregoire L, Lancaster WD, Carey TE, et al. Human papillomavirus DNA sequences in cell lines derived `from head and neck squamous cell carcinomas. Otolaryngol Head Neck Surg 1991;104(3):303–10.
[70] Krause CJ, Carey TE, Ott RW, Hurbis C, Mcclatchey KD, Regezi JA. Human squamous cell carcinoma: establishment and characterization of new permanent cell lines. Arch Otolaryngol 1981;107:703–10.
[71] Carey TE, Kimmel KA, Schwartz DR, Richter DE, Baker SR, Krause CJ. Antibodies to human squamous cell carcinoma. Otolaryngol Head Neck Surg 1983;91:482–91.
[72] Tang AL, Hauff SJ, Owen JH, Graham MP, Czerwinski MJ, Park JJ, et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. Head Neck 2012;34:1480–91.

EPIGENETISCHES *PRIMING* VON UCC-ZELLEN MITTELS DECITABIN VERSTÄRKT DIE ZYTOTOXIZITÄT VON EGFR UND CD44v6 CAR-T-ZELLEN

Titel: Epigenetic priming of bladder cancer cells with decitabine increases cytotoxicity of human EGFR and CD44v6 CAR engineered T-cells

Autoren: Camilla M. Grunewald*, **Corinna Haist***, Carolin König, Patrick Petzsch, Arthur Bister, Elfriede Nößner, Constanze Wiek, Kathrin Scheckenbach, Karl Köhrer, Günter Niegisch#, Helmut Hanenberg#, Michèle J. Hoffmann#

* Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen

Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen

Eigenleistung: 40 %

- Planung, Durchführung und Auswertung der Experimente, Erstellung der Manuskriptabbildungen, Schreiben und Korrektur des Manuskriptes

Veröffentlicht in: Frontiers in Immunology

Impact Factor: 7,561

Status: veröffentlicht

DOI: 10.3389/fimmu.2021.782448.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 November 2021
doi: 10.3389/fimmu.2021.782448

Epigenetic Priming of Bladder Cancer Cells With Decitabine Increases Cytotoxicity of Human EGFR and CD44v6 CAR Engineered T-Cells

Camilla M. Grunewald[1‡], Corinna Haist[2,3‡], Carolin König[1], Patrick Petzsch[4†], Arthur Bister[2,3], Elfriede Nößner[5], Constanze Wiek[2], Kathrin Scheckenbach[2], Karl Köhrer[4†], Günter Niegisch[1§], Helmut Hanenberg[2,3§] and Michèle J. Hoffmann[1§]*

[1] Department of Urology, Medical Faculty, Heinrich Heine University Duesseldorf, Duesseldorf, Germany, [2] Department of Otorhinolaryngology and Head and Neck Surgery, Medical Faculty, Heinrich Heine University Duesseldorf, Duesseldorf, Germany, [3] Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Essen, Germany, [4] Biological and Medical Research Center (BMFZ), Heinrich Heine University Duesseldorf, Duesseldorf, Germany, [5] Immunoanalytics: Tissue Control of Immunocytes, German Research Center for Environmental Health, Helmholtz Zentrum München, Munich, Germany

Background: Treatment of B-cell malignancies with CD19-directed chimeric antigen receptor (CAR) T-cells marked a new era in immunotherapy, which yet has to be successfully adopted to solid cancers. Epigenetic inhibitors of DNA methyltransferases (DNMTi) and histone deacetylases (HDACi) can induce broad changes in gene expression of malignant cells, thus making these inhibitors interesting combination partners for immunotherapeutic approaches.

Methods: Urothelial carcinoma cell lines (UCC) and benign uroepithelial HBLAK cells pretreated with the DNMTi decitabine or the HDACi romidepsin were co-incubated with CAR T-cells directed against EGFR or CD44v6, and subsequent cytotoxicity assays were performed. Effects on T-cell cytotoxicity and surface antigen expression on UCC were determined by flow cytometry. We also performed next-generation mRNA sequencing of inhibitor-treated UCC and siRNA-mediated knockdown of potential regulators of CAR T-cell killing.

Results: Exposure to decitabine but not romidepsin enhanced CAR T-cell cytotoxicity towards all UCC lines, but not towards the benign HBLAK cells. Increased killing could neither be attributed to enhanced target antigen expression (EGFR and CD44v6) nor fully explained by changes in the T-cell ligands PD-L1, PD-L2, ICAM-1, or CD95. Instead, gene expression analysis suggested that regulators of cell survival and apoptosis were differentially induced by the treatment. Decitabine altered the balance between survival and apoptosis factors towards an apoptosis-sensitive state associated with increased CAR T-cell killing, while romidepsin, at least partially, tilted this balance in the opposite

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Edited by:
Sarwish Rafiq, Emory University, United States

Reviewed by:
Hamid Reza Mirzaei, Tehran University of Medical Sciences, Iran
Muhammad Sadeqi Nezhad, Islamic Azad University of Gorgan, Iran

***Correspondence:**
Camilla M. Grunewald
camillamarisa.grunewald@med.uni-duesseldorf.de

†ORCID:
Patrick Petzsch
orcid.org/0000-0002-8355-5524
Karl Köhrer
orcid.org/0000-0003-3644-2022

‡These authors have contributed equally to this work and share first authorship

§These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *24 September 2021*
Accepted: *26 October 2021*
Published: *17 November 2021*

Citation:
Grunewald CM, Haist C, König C, Petzsch P, Bister A, Nößner E, Wiek C, Scheckenbach K, Köhrer K, Niegisch G, Hanenberg H and Hoffmann MJ (2021) Epigenetic Priming of Bladder Cancer Cells With Decitabine Increases Cytotoxicity of Human EGFR and CD44v6 CAR Engineered T-Cells. Front. Immunol. 12:782448. doi: 10.3389/fimmu.2021.782448

direction. Knockdown experiments with siRNA in UCC confirmed BID and BCL2L1/BCLX as two key factors for the altered susceptibility of the UCC.

Conclusion: Our data suggest that the combination of decitabine with CAR T-cell therapy is an attractive novel therapeutic approach to enhance tumor-specific killing of bladder cancer. Since BID and BCL2L1 are essential determinants for the susceptibility of a wide variety of malignant cells, their targeting might be additionally suitable for combination with immunotherapies, e.g., CAR T-cells or checkpoint inhibitors in other malignancies.

Keywords: epigenetic inhibitors, bladder cancer, chimeric antigen receptor, immunotherapy, T-cell

INTRODUCTION

In recent years, the field of tumor immunotherapy has evolved rapidly (1). One of the most exciting approaches is the use of autologous patient-derived T-cells that have been genetically modified to express chimeric antigen receptors (CARs). Such CAR molecules combine the antigen-binding properties of monoclonal antibodies with the lytic capacity of T-cells (2). Remarkable remission rates in clinical trials using CAR T-cells directed against $CD19^+$ B-cell malignancies led to FDA approval of the first CAR T-cell therapies for patients with relapsed/refractory acute lymphoblastic leukemia (ALL) or diffuse large B-cell lymphoma (DLBCL) and for patients with primary mediastinal B-cell lymphoma (PMBCL) in 2017. In addition to Yescarta (axicabtagene ciloleucel) and Kymriah (tisagenlecleucel), two other CD19-targeted CAR T-cell therapies, namely, Tecartus (brexuscabtagene autoleucel) and Breyanzi (lisocatbagene maraleucel), were approved in 2020 and 2021, respectively. Finally, the first BCMA-targeted CAR T-cell therapy, Abecma (idecabtagene vicleucel), for relapsed/refractory multiple myeloma received approval in March 2021 (3).

The exceptional success in hematological malignancies could not be transferred to solid cancers, due to issues with T-cell trafficking, immunosuppressive tumor microenvironment, target antigen heterogeneity, and intrinsic regulatory mechanisms of T-cells in these malignancies (4). Although no definite clinical data on CAR T-cell therapy has been published yet for its use in bladder cancer, several early phase I/II clinical trials are ongoing targeting the prostate-specific membrane antigen (PSMA), the human epidermal growth factor receptor 2 (HER2), Nectin4/FAP, NKG2D ligands, and the receptor tyrosine kinase-like orphan receptor 2 (ROR2), respectively (NCT03185468, NCT03740256, NCT 03932565, NCT03018405, NCT03960060[1]).

Epigenetic dysregulation caused by DNA hypermethylation through DNA methyltransferases (DNMTs) and histone hypoacetylation catalyzed by histone deacetylases (HDACs) leads to silencing of key genes and thereby determines the phenotype of urothelial carcinoma of the bladder (UC) with regard to pathogenesis, tumor biology, and outcome to standard treatment (5). Novel therapeutic strategies directed towards these epigenetic drivers include inhibitors of DNMTs (DNMTi, e.g., decitabine) and HDACs (HDACi, e.g., romidepsin). Importantly, both of these drugs were already approved for the treatment of certain hematological malignancies. Besides induction of broad gene expression changes affecting various cellular processes, epigenetic inhibitors (epidrugs) can also remodel the differentiation and the immune phenotypes of both cancer and immune cells (6, 7). Epidrugs can also influence key components of apoptosis signaling in cells, e.g., expression of the FAS receptor (CD95) is regulated by DNA methylation (8). Concurringly, epidrugs have been shown to resensitize tumors to previously failed therapies and to affect both the cancer cells as well as the tumor microenvironment (9). We therefore considered it an interesting approach to prime UC for immune-oncological approaches like CAR T-cell therapy, especially as we previously characterized the functional importance of individual HDAC isoenzymes and their potential as therapeutic targets in UC (10). So far, only a few clinical trials evaluate the use of epidrugs in bladder cancer as part of combination therapies (11).

To determine whether epigenetic pretreatment of UC cell lines (UCC) might affect their susceptibility towards cytotoxic killing by CAR T-cells, we developed a combined treatment protocol involving UCC treatment with either the DNMTi decitabine (DEC) or the HDACi romidepsin (ROM). We used previously established treatment conditions of 3 nM ROM for 3 days (10), whereas DEC was applied in a low-dose/long-term protocol (100 nM DEC for 7 days), following emerging data that administration of low doses of an DNMTi might enhance the desired epigenetic effects whilst reducing toxicity (12) being comparably well tolerated in patients, e.g., with low-risk myelodysplastic syndrome (13). As target antigens, we chose two different surface molecules on UCC: The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein and member of the ERBB family of surface proteins that are associated with cell migration, adhesion, and proliferation. EGFR expression is highly enriched in UC tissues and strongly associated with certain tumor grades and stages as well as risk of recurrence (14). Although there is no data for CAR T-cells directed against EGFR in UC, cetuximab as the most prominent anti-EGFR antibody is currently discussed as radiosensitizer (15), albeit prior phase II data on the use of cetuximab in metastatic UC showed limited activity of the antibody as a single modality treatment (16). As a second target antigen structure, we selected CD44v6, a splicing variant of the cell surface adhesion receptor CD44, that is overexpressed on a large variety of malignant cells (17). CD44v6 expression is associated with tumor cell invasion, metastasis, and disease progression and has been correlated with increased tumor

[1] www.clinicaltrials.gov

grade and stage in UC (18). Similar to EGFR, clinically relevant expression of CD44v6 on normal epithelial tissue has mainly been reported in skin and oral mucosa (19).

In this study, we identified CD44v6 and EGFR as promising target antigens for CAR T-cell therapy of UC and demonstrated that the specific killing capacity of CAR T-cells against malignant UCC is strongly influenced by expression of pro- and anti-apoptotic genes in the malignant cells that can be modulated by epigenetic treatment strategies.

MATERIALS AND METHODS

Cell Culture

We used four UCC, namely, RT-112, BFTC905, VM-CUB-1, and UM-UC-3 (10). HBLAK was used as normal urothelial control cell line (20). All cell lines were regularly authenticated by STR profiling, checked for mycoplasma contamination, and cultured as described (21). Human embryonic kidney cells (HEK293T) were obtained from DSMZ (Braunschweig, Germany) and cultured in DMEM GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (all Thermo Fisher Scientific, Schwerte, Germany).

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy adult volunteers by density-gradient centrifugation (Cytiva, Marlborough, MA, USA). Blood donors gave informed consent according to the protocol (#2019-623) approved by the local ethics committee/IRB in Düsseldorf. Prior to transduction, T-cells were prestimulated with immobilized antibodies against CD3 (OKT3, Ortho Biotech, Neuss, Germany) and CD28 (BD Biosciences Pharmingen, San Diego, CA, USA), as well as 100 IU/ml Interleukin-2 (IL-2, Proleukin, Novartis, Basel, Switzerland) in IMDM (Sigma, MO, USA) containing 10% FBS, 1% P/S, 1% glutamine as previously described (22, 23).

In Vitro Treatment of Cell Lines With DEC or ROM and siRNA Transfection

5-Aza-2'-Desoxycytidin (DEC) was purchased from Sigma-Aldrich (Steinheim, Germany) and romidepsin (ROM) from Selleckchem (Houston, TX, USA) and dissolved in DMSO. Control cells were treated with corresponding DMSO concentrations. Since DEC is known to have only an *in vitro* half-life of 5–16 h at 37°C, 100 nM DEC was freshly added every 24 h for 3 days during medium change. Hereafter, cells were cultured for 4 additional days, washed, and passaged into 96-well plates prior to co-culture with CAR T-cells (24). For ROM treatment, cells were cultured in the same medium containing 3 nM ROM for 3 days according to common ROM treatment protocols (25). ROM solution is stable at room temperature for about 24 h. Thus, we expected the T-cells not to be touched by active ROM when these were added to treated UCC for co-culture after 72 h. As a control, we performed washout experiments demonstrating no difference between samples with or without ROM washout 72 h after UCC treatment before adding T-cells for co-culture.

UCC were transfected with siRNA as described (21) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) and 8 nM of the ON-TARGETplus SMARTPool (Dharmacon, GE Healthcare, Freiburg, Germany), comprising a set of four individual siRNAs against each of the targets TRADD, DAXX, BCL2L1, and BID or the non-targeting control pool (**Supplementary Table S1**).

Flow Cytometry

Surface expression of antigens was assessed 3 days (ROM) and 7 days (DEC) after treatment by immunofluorescence staining and flow cytometry (MACSQuant Analyzer 10; Miltenyi Biotec, Bergisch Gladbach, Germany). Likewise, T-cell phenotype was determined by immunofluorescence staining with subsequent flow cytometric analysis. Antibodies and counterstaining are listed in **Supplementary Table S1**. Data were analyzed using the FlowJo™ Software (v10.0.7). Results were expressed as percentage of positive cells and median fluorescence intensity (MFI). Values of unstained cells were subtracted from values of stained cells.

Generation of Lentiviral Constructs and T-Cell Transduction, Selection, and Functional Assays

The CD19 and CD44v6 CAR constructs were described previously (26–29). The EGFR CAR lentiviral vector contains optimized sequences for the heavy-chain and light-chain variable region segments derived from the monoclonal antibody of Cetuximab (30). In comparison to a Cetuximab-based CAR construct published previously (31), the light chain of Cetuximab in our construct was shortened at amino acid (aa) 108 and the heavy chain at aa 128 (Haist et al. submitted). HEK293T cells were transfected with polyethylenimine (PEI, Sigma-Aldrich, St. Louis, MO, USA) using 6 µg HIV1 helper plasmid (gag-pol-rev), 6 µg vesicular stomatitis virus glycoprotein (VSV-G) envelope, and 6 µg lentiviral CAR construct plasmid for the generation of recombinant lentiviral particles (26). The next day, the medium was changed to IMDM, and then after additional 16–20 h and 0.45 µm filtration, the culture supernatants were directly used for transduction.

After prestimulation on immobilized CD3/CD28 monoclonal antibodies, T-cells were transduced with lentiviral particles on the recombinant fibronectin fragment Retronectin® as described (26, 32). Subsequently, T-cells were cultured in medium containing 100 IU/ml IL-2 for 72 h. To obtain >98% pure CAR T-cells, transduced T-cells were incubated with magnetic microbeads coupled to the CD34 QBEND10 antibody (Miltenyi Biotec) (**Supplementary Table S1**), which recognizes a 99 amino acid sequence that we have included as novel hinge domain in our CARs (29) (Bister et al., in press; patent EP3293199). Subsequently, CD34 microbead-stained T-cells were purified on MACS MS columns according to the manufacturer's instructions (Miltenyi Biotec).

For cytotoxicity assays, UCC were pretreated with DEC or ROM or cultured in the presence of DMSO and seeded in U-bottom 96-well plates. On day 3 of ROM and day 7 of DEC pretreatment or day 2 after siRNA transfection, cells were counted and CD19, EGFR, and CD44v6 CAR T-cells added at different effector to target (E:T) cell ratios (3:1, 1:1, 0.3:1, 0.1:1,

0.03:1, and 0.01:1). Prestimulated non-transduced T-cells served as additional controls. After 16 h of co-culture at 37°C, the non-adherent T-cells and the dead tumor cells were carefully removed by washing steps. Remaining adherent UCCs were incubated with the CellTiter 96® AQueous One Solution Cell Proliferation substrate according to manufacturer specifications (Promega, Fitchburg, WI, USA), and viability was determined on a TECAN sunrise (Tecan Group AG, Männedorf, Switzerland). The percent lysis was calculated as follows:

$$100\,\% - (\textit{Absorption of targets incubated with T-cells} / \textit{Absorption of reference targets}) \times 100$$

For the spheroid model, 1×10^5 untreated or DEC-treated UM-UC-3 or BFTC905 cells were seeded in ultra-low attachment U-bottom 96-well plates (Corning, Wiesbaden, Germany). Twenty-four hours later, spheroid formation was confirmed by microscope, and CD19, EGFR, CD44v6 CAR T-cells or prestimulated non-transduced T-cells from three different donors were added at E:T ratios of 1:1, 1:2, or 1:4. After 16 h of co-culture, CellTiter-Glo® 3D Cell Viability substrate (Promega) was added according to manufacturer specifications and viability determined on a Wallac VICTOR 2 (Perkin-Elmer, Waltham, MA, USA). The percent specific lysis was calculated as follows:

$$100\,\% - (\textit{Luminescence of spheroids with CAR T-cells} / \textit{Luminescence of spheroids with non-transduced T-cells}) \times 100$$

To analyze cytokine secretion by T-cells, supernatants of the wells containing the 1:1 E:T ratio of effector to target cells were harvested and stored at −20°C. Then 50 µl of each supernatant was analyzed using the MACSPlex Cytotoxic T/NK cell kit (Miltenyi Biotec) and measured on a MACSQuant Analyzer 10 according to the manufacturer's instructions.

Next-Generation RNA Sequencing

Total RNA was extracted from BFTC905 and UM-UC-3 cells treated with 100 nM DEC using the RNAeasy Mini Kit (Qiagen, Hilden, Germany). DMSO-treated cells were harvested in parallel as controls. RNA was quantified using Qubit RNA HS Assays (Thermo Fisher Scientific). Quality was confirmed by capillary electrophoresis using Fragment Analyzer and Total RNA Standard Sensitivity Assay (Agilent Technologies, Santa Clara, CA, USA). Library preparation and next-generation sequencing were performed as described (21). Multigroup comparisons were calculated using the Empirical Analysis of DGE (version 1.1, cutoff = 5) after grouping of samples (three biological replicates each) according to their respective experimental condition. The resulting p-values were adjusted for multiple testing by FDR and Bonferroni-correction. A p-value of ≤ 0.05 was considered significant. Cutoff for differential gene expression was set to 1.5-fold. Further analysis and data visualization were performed using Microsoft Excel and Graph Pad Prism 8. Venn diagrams were prepared with the online tool Venny 2.0 (33). GO group analysis was performed using the online tool DAVID (34).

Western Blot Analysis

Proteins were extracted 72 h after siRNA transfection and used for Western Blot analysis as described (21). Expression of knockdown targets was detected by antibodies listed in **Supplementary Table S1**.

Statistical Analysis

All experiments were repeated at least three times. Significance between groups was analyzed by means of Graph Pad Prism 9 using 2-way ANOVA (analysis of variances) with Dunnett's correction for multiple comparison. P-values of < 0.05 were considered significant and denoted with an asterisk.

RESULTS

The CAR Target Antigens EGFR and CD44v6 Are Expressed on Urothelial Cells

In order to determine the role of epigenetic treatment for immunotherapy of bladder cancer, we used a representative set of human urothelial carcinoma cell lines, RT-112, BFTC905, VM-CUB-1, and UM-UC-3, which covers the heterogeneity of urothelial cancer. HBLAK (20), a non-malignant urothelial cell line, was employed as control. First, we evaluated surface expression of two potentially suitable target antigens on our cell lines, EGFR and CD44v6. All UCC expressed high levels of EGFR (MFI 4.2-21.0, 74–93% positive cells), while the expression level was clearly lower on HBLAK cells (MFI 1.3, 33% positive cells, **Figure 1**). In contrast, CD44v6 expression was similar for all five cell lines (MFI 0.3–1.8, 23–48% positive cells).

High-Level Expression of EGFR and CD44v6 CARs on Normal Allogeneic T-Cells After Lentiviral Transduction Followed by MACS Enrichment

To generate CAR T-cells, we employed previously established CARs against EGFR, CD44v6, and CD19 (29) (Haist et al. submitted). The standard lentiviral vectors (26) expressed second-generation CARs containing the CD8 leader peptide, the single-chain variable fragments (scFvs) of a EGFR-, CD44v6-, or CD19-specific monoclonal antibody, an extracellular hinge region using 99 amino acids from human CD34 (29), the CD28 transmembrane and co-stimulatory domains as well as the CD3ζ signaling domain (**Figure 2A**). Three days after transduction, CAR-expressing T-cells were purified. Representative samples for the purification steps (Pre MACS, flow-through, Post MACS) were analyzed by flow cytometry (**Figure 2B**), demonstrating transduction efficiencies between 61.7 and 65.5% prior to enrichment and ≥98% strongly CAR positive T-cells after the enrichment step. MACS-selected CAR T-cells were further characterized for the CD4/CD8 ratio and the memory phenotype by flow cytometry. While the phenotype of CD19, EGFR, and CD44v6 CAR T-cells did not differ from the phenotype of non-transduced T-cells expanded under identical conditions in parallel (**Supplementary Figures S1A, B**), it is noteworthy that the majority of the T-cells were central memory cells (CM; **Supplementary Figure S1B**), as defined by co-expression of CD62L and CD45RO (35).

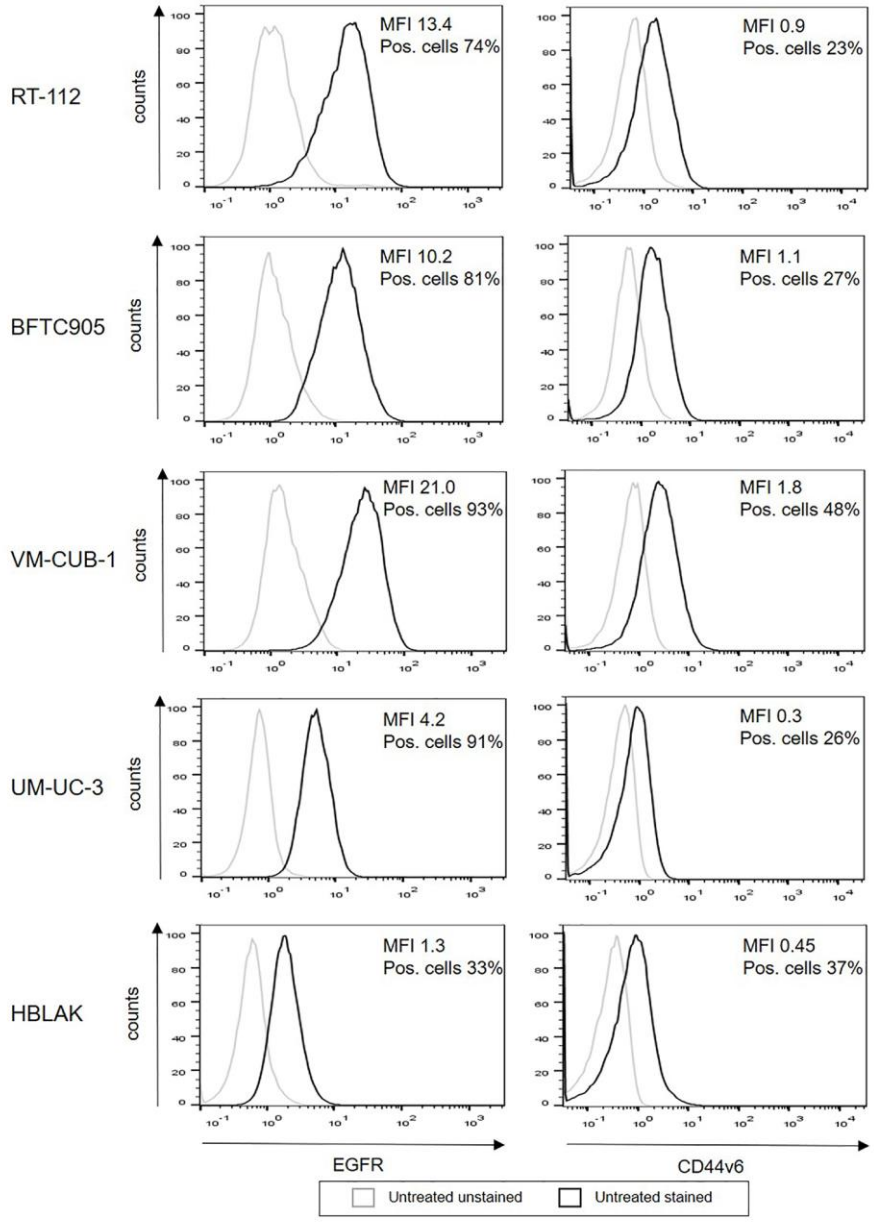


FIGURE 1 | Surface expression of EGFR and CD44v6 on untreated UCC and HBLAK. The UCC RT-112, BFTC905, VM-CUB-1, UM-UC-3, and benign HBLAK were stained with the indicated antibodies and analyzed by flow cytometry. The figure shows representative histograms out of at least three independent experiments for each cell line. Histogram of unstained cells appear in gray, stained cells in black.

Primary Human T-Cells Expressing EGFR and CD44v6 CARs Effectively Kill UCC

To assess the specific cytotoxicity, ≥98% MACS-enriched EGFR and CD44v6 CAR T-cells were co-incubated with the four UCC and HBLAK in different E:T ratios (3:1 to 0.01:1). Negative controls in these assays were non-transduced T-cells as well as CD19 CAR T-cells. We initially confirmed that our flow cytometry-based (26) and our 96-well plate cytotoxicity assay (29) provided similar results (**Supplementary Figure S2A**). As the plate cytotoxicity assay facilitated a much higher throughput of samples, all consecutive

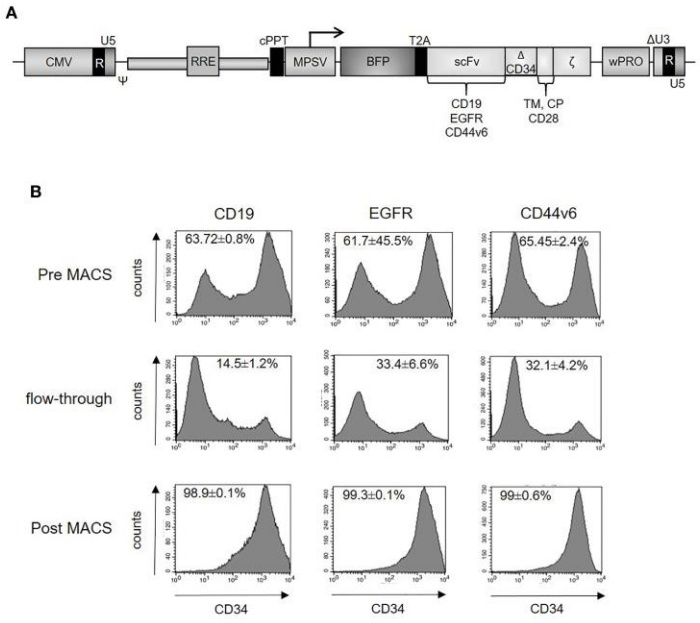


FIGURE 2 | **(A)** Schematic design of the lentiviral vector for expression of the second-generation CARs. The constructs contain CD19, EGFR or CD44v6 scFv, CD34 hinge domain, CD28 transmembrane and intracellular domain, CD3ζ cytosolic domain and blue fluorescent protein (BFP). **(B)** Enrichment of CAR-positive T-cells. Primary human T-cells were lentivirally transduced with constructs expressing CD19, EGFR, or CD44v6 CARs. Three days after transduction, CAR-positive T-cells were stained with CD34 microbeads (Miltenyi) and finally enriched by magnetic cell sorting. Different fractions (pre, flow-through, post MACS) were analyzed by flow cytometry using the QBEND-PE antibody (**Supplementary Table S1**). CAR expression (x-axis) is plotted against the absolute cell count (y-axis). Illustrations were gated with the percentage of positive cells. Data are depicted as mean ± SEM for three different experiments.

cytotoxicity assays were analyzed with this methodology. The lysis curves obtained with the 96-well plate assay revealed that the EGFR and CD44v6 CAR T-cells efficiently and specifically killed RT-112, VM-CUB-1, and UM-UC-3 cells to similar degrees at comparable E:T ratios (**Figure 3**). Notably, BFTC905 cells, despite robustly expressing both target antigens (MFI 10.2 and 1.1, 81% and 27% positive cells, respectively) were killed less efficiently. HBLAK cells were killed more efficiently at higher effector-to-target-cell ratios (1:1 and 3:1), despite having low target antigen expression levels (MFI 1.3 and 0.45, 33% and 37% positive cells, respectively). Therefore, these results clearly demonstrated that the degree of CAR T-cell cytotoxicity is not simply dependent on the expression level of the target antigens.

Epigenetic Treatment of UCC Influences CAR T-Cell Cytotoxicity

To investigate whether cytotoxicity of CAR T-cells can be influenced by treatment of the target cells with epidrugs, UCC and HBLAK were pretreated with either DEC, ROM, or DMSO as control and subsequently incubated with CAR T-cells. Viability analysis revealed that DEC pretreatment (white square) sensitized all four UCC more towards EGFR and CD44v6 CAR T-cell-mediated cytotoxicity when compared to DMSO-treated cells (black rhombus) (**Figure 3**). Especially BFTC905 cells, which were insufficiently killed by EGFR and CD44v6 CAR T-cells, were considerably better killed after DEC with killing values augmented from 18 ± 5 to 42 ± 19% (EGFR) and from 8 ± 2 to 47 ± 14% (CD44v6) at the 3:1 ratio, respectively. UM-UC-3 and VM-CUB-1 cells were also killed considerably more effective when pretreated with DEC. Cytotoxicity of HBLAK cells was not clearly affected by pretreatment with neither inhibitor, suggesting increased tumor-specific killing of CAR T-cells after DEC treatment. In contrast, ROM treatment (orange circle) resulted in unchanged (BFTC905, RT-112) or even decreased (UM-UC-3, VM-CUB-1) specific cytotoxic activity for both CAR T-cells. Even though ROM is not stable for 72 h incubation time, we performed additional washout experiments to demonstrate that reduced cytotoxicity after ROM pretreatment did not result from negative impact of the compound on CAR T-cells (**Supplementary Figure S2B**).

Cancer cell spheroids can partially fill the gap between conventional 2D *in vitro* assays and animal models, as these spheroids better model the infiltration of immune effector cells into solid cancer tissues (36). To confirm our findings in a more advanced model, we therefore used DEC-treated and untreated UM-UC-3 and BFTC905 spheroids and performed subsequent cytotoxicity assays after co-incubation with EGFR, CD44v6, and CD19 CAR or non-transduced T-cells, similar to the 2D experiments described above. As shown in **Supplementary Figures S3A, B**, EGFR and CD44v6 CAR T-cells killed cells

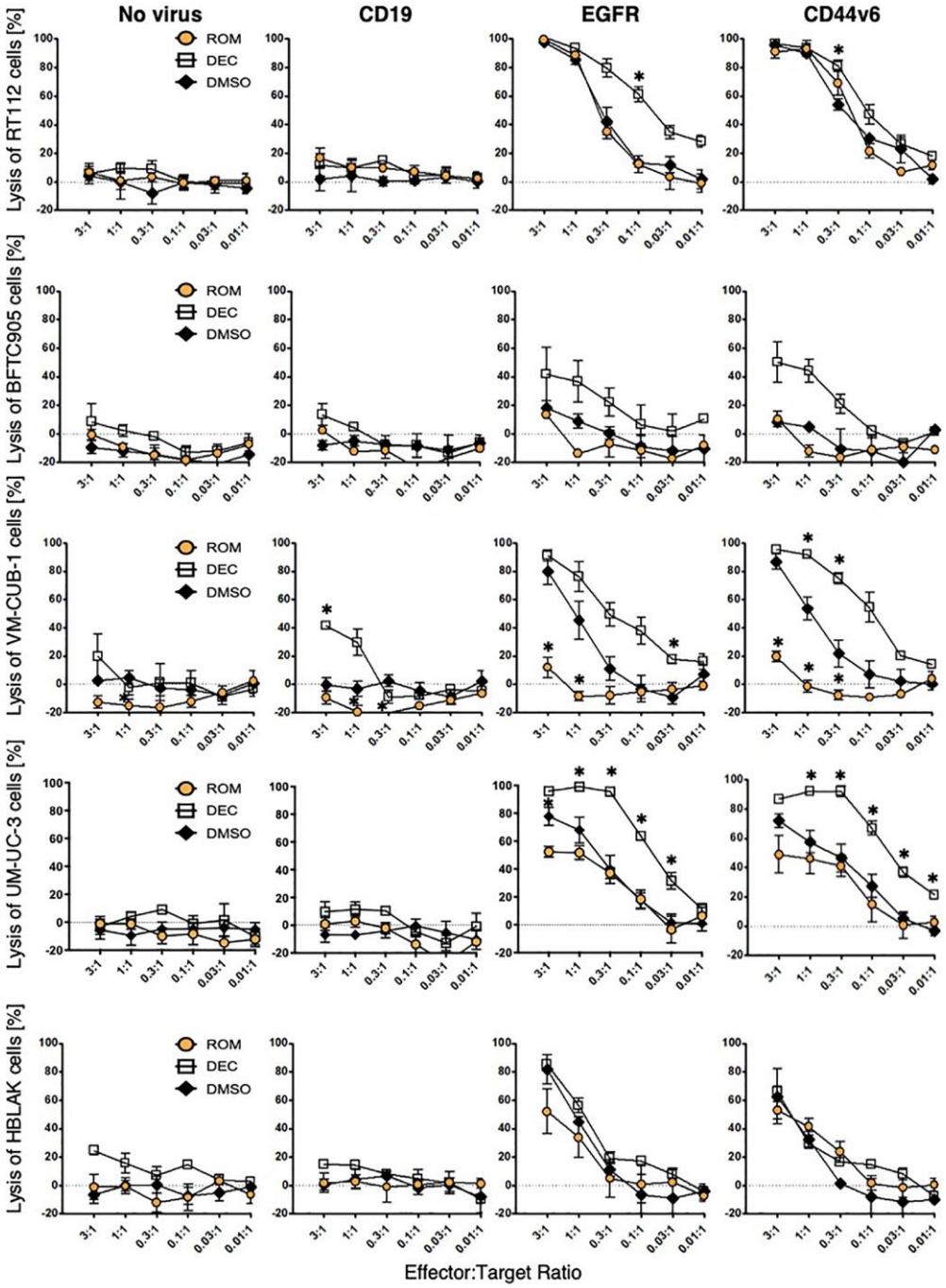


FIGURE 3 | CAR T-cell cytotoxicity can be modulated by epigenetic treatment. UCC and HBLAK cells were pretreated with 100 nM decitabine (white square), 3 nM romidepsin (orange circle), or DMSO (black rhombus) and subsequently co-cultured with CD19-, EGFR-, or CD44v6 CAR T-cells or untransduced (no virus) T-cells. After 16 h co-culture, lysis was determined by MTT assay. Graphs depict the mean values (± SD) for percentage of lysed cells from at least three independent experiments. Statistical significance (p-value <0.05) is denoted by asterisk and was determined by two-way ANOVA and Dunnett's correction for multiple comparisons.

in the spheroids of both cell lines at comparable efficiencies, as observed in the 2D *in vitro* cultures (**Figure 3**). However, the effects for DEC-pretreated BFTC905 were generally less pronounced. The secretion profiles for granzyme B, TNFα, and GM-CSF in the culture supernatants very well reflected the excellent killing of UM-UC-3 cells and the much lower killing of BFTC905 cells by EGFR and CD44v6 CAR T-cells, while the cytokine profiles for CD19 CAR T-cells were similar to those of non-transduced T-cells (**Supplementary Figures S3C, D**). Importantly, the DEC pretreatment of both UC cell lines did not result in increased cytokine secretions by the CAR T-cells, suggesting that the increased lysis is more likely due to a tumor-intrinsic than a T-cell-mediated effect.

Expression Levels of the Target Antigens, Immune Checkpoints, and Adhesion Molecules Do Not Correlate Well With the Killing Efficacy

Increased target antigen expression would provide a straightforward explanation for enhanced CAR T-cell cytotoxicity following DEC treatment. EGFR and CD44v6 expression levels increased strongly in BFTC905 cells after DEC treatment (**Figure 4**), which correlated well with the improved killing observed with both CAR T-cells. However, despite an even stronger increase in CD44v6 expression and comparable EGFR expression after ROM compared to DEC treatment, no enhanced killing was detected when using ROM-treated BFTC905 cells. RT112 cells were killed to similar degrees after all treatments by both CARs, in correlation to the unaltered target expression levels. Simultaneously, we observed increases in EGFR surface expression after ROM treatment in UM-UC-3 cells, but reduced cytotoxicity. Therefore, the increased cytotoxicity after DEC pretreatment cannot simply be explained by increased levels of target antigen expression.

It is well established that inhibitory immune checkpoints can regulate CAR T-cell cytotoxicity (37). We therefore analyzed whether treatment with DEC or ROM led to altered expression of PD-L1 or PD-L2. As shown in **Figure 4**, UM-UC-3 cells showed increased expression of PD-L1 and PD-L2 after ROM treatment compared to DEC or DMSO. This increase inversely correlated with the decrease in CAR T-cell cytotoxicity after ROM treatment. In contrast, DEC pretreatment of BFTC905 cells increased the PD-L1 and PD-L2, and ROM, the PD-L2 expression levels. Therefore, the higher expression of inhibitory checkpoint molecules did not explain the increased cytotoxicity observed after DEC but not after ROM pretreatment.

Recent work from Kantari-Mimoun et al. on CAR T-cell function indicated that the adhesion molecule ICAM-1 (intracellular adhesion receptor-1) might be involved in the recognition of malignant cells by CAR T-cells (38). ICAM-1 expression was significantly induced in all cell lines by DEC and in VM-CUB1 and BFTC905 cells also by ROM (**Figure 4**). While this increase may have contributed to enhanced CAR T-cell lysis, it did not appear to be an important determinant for the cytotoxicity, since DEC and ROM pretreatment both caused increased ICAM-1 expression in BFTC905 cells, whereas killing was only improved by DEC.

Finally, the FAS (CD95) and FASL axis is another mechanistic pathway by which CAR T-cells can mediate tumor cell killing (39). Accordingly, we detected a significant induction of CD95 expression after DEC treatment in RT-112, BFTC905, and HBLAK cells (**Figure 4**), which only partially explained the variations in the cytotoxicity curves (**Figure 3**).

Epigenetic Treatment Alters the Balance of Survival and Apoptosis Signaling in UCC

To identify other target cell factors that might contribute to the differences in CAR T-cell cytotoxicity, we performed next-generation RNA sequencing of BFTC905 and UM-UC-3 cells after treatment with DEC. High-throughput data from UM-UC-3 and VMCUB-1 cells treated with 3 nM ROM for 72 h were already available from an earlier publication (10) (GEO accession GSE70120).

Overall, 1,553 genes were differentially expressed in BFCT905 cells after 7 days of DEC treatment compared to only 927 genes in UM-UC-3 cells. A comparable number of genes was downregulated in both cell lines after DEC treatment (**Supplementary Figures S4A, B**). To identify immune response-associated factors that were altered by epidrugs, we developed a list of candidate factors (n=143) based on literature reviews (40). However, only few of these candidate genes significantly deviated in their expression upon DEC treatment (1.5-fold change, $p \leq 0.05$), and none of the genes with robust changes could easily explain the altered CAR T-cell killing efficacy (**Supplementary Figure S4C**).

To analyze the effect of DEC treatment in an unbiased way, we determined the overlap of differentially expressed genes between both cell lines and performed GO analysis on this gene set (**Supplementary Figure S4B**, **Figure 5A** and **Supplementary Tables S2–5**). This overlap of genes was surprisingly small, and enrichment in GOs could not explain differences in CAR T-cell killing (**Supplementary Tables S2–5**). However, we found intrinsic transcriptomic differences between BFTC905 and UM-UC-3 cells that could affect the balance between cell survival and apoptosis. Untreated BFTC905 cells had a more pronounced pro-survival and anti-apoptotic profile compared to UM-UC-3 cells. These findings could readily explain why untreated UM-UC-3 cells were better killed by CAR T-cells.

Using GSEA gene set analysis, we merged the candidate gene lists for PI3K-AKT survival and apoptosis signaling (extrinsic and intrinsic), where genes could be either induced or downregulated, depending on their pro- or anti-apoptotic functions, and displayed fold change expression after DEC and ROM treatment as heatmaps. As shown in **Supplementary Figure S5**, many genes that were induced or remained unchanged by DEC were downregulated by ROM and vice versa. Especially survival and apoptosis genes were differentially altered by DEC and ROM, and differences in gene expression between BFTC905 and UM-UC-3 cells became more obvious after DEC treatment. We next divided the differentially expressed apoptosis-related genes into two groups: Genes in the first group were expressed at lower levels in DMSO-treated

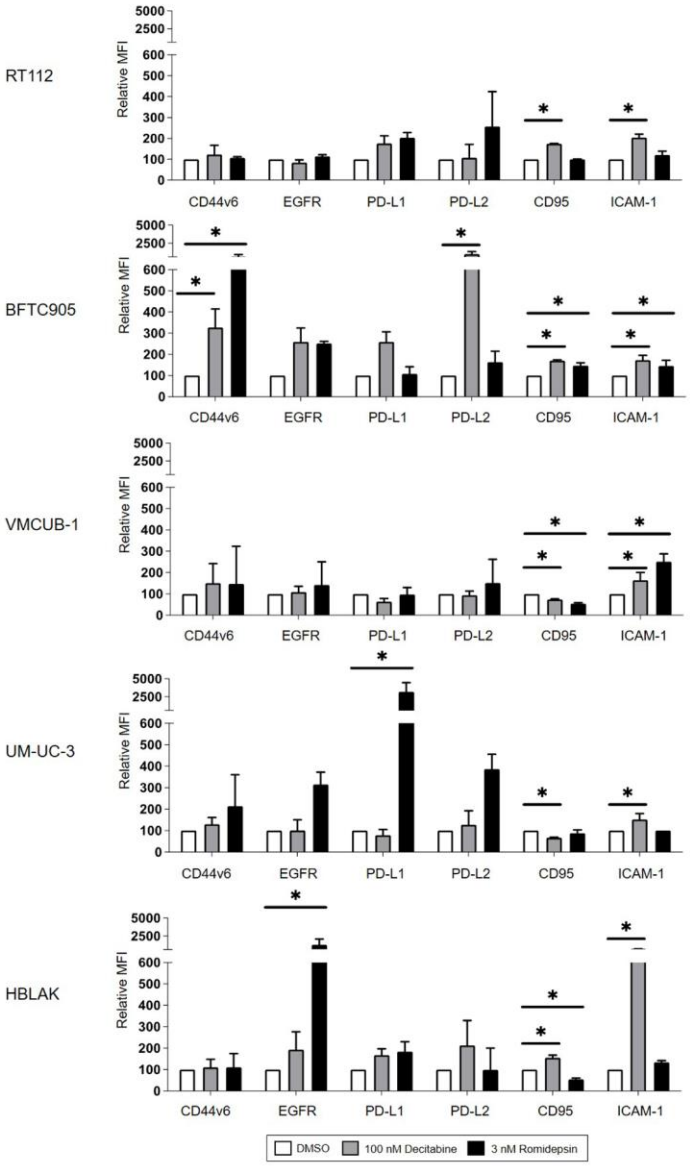


FIGURE 4 | Cell surface protein expression of CD44v6, EGFR, PD-L1, PD-L2, CD95, and ICAM-1 on target cells after DMSO, DEC, or ROM treatment. The graphs represent changes in median fluorescence intensity (MFI) of each marker normalized to DMSO control. White bars represent the values for DMSO-treated cells, and gray and black bars represent results of DEC- or ROM-treated cells, respectively. Results are shown as the mean (± SD) of at least three independent experiments; values of unstained cells are subtracted. p-values were calculated by means of two-way ANOVA and subsequent Dunnett's correction for multiple comparisons. Statistical significance (p-value ≤ 0.05) is denoted by asterisk.

BFTC905 compared to DMSO-exposed UM-UC-3 cells. Their induction by DEC might therefore be responsible for pro-apoptotic effects associated with the increased killing of UCC by CAR T-cells after DEC treatment. The second group included apoptosis genes that were at least twofold higher expressed in DMSO-treated BFTC905 cells compared to UM-UC-3 and therefore might exert anti-apoptotic functions, thus at least partially providing protection against CAR T-cell-mediated killing. In order to visualize the differences between the two cell lines and treatments in a bar diagram, the expression levels were normalized to the values of BFTC905 DMSO-treated cells. We also displayed the differentially expressed survival genes in a similar

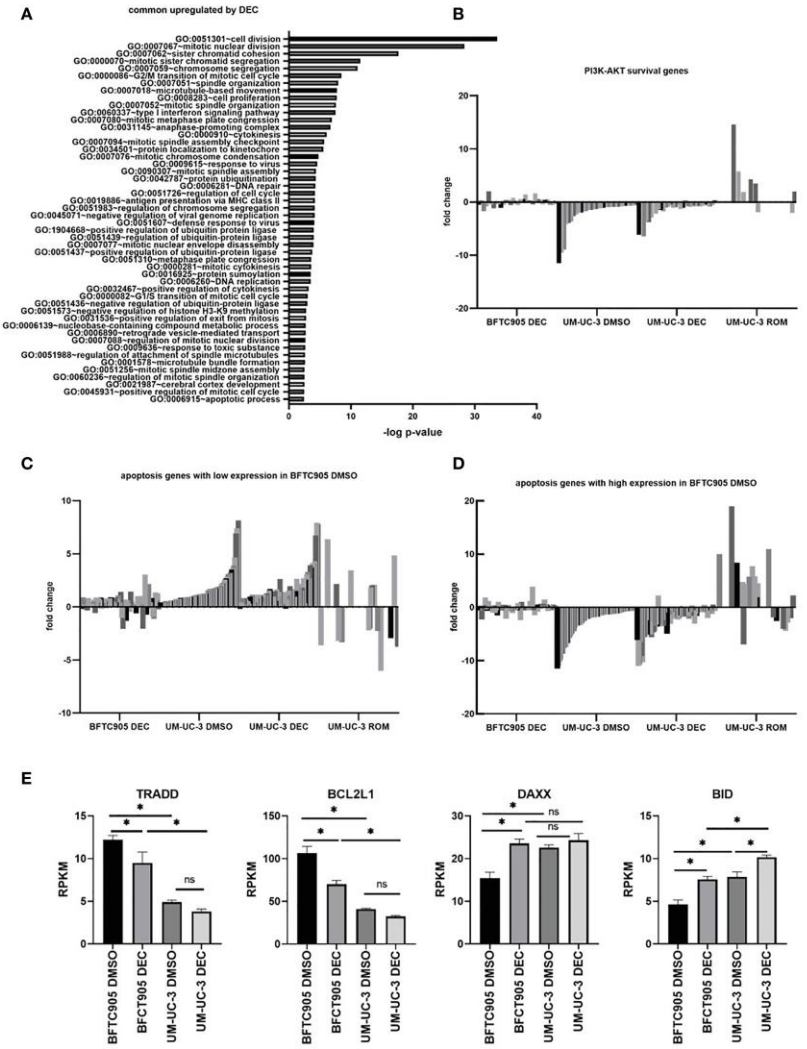


FIGURE 5 | Gene expression changes in UCC BFTC905 and UM-UC-3 after DEC treatment determined by RNA sequencing. **(A)** GO group analysis was performed for 207 genes commonly induced by DEC in both UCC (d7) using the online tool DAVID (34, 41). Only the GOs with p<0.004 are displayed. **(B)** Fold change expression of PI3K-AKT survival genes (shades of gray) in indicated samples relative to BFTC905 DMSO used for normalization. Gene lists were downloaded from the Broad Institute GSEA gene sets database (https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=CP : KEGG). Genes names and expression values are given in **Supplementary Table S6**. **(C)** Fold change expression of apoptosis genes in indicated samples relative to BFTC905 DMSO. Apoptosis genes with lower expression in BFTC905 DMSO compared to UM-UC-3 DMSO are displayed. See **Supplementary Table S7** for gene names and expression values. **(D)** Fold change expression of apoptosis genes in indicated samples relative to BFTC905 DMSO. Apoptosis genes with higher expression in BFTC905 DMSO compared to UM-UC-3 DMSO are displayed. See **Supplementary Table S8** for gene names and expression values. **(E)** Mean RPKM expression values from RNA sequencing analysis obtained from three replicates are visualized as bar graphs for the four apoptosis regulators that were chosen for further functional analysis by siRNA knockdown. Statistical significance (p-value <0.05) is denoted by asterisk and was determined by two-way ANOVA and Dunnett's correction for multiple comparisons. ns, not significant.

manner. Our analyses clearly demonstrated a difference between BFTC905 and UM-UC-3 cells with regard to expression of cell survival genes (**Figure 5B**, see **Supplementary Table S6** for gene names and detailed expression levels). Survival genes were more strongly expressed in BFTC905 cells compared to UM-UC-3 cells and only marginally induced by DEC in both cell lines. In contrast, pro-apoptotic genes, which were weakly expressed in untreated BFTC905 cells compared to UM-UC-3, were indeed upregulated by DEC treatment in both cell lines (**Figure 5C** and **Supplementary Table S7**), thus providing an explanation why this treatment was

associated with improved CAR T-cell killing of UCC. In contrast, ROM treatment of UM-UC-3 cells strongly induced pro-survival signaling genes (**Figure 5B**) and reduced some genes from the pro-apoptotic group (**Figure 5C**), which would explain the reduced CAR T-cell killing of ROM pre-treated UCC. The second group of apoptosis genes (**Figure 5D** and **Supplementary Table S8**) was more strongly expressed in untreated BFTC905 cells compared to UM-UC-3 cells, presumably protecting them from apoptosis. Many of them were downregulated by DEC in both UCC, thereby potentially facilitating cell death induction. These genes responded conversely to ROM, thus more likely protecting the cells from the CAR T-cell cytotoxicity.

Based on these *in silico* analyses, the different killing efficacies can be explained by differential changes in the balance between survival and apoptosis induced by the two epigenetic inhibitors. To mechanistically confirm the functional role of apoptosis regulators in determining the susceptibility of DEC-treated UCC toward CAR T-cell-induced cell death, we selected four candidates from the RNA sequencing data for further knockdown experiments (**Supplementary Tables S7, 8**). These candidates were chosen considering their regulatory function in intrinsic and extrinsic apoptosis signaling, their expression differences between untreated BFTC905 and UM-UC-3 cells, and also their response to DEC treatment. We chose two anti-apoptotic genes with higher expression in BFTC905 cells compared to UM-UC-3 that were reduced in expression by DEC, *TRADD* and *BCL2L1* with the latter encoding for BCLX (**Figure 5E**). Likewise, we chose two pro-apoptotic genes that were weakly expressed in BFTC905 compared to UM-UC-3 cells and that were induced by DEC, *DAXX* and *BID* (**Figure 5E**).

BCL2L1 and BID Are Important Target Cell Susceptibility Factors for CAR T-Cell Killing

Next, we performed siRNA knockdown of these four apoptosis regulators. We transfected UM-UC-3 cells with all four siRNAs pools as well as a pool of non-targeting control siRNAs. BFTC905 cells were only transfected with TRADD, BCL2L1, and the control siRNA pool, as the already low CAR T-cell cytotoxicity for unmodified BFTC905 (**Figure 3**) would not have permitted to reliably detect further diminished cytotoxic activity. The two siRNA-transfected UCC were submitted to CAR T-cell cytotoxicity assays and harvested on the same day for Western Blot analysis.

Cytotoxicity analysis demonstrated that knockdown of anti-apoptotic BCL2L1 mRNA (black rhombus) strongly increased the killing of UM-UC-3 and BFTC905 cells, while the TRADD knockdown (white square) had no effect (**Figures 6C, D, G, H**). Notably, while the pro-apoptotic DAXX knockdown (white circle) did not alter cytotoxicity of CAR T-cells towards UM-UC-3 cells (**Figures 6C, D**), the BID knockdown (green square) almost completely abolished the cytotoxicity of CAR T-cells for UM-UC-3 cells (**Figures 6C, D**). Negative controls were not significantly affected by knockdown (**Figures 6A, B, E, F**). Hence, two of the four candidate genes, *BCL2L1* and *BID*, were identified as essential determinants for the susceptibility of DEC-pretreated UCC towards CAR T-cell-induced cytotoxicity, thus providing a compelling new approach to influence the susceptibility of UCC for immunotherapy strategies. Western blot analysis revealed highly efficient knockdown for TRADD, BCL2L1, DAXX, and BID (**Figure 6I**).

DISCUSSION

Epigenetic changes are characteristic for all cancers and essential for accretion of the 10 properties of malignant cells proposed as hallmarks of cancer, which also includes anti-apoptotic signaling (42). Therefore, our main objective here was to investigate whether pretreatment of cancer cells with epidrugs improves the cytotoxicity and target specificity of immunotherapy with CAR T-cells as effector cells.

As the search for ideal target antigens to achieve specific antitumor activity still is a major challenge for solid cancers, we evaluated well-established surface molecules, EGFR and CD44v6, as possible target antigens for CAR T-cell therapy of UC. Both antigens are overexpressed on solid cancers (17, 19), and we already had constructed second-generation CARs in lentiviral vectors that efficiently killed human head and neck squamous cell carcinomas (29) (Haist et al. submitted). Using a set of UCC we demonstrated that EGFR was robustly expressed on all malignant UCC and that the expression levels were higher compared to non-malignant uroepithelial HBLAK cells. CD44v6 was also expressed on all cell lines, albeit with comparable levels between UCC and HBLAK. EGFR and CD44v6 CAR T-cells efficiently killed the cells of three UCC and also HBLAK, although the killing of cells of the non-malignant cell line was less efficient and required higher E:T ratios. We also noted that the cancer line BFTC905, even though it robustly expressed both target antigens, was only marginally killed by the EGFR and CD44v6 CAR T-cells from several donors. In conclusion, the killing efficacy of CAR T-cells did not correlate well with the target antigen expression patterns on the UC cell lines and therefore had to be influenced/determined by other mechanisms.

Multiple lines of evidence demonstrate that epidrugs like DNMTi (DEC) and HDACi (ROM) can exhibit profound immune-modulating effects on several levels (40, 43, 44), including both epigenetic modulation of the immunosuppressive tumor microenvironment as well as direct modulation of tumor cells. However, in order to clearly identify the cellular pathway in the tumor cells responsible for the susceptibility to CAR T-cells, we pretreated the UCC for several days with the epidrugs and then only later added the transduced immune effector cells for the overnight cytotoxic assays. Interestingly, DEC pretreatment increased the CAR T-cell cytotoxicity towards all UCC, but not towards the non-malignant HBLAK cells, thus suggesting a tumor-specific modulation of gene expression in malignant cells. This malignant cell-specific increased killing is an important finding here, as *on-target off-tumor* effects due to expression of the target antigen on normal cells is a well-recognized problem/side-effect of CAR T-cell therapy and poses a significant clinical challenge. Major strategies currently pursued to increase the tumor specificity of CAR T-cell therapy are the use of CARs with reduced affinity, which might not kill normal cells with lower

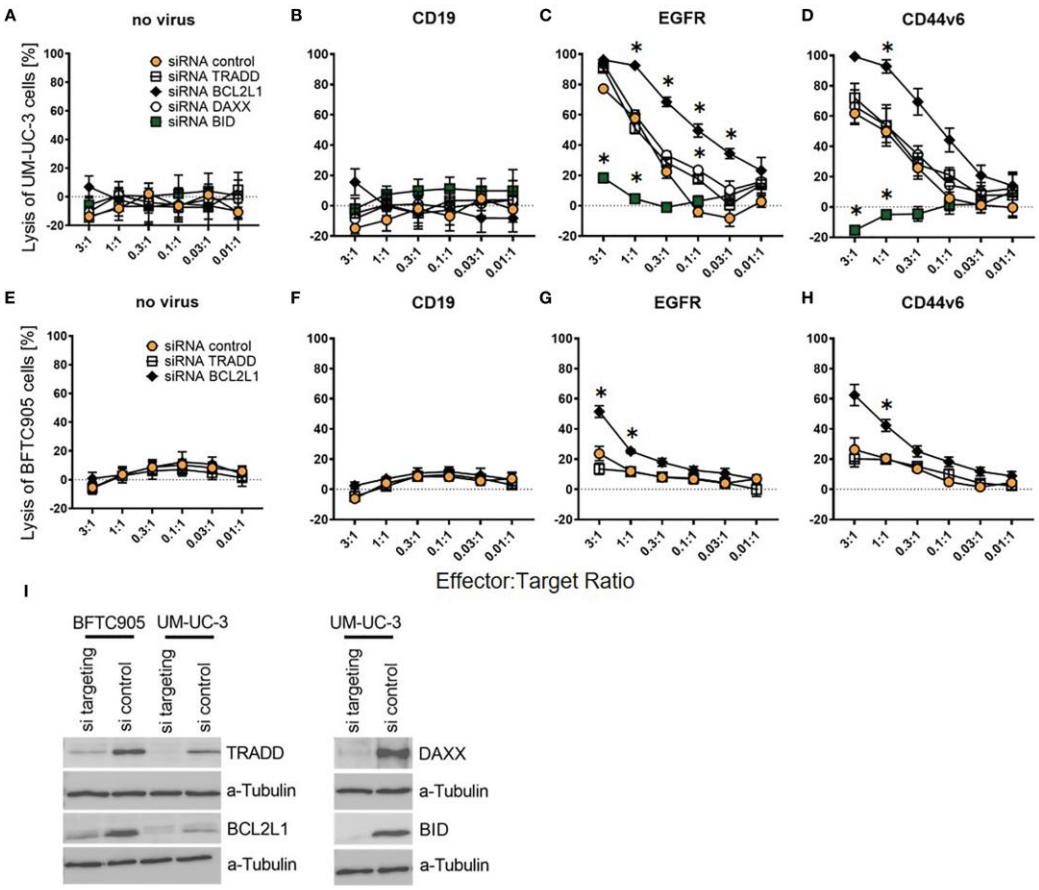


FIGURE 6 | Killing efficiency of EGFR and CD44v6 CAR T-cells against UM-UC-3 and BFTC905 cells after siRNA knockdown of apoptosis regulating genes. **(A)** Cytotoxicity of untransduced (no virus), CD19, EGFR, and CD44v6 CAR T-cells against UM-UC-3 **(A–D)** and BFTC905 **(E–H)** cells transfected with indicated siRNA was determined. Graphs depict the mean values (± SD) of three independent experiments. Values for different effector to target ratios (E:T ratio 3:1–0.01:1) are given. **(I)** Western Blot analysis of TRADD (34 kDa), BCL2L1 (26 kDa), BID (22 kDa), and DAXX (110 kDa) protein expression after the respective siRNA knockdown (si targeting) compared to control cells (si control). Statistical significance (p-value <0.05) is denoted by asterisk and was determined by two-way ANOVA and Dunnett's correction for multiple comparisons.

target antigen expression levels, or injection of high-affinity CAR T-cells directly into the tumor tissue (31, 45, 46). The specific effect of demethylating agents on malignant cells that we describe here might be another relatively simple way to increase the *on-tumor* effects of CAR T-cells. We have confirmed the DEC sensitizing effects with two different CARs, EGFR and CD44v6, for multiple UCC in 2D and for two also in 3D cultures, achieving comparable killing. Noteworthy is also that for some of the DEC-pretreated cell lines, the control T-cells (no virus, CD19) demonstrated slightly increased killing efficacy at higher effector-to-target ratios. This non-specific cytotoxicity most likely occurred due to the maximum stimulation of T-cells prior to transduction and was not caused by a direct effect of DEC, as due to passaging of pretreated UCC and also the short half-life of DEC in culture (24), the T-cells were never in contact with the compound.

We next wanted to decipher the mechanism responsible for the improved killing efficacy after DEC treatment in all UCC and to understand why untreated BFTC905 cells were hardly killed, despite their strong target antigen expression comparable to the other UCC. Therefore, we analyzed the expression of molecules that are known to influence the interaction between T-cells and cancer cells. Importantly, the expression of the two target antigens did not consistently increase in the examined cell lines and therefore could not explain the improved killing after DEC. Most strikingly, both target antigens increased under the epigenetic treatments in BFTC905 cells; however, killing was only improved by exposure to DEC and not to ROM. Although expression of PD-L1, PD-L2, and ICAM-1 increased after treatment with DEC, we could not detect a consistent correlation between CAR T-cell cytotoxicity and changes in the

protein expression levels as assessed by flow cytometry. T-cell cytotoxicity is partially mediated by the interaction between FAS ligand on T-cells and the death receptor FAS (CD95) on the malignant cells (39). FAS was similarly expressed on all UCC at baseline and further increased by DEC in individual cell lines. In particular, BFTC905 cells responded to both epigenetic inhibitors by increasing the protein expression, but were more sensitive to CAR T-cells after DEC and less after ROM treatment.

For a more unbiased screening approach to identify factors determining the susceptibility towards CAR T-cell cytotoxicity, we performed RNA sequencing analysis of two DEC-treated UCC, UM-UC-3 as a representative candidate for the effects of DEC and ROM and BFTC905 as outlier with almost no CAR T-cell killing at baseline. The GO term analysis demonstrated that DEC shifted the balance between survival/pro- and anti-apoptotic genes towards an expression profile that could facilitate the induction of cell death by cytotoxic CAR T-cells. ROM treatment shifted this balance into the opposite direction, thus providing a possible explanation for its poor effects on CAR T-cell killing efficacy. Although Yang et al. also reported divergent effects of DEC and ROM on apoptosis-related genes in metastatic human colon carcinoma cells (47), the impact of these substances on other cancer entities appeared to be cancer-type specific: ROM treatment induced G2/M phase arrest and apoptosis *via* activation of ERK-MAPK and JNK-MAPK pathways in hepatocellular cancer (48), while ROM induced apoptosis in non-small cell lung cancer cells by inhibition of RAF-MEK-ERK PI3K/AKT signaling and by downregulation of anti-apoptotic genes and upregulation of the pro-apoptotic BAX (49).

Based on our *in silico* analysis, we chose four genes that were strongly deregulated by DEC treatment and performed specific siRNA knockdown in BFTC905 and UM-UC-3 cells. The co-culture of treated cells with EGFR and CD44v6 CAR T-cells identified BCL2L1 and BID, both members of the BCL-2 family of death regulators, as key cellular factors whose modulation can ameliorate the T-cell cytotoxicity towards UCC. Mechanistically, pretreatment of UCC with DEC induced the expression of the pro-apoptotic BID, which can counterbalance the function of anti-apoptotic BCL-2 like proteins (50), thus promoting increased cytotoxicity of CAR T-cells. Concurringly, knockdown of BID in the target cells by siRNA rendered them resistant to CAR T-cells. In addition, DEC reduced the expression of anti-apoptotic BCL2L1 (also known as BCLX), thereby further altering the balance towards apoptosis induction by CAR T-cells. Concurringly, siRNA knockdown of BCL2L1 improved the CAR T-cell cytotoxicity similar to DEC pretreatment of UCC, particularly of BFTC905 cells.

Due to our study design, the CAR T-cells were not exposed to DEC. This is reflected by the findings that the cytokine profiles in the supernatants of the spheroid experiments were similar for the DEC-treated and untreated cells, when using the CAR T-cells with *on-target* CARs. Although BFTC905 cells were not efficiently killed, the induction of granzyme B and TNFa over the control wells clearly indicated that the EGFR and CD44v6 CAR T-cells specifically recognized their target antigens. Obviously, pretreatment of tumor cells with DEC prior to CAR T-cell therapy does not reflect the *in vivo* situation in patients, as epidrugs will also directly affect the CAR T-cells themselves and indirectly will alternate the whole tumor microenvironment (TME) (7). However, based on the mechanistic study performed here, demonstrating that DEC treatment sensitizes UC tumor cells towards CAR T-cell cytotoxicity, the combination of both approaches appears attractive. Importantly, the effects of low-dose DEC treatment on CAR T-cells were already reported to be promising, namely, augmenting cytokine production and increased lytic antitumor activity of CAR T-cells *in vitro* and *in vivo*. In addition, DEC induces the expression of memory-related genes and reduction of exhaustion-related genes. Inhibition of epigenetic modifiers is also associated with long-persisting CAR T-cells due to enrichment of a memory-like phenotype (7, 51). Finally, low-dose DEC treatment may also positively influence the TME *via*, e.g., the induction of chemokines like CXCL9 and -10, thereby promoting T-cell infiltration (52), or *via* the decrease of immunosuppressive cells in the microenvironment like myeloid-derived suppressor cells (MDSCs) (53). Thus, systemic low-dose DEC treatment, such as already used for MDS treatment (13), will most likely have benefits *in vivo* beyond the direct influence on the UC.

In conclusion, our study provides an exciting rationale for combining DEC with CAR T-cell therapy for bladder cancer. To our knowledge, only one study using murine solid cancer cells reported that siRNA interference with BCL-2, BCLXL, or BAX can determine the sensitivity of solid tumor cells towards cytotoxicity of T-cells (54). Interestingly, loss of BID, together with FADD (Fas associated *via* death domain) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), was reported to contribute to resistance of lymphoma cells to CD19 CAR T-cells (55). Nevertheless, considering the role of BCL-2 family members in B-cell lymphoma therapy (56), it is not surprising that combinations of CAR T-cell therapy with compounds targeting apoptosis regulators, like BH3 mimetics (e.g., ABT-737) or pan-BCL-2 inhibitors, are currently discussed for mature B-cell malignancies. Our results provide a rationale for extending this combinatorial approach to solid cancers in the future, especially if members of the BCL-2 family are dysregulated in the malignant cells (50, 57).

DATA AVAILABILITY STATEMENT

Next generation sequence data is available *via* the GEO repository (accession number GSE164862). All other data relevant to this study is included in the article or uploaded as **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CG, CH, MH, HH, GN, and EN contributed to conception and design of the study. CG, CH, MH, CK, AB, PP, and KK performed experiments and statistical analyses. CG wrote the first draft of the manuscript. MH, CH, HH, GN, CW, and KS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported, in part, by the Foschungskommission of the Medical Faculty of the Heinrich Heine University Duesseldorf to CG, the Duesseldorf School of Oncology DSO to AB, and the European Regional Development Fund (EFRE-0801320, iCAN33) to HH.

ACKNOWLEDGMENTS

Computational support of the Zentrum für Informations- und Medientechnologie, especially the HPC team (High Performance Computing) at the Heinrich-Heine University, is acknowledged. The authors would like to thank Björn Stork for the very helpful discussions on regulation of apoptosis. Technical assistance by Christiane Hader is also acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.782448/full#supplementary-material

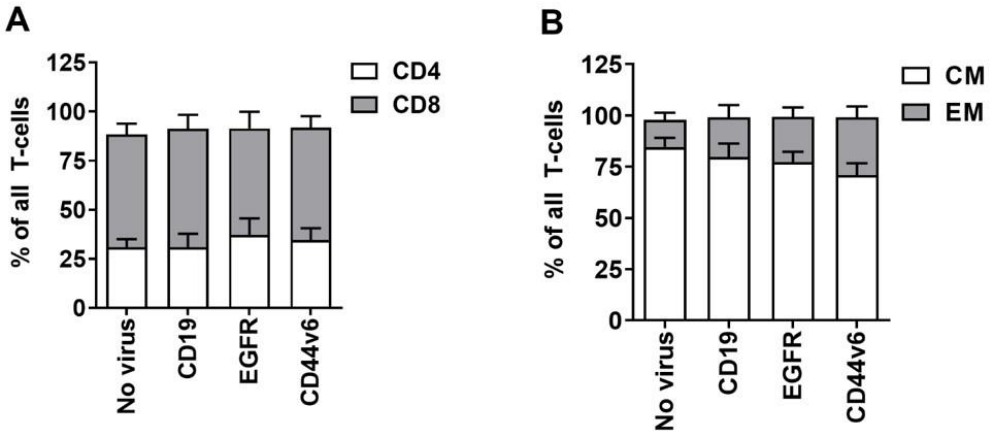
REFERENCES

1. Esfahani K, Roudaia L, Buhlaiga N, Del Rincon SV, Papneja N, Miller WH Jr. A Review of Cancer Immunotherapy: From the Past, to the Present, to the Future. *Curr Oncol* (2020) 27:S87–97. doi: 10.3747/co.27.5223
2. Lindner SE, Johnson SM, Brown CE, Wang LD. Chimeric Antigen Receptor Signaling: Functional Consequences and Design Implications. *Sci Adv* (2020) 6:eaaz3223. doi: 10.1126/sciadv.aaz3223
3. Alexander M, Culos K, Roddy J, Shaw JR, Bachmeier C, Shigle TL, et al. Chimeric Antigen Receptor T Cell Therapy: A Comprehensive Review of Clinical Efficacy, Toxicity, and Best Practices for Outpatient Administration. *Transplant Cell Ther* (2021) 27:558–70. doi: 10.1016/j.jtct.2021.01.014
4. Watanabe K, Kuramitsu S, Posey AD Jr, June CH. Expanding the Therapeutic Window for CAR T Cell Therapy in Solid Tumors: The Knowns and Unknowns of CAR T Cell Biology. *Front Immunol* (2018) 9:2486. doi: 10.3389/fimmu.2018.02486
5. Porten SP. Epigenetic Alterations in Bladder Cancer. *Curr Urol Rep* (2018) 19:102. doi: 10.1007/s11934-018-0861-5
6. Jones PA, Issa JP and Baylin S. Targeting the Cancer Epigenome for Therapy. *Nat Rev Genet* (2016) 17:630–41. doi: 10.1038/nrg.2016.93
7. You L, Han Q, Zhu L, Zhu Y, Bao C, Yang C, et al. Decitabine-Mediated Epigenetic Reprograming Enhances Anti-Leukemia Efficacy of CD123-Targeted Chimeric Antigen Receptor T-Cells. *Front Immunol* (2020) 11:1787. doi: 10.3389/fimmu.2020.01787
8. Watson CJ, O'Kane H, Maxwell P, Sharaf O, Petak I, Hyland PL, et al. Identification of a Methylation Hotspot in the Death Receptor Fas/CD95 in Bladder Cancer. *Int J Oncol* (2012) 40:645–54. doi: 10.3892/ijo.2011.1250
9. Grunewald CM, Schulz WA, Skowron MA, Hoffmann MJ, Niegisch G. Tumor Immunotherapy—The Potential of Epigenetic Drugs to Overcome Resistance. *Trans Cancer Res* (2018) 7:1151–60. doi: 10.21037/tcr.2018.06.24
10. Pinkerneil M, Hoffmann MJ, Deenen R, Köhrer K, Arent T, Schulz WA, et al. Inhibition of Class I Histone Deacetylases 1 and 2 Promotes Urothelial Carcinoma Cell Death by Various Mechanisms. *Mol Cancer Ther* (2016) 15:299–312. doi: 10.1158/1535-7163.MCT-15-0618
11. Faleiro I, Leao R, Binnie A, de Mello RA, Maia AT, Castelo-Branco P. Epigenetic Therapy in Urologic Cancers: An Update on Clinical Trials. *Oncotarget* (2017) 8:12484–500. doi: 10.18632/oncotarget.14226
12. Tsai H, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient Low Doses of DNA-Demethylating Agents Exert Durable Antitumor Effects on Hematological and Epithelial Tumor Cells. *Cancer Cell* (2012) 21:430–46. doi: 10.1016/j.ccr.2011.12.029
13. Jabbour E, Short NJ, Montalban-Bravo G, Huang X, Bueso-Ramos C, Qiao W, et al. Randomized Phase 2 Study of Low-Dose Decitabine *vs* Low-Dose Azacitidine in Lower-Risk MDS and MDS/MPN. *Blood* (2017) 130:1514–22. doi: 10.1182/blood-2017-06-788497
14. Li W, Wang Y, Tan S, Rao Q, Zhu T, Huang G, et al. Overexpression of Epidermal Growth Factor Receptor (EGFR) and HER-2 in Bladder Carcinoma and Its Association With Patients' Clinical Features. *Med Sci Monit* (2018) 24:7178–85. doi: 10.12659/MSM.911640
15. James ND, Pirrie S, Liu W, Ford D, Zarkar A, Southgate E, et al. A Phase I/II Feasibility Study of Cetuximab With 5FU and Mitomycin C or Cisplatin With Concurrent Radiotherapy in Muscle Invasive Bladder Cancer. *J Clin Oncol* (2020) 38(6_suppl):491–1. doi: 10.1200/JCO.2020.38.6_suppl.491
16. Wong Y, Litwin S, Vaughn D, Cohen S, Plimack ER, Lee J, et al. Phase II Trial of Cetuximab With or Without Paclitaxel in Patients With Advanced Urothelial Tract Carcinoma. *J Clin Oncol* (2012) 30:3545–51. doi: 10.1200/JCO.2012.41.9572
17. Heider KH, Kuthan H, Stehle G, Munzert G. CD44v6: A Target for Antibody-Based Cancer Therapy. *Cancer Immunol Immunother* (2004) 53:567–79. doi: 10.1007/s00262-003-0494-4
18. Anand V, Khandelwal M, Appunni S, Gupta N, Seth A, Singh P, et al. CD44 Splice Variant (CD44v3) Promotes Progression of Urothelial Carcinoma of Bladder Through Akt/ERK/STAT3 Pathways: Novel Therapeutic Approach. *J Cancer Res Clin Oncol* (2019) 145:2649–61. doi: 10.1007/s00432-019-03024-9
19. Yano S, Kondo K, Yamaguchi M, Richmond G, Hutchison M, Wakeling A, et al. Distribution and Function of EGFR in Human Tissue and the Effect of EGFR Tyrosine Kinase Inhibition. *Anticancer Res* (2003) 23:3639–50.
20. Hoffmann MJ, Koutsogiannouli E, Skowron MA, Pinkerneil M, Niegisch G, Brandt A, et al. The New Immortalized Uroepithelial Cell Line HBLAK Contains Defined Genetic Aberrations Typical of Early Stage Urothelial Tumors. *Bladder Cancer* (2016) 2:449–63. doi: 10.3233/BLC-160065
21. Lang A, Yilmaz M, Hader C, Murday S, Kunz X, Wagner N, et al. Contingencies of UTX/KDM6A Action in Urothelial Carcinoma. *Cancers (Basel)* (2019) 11:481. doi: 10.3390/cancers11040481
22. Wiek C, Schmidt EM, Roellecke K, Freund M, Nakano M, Kelly EJ, et al. Identification of Amino Acid Determinants in CYP4B1 for Optimal Catalytic Processing of 4-Ipomeanol. *Biochem J* (2015) 465:103–14. doi: 10.1042/BJ20140813
23. Schmidt EM, Wiek C, Parkinson OT, Roellecke K, Freund M, Gombert M, et al. Characterization of an Additional Splice Acceptor Site Introduced Into CYP4B1 in Hominoidae During Evolution. *PloS One* (2015) 10:e0137110. doi: 10.1371/journal.pone.0137110
24. Liu Z, Marcucci G, Byrd JC, Grever M, Xiao J, Chan KK. Characterization of Decomposition Products and Preclinical and Low Dose Clinical Pharmacokinetics of Decitabine (5-Aza-2'-Deoxycytidine) by a New Liquid Chromatography/Tandem Mass Spectrometry Quantification Method. *Rapid Commun Mass Spectrom* (2006) 20:1117–26. doi: 10.1002/rcm.2423
25. Nakajima H, Kim YB, Terano H, Yoshida M, Horinouchi S. FR901228, A Potent Antitumor Antibiotic, Is a Novel Histone Deacetylase Inhibitor. *Exp Cell Res* (1998) 241:126–33. doi: 10.1006/excr.1998.4027
26. Roellecke K, Virts EL, Einholz R, Edson KZ, Altvater B, Rossig C, et al. Optimized Human CYP4B1 in Combination With the Alkylator Prodrug 4-Ipomeanol Serves as a Novel Suicide Gene System for Adoptive T-Cell Therapies. *Gene Ther* (2016) 23:615–26. doi: 10.1038/gt.2016.38
27. Roessig C, Scherer SP, Baer A, Vormoor J, Rooney CM, Brenner MK, et al. Targeting CD19 With Genetically Modified EBV-Specific Human T Lymphocytes. *Ann Hematol* (2002) 81(Suppl 2):S42–43.

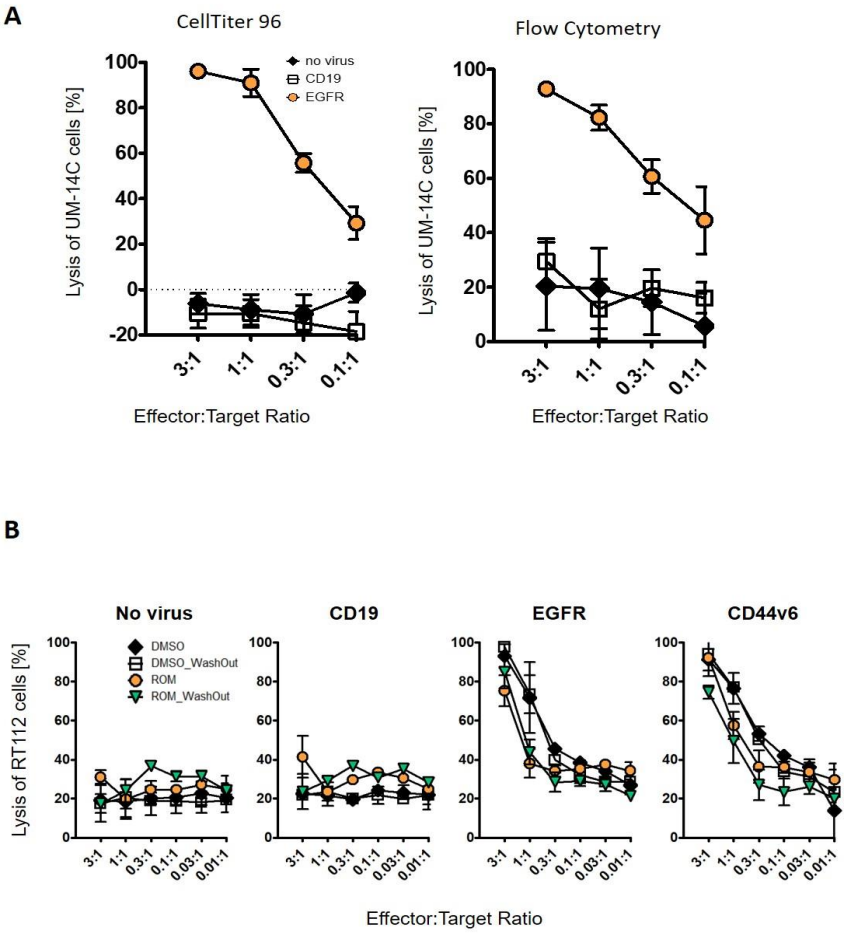
28. Rischer M, Pscherer S, Duwe S, Vormoor J, Jürgens H, Rossig C. Human Gammadelta T Cells as Mediators of Chimaeric-Receptor Redirected Anti-Tumour Immunity. *Br J Haematol* (2004) 126:583–92. doi: 10.1111/j.1365-2141.2004.05077.x
29. Haist C, Schulte E, Bartels N, Bister A, Poschinski Z, Ibach T, et al. CD44v6-Targeted CAR T-Cells Specifically Eliminate CD44 Isoform 6 Expressing Head/Neck Squamous Cell Carcinoma Cells. *Oral Oncol* (2021) 116:105259. doi: 10.1016/j.oraloncology.2021.105259
30. Ayoub D, Jabs W, Resemann A, Evers W, Evans C, Main L, et al. Correct Primary Structure Assessment and Extensive Glyco-Profiling of Cetuximab by a Combination of Intact, Middle-Up, Middle-Down and Bottom-Up ESI and MALDI Mass Spectrometry Techniques. *MAbs* (2013) 5:699–710. doi: 10.4161/mabs.25423
31. Caruso HG, Hurton LV, Najjar A, Rushworth D, Ang S, Olivares S, et al. Tuning Sensitivity of CAR to EGFR Density Limits Recognition of Normal Tissue While Maintaining Potent Antitumor Activity. *Cancer Res* (2015) 75:3505–18. doi: 10.1158/0008-5472.CAN-15-0139
32. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA, et al. Colocalization of Retrovirus and Target Cells on Specific Fibronectin Fragments Increases Genetic Transduction of Mammalian Cells. *Nat Med* (1996) 2:876–82. doi: 10.1038/nm0896-876
33. Oliveros JC. *Venny. An Interactive Tool for Comparing Lists With Venn's Diagrams*. (2007). Available at: https://bioinfogp.cnb.csic.es/tools/venny_old/venny.php.
34. Huang D, Sherman BT, Lempicki RA. Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nat Protoc* (2009) 4:44–57. doi: 10.1038/nprot.2008.211
35. Golubovskaya V, Wu L. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers (Basel)* (2016) 8:36. doi: 10.3390/cancers8030036
36. Zanoni M, Cortesi M, Zamagni A, Arienti C, Pignatta S, Tesei A. Modeling Neoplastic Disease With Spheroids and Organoids. *J Hematol Oncol* (2020) 13:97. doi: 10.1186/s13045-020-00931-0
37. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, et al. Human CAR T Cells With Cell-Intrinsic PD-1 Checkpoint Blockade Resist Tumor-Mediated Inhibition. *J Clin Invest* (2016) 126:3130–44. doi: 10.1172/JCI83092
38. Kantari-Mimoun C, Barrin S, Vimeux L, Haghiri S, Gervais C, Joaquina S, et al. CAR T Cell Entry Into Tumor Islets Is a Two-Step Process Dependent on IFNγ and ICAM-1. *Cancer Immunol Res* (2020). doi: 10.1101/2020.05.27.119198
39. Benmebarek MR, Karches CH, Cadilha BL, Lesch S, Endres S, Kobold S. Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. *Int J Mol Sci* (2019) 20:1283. doi: 10.3390/ijms20061283
40. Li H, Chiappinelli KB, Guzzetta AA, Easwaran H, Yen RC, Vatapalli R, et al. Immune Regulation by Low Doses of the DNA Methyltransferase Inhibitor 5-Azacitidine in Common Human Epithelial Cancers. *Oncotarget* (2014) 5:587–98. doi: 10.18632/oncotarget.1782
41. Huang D, Sherman BT, Lempicki RA. Bioinformatics Enrichment Tools: Paths Toward the Comprehensive Functional Analysis of Large Gene Lists. *Nucleic Acids Res* (2009) 37:1–13. doi: 10.1093/nar/gkn923
42. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
43. Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, et al. Inhibiting DNA Methylation Causes an Interferon Response in Cancer *via* Dsrna Including Endogenous Retroviruses. *Cell* (2017) 169:361. doi: 10.1016/j.cell.2017.03.036
44. Mazzone R, Zwergel C, Mai A, Valente S. Epi-Drugs in Combination With Immunotherapy: A New Avenue to Improve Anticancer Efficacy. *Clin Epigenet* (2017) 9:59. doi: 10.1186/s13148-017-0358-y
45. van Schalkwyk MC, Papa SE, Jeannon JP, Guerrero Urbano T, Spicer JF, Maher J, et al. Design of a Phase I Clinical Trial to Evaluate Intratumoral Delivery of Erbb-Targeted Chimeric Antigen Receptor T-Cells in Locally Advanced or Recurrent Head and Neck Cancer. *Hum Gene Ther Clin Dev* (2013) 24:134–42. doi: 10.1089/humc.2013.144
46. Brudno JN, Lam N, Vanasse D, Shen YW, Rose JJ, Rossi J, et al. Safety and Feasibility of Anti-CD19 CAR T Cells With Fully Human Binding Domains in Patients With B-Cell Lymphoma. *Nat Med* (2020) 26:270–80. doi: 10.1038/s41591-019-0737-3
47. Yang D, Torres CM, Bardhan K, Zimmerman M, McGaha TL, Liu K, et al. Decitabine and Vorinostat Cooperate to Sensitize Colon Carcinoma Cells to Fas Ligand-Induced Apoptosis *In Vitro* and Tumor Suppression *In Vivo*. *J Immunol* (2012) 188:4441–9. doi: 10.4049/jimmunol.1103035
48. Sun WJ, Huang H, He B, Hu DH, Li PH, Yu YJ, et al. Romidepsin Induces G2/M Phase Arrest *via* Erk/Cdc25c/Cdc2/Cyclinb Pathway and Apoptosis Induction Through JNK/C-Jun/Caspase3 Pathway in Hepatocellular Carcinoma Cells. *Biochem Pharmacol* (2017) 127:90–100. doi: 10.1016/j.bcp.2016.12.008
49. Yu XD, Wang SY, Chen GA, Hou CM, Zhao M, Hong JA, et al. Apoptosis Induced by Depsipeptide FK228 Coincides With Inhibition of Survival Signaling in Lung Cancer Cells. *Cancer J* (2007) 13:105–13. doi: 10.1097/PPO.0b013e318046eedc
50. Roberts AW, Huang D. Targeting BCL2 With BH3 Mimetics: Basic Science and Clinical Application of Venetoclax in Chronic Lymphocytic Leukemia and Related B Cell Malignancies. *Clin Pharmacol Ther* (2017) 101:89–98. doi: 10.1002/cpt.553
51. Wang Y, Tong C, Dai H, Wu Z, Han X, Guo Y, et al. Low-Dose Decitabine Priming Endows CAR T Cells With Enhanced and Persistent Antitumour Potential *via* Epigenetic Reprogramming. *Nat Commun* (2021) 12:409. doi: 10.1038/s41467-020-20696-x
52. Peng D, Kryczek I, Nagarsheth N, Zhao L, Wei S, Wang W, et al. Epigenetic Silencing of TH1-Type Chemokines Shapes Tumour Immunity and Immunotherapy. *Nature* (2015) 527:249–53. doi: 10.1038/nature15520
53. Mikyskova R, Indrova M, Vlkova V, Bieblova J, Simova J, Parackova Z, et al. DNA Demethylating Agent 5-Azacytidine Inhibits Myeloid-Derived Suppressor Cells Induced by Tumor Growth and Cyclophosphamide Treatment. *J Leukoc Biol* (2014) 95:743–53. doi: 10.1189/jlb.0813435
54. Bauer C, Hees C, Sterzik A, Bauernfeind F, Mak'Anyengo R, Duewell P, et al. Proapoptotic and Antiapoptotic Proteins of the Bcl-2 Family Regulate Sensitivity of Pancreatic Cancer Cells Toward Gemcitabine and T-Cell-Mediated Cytotoxicity. *J Immunother* (2015) 38:116–26. doi: 10.1097/CJI.0000000000000073
55. Larson RC, Maus MV. Recent Advances and Discoveries in the Mechanisms and Functions of CAR T Cells. *Nat Rev Cancer* (2021) 21:145–61. doi: 10.1038/s41568-020-00323-z
56. Wu H, Medeiros LJ and Young KH. Apoptosis Signaling and BCL-2 Pathways Provide Opportunities for Novel Targeted Therapeutic Strategies in Hematologic Malignances. *Blood Rev* (2018) 32:8–28. doi: 10.1016/j.blre.2017.08.004
57. Karlsson H. Approaches to Augment CAR T-Cell Therapy by Targeting the Apoptotic Machinery. *Biochem Soc Trans* (2016) 44:371–6. doi: 10.1042/BST20150253

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.

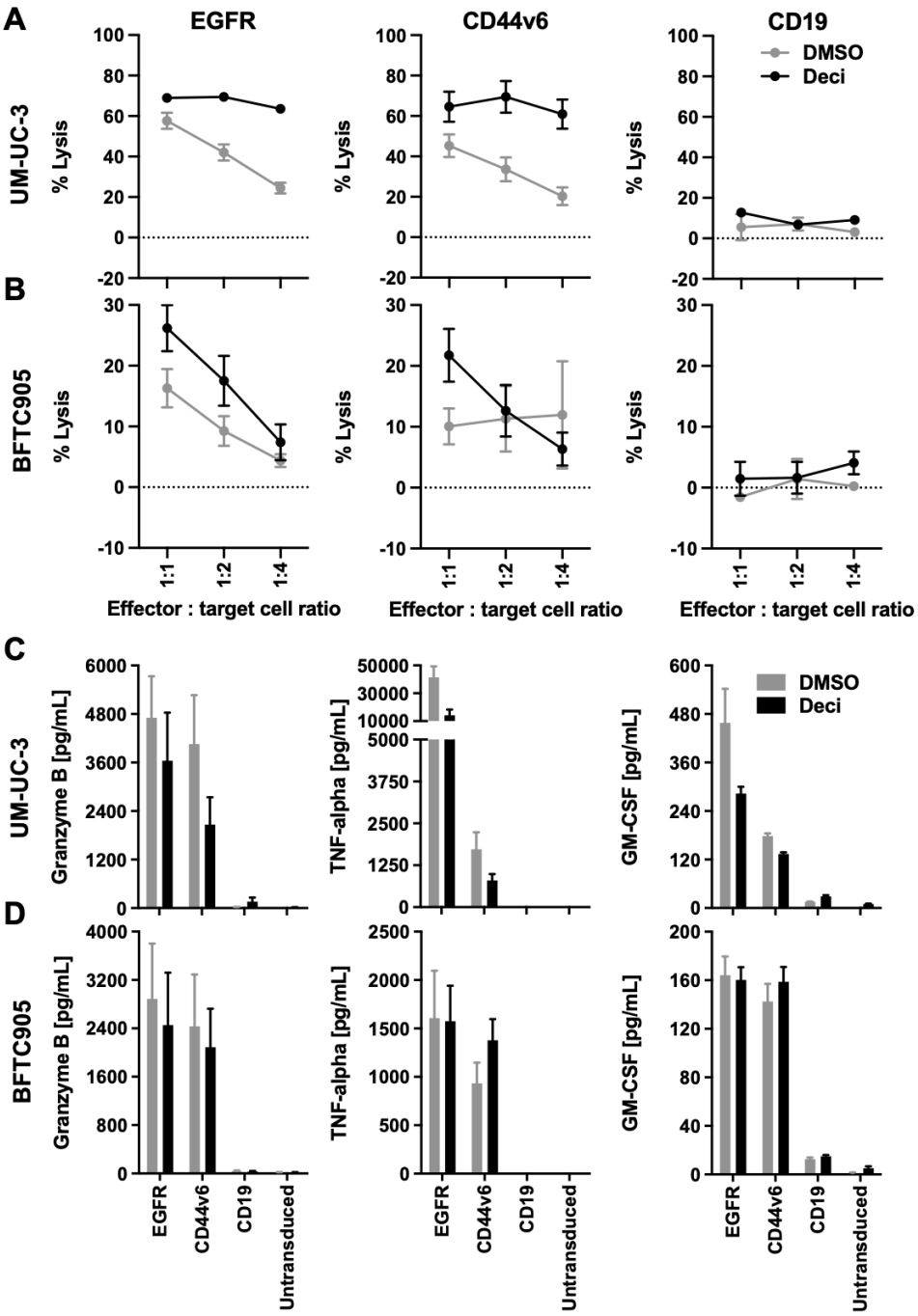
Supplementary Figures



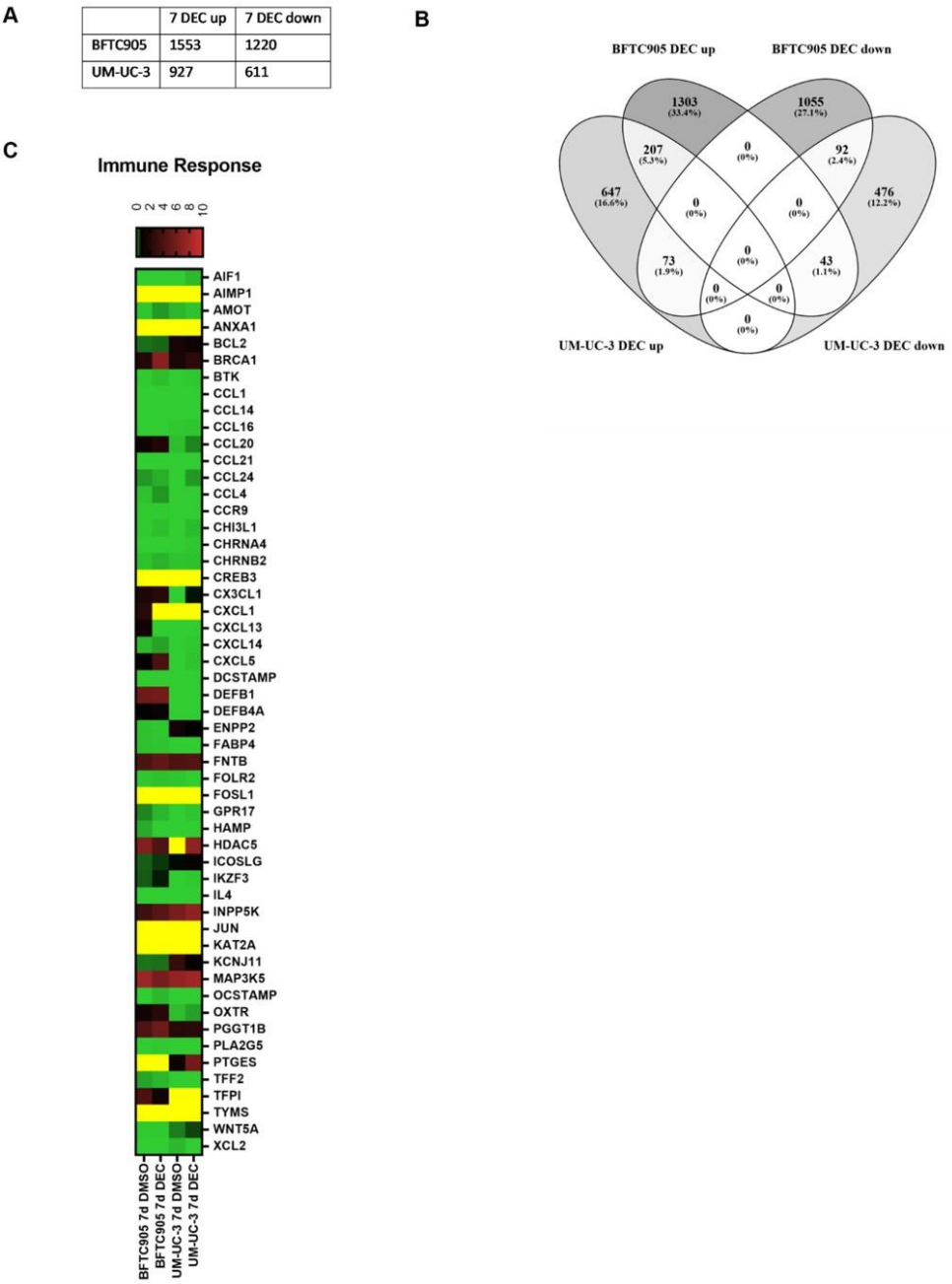
Supplementary Figure S1. Phenotype characterization of CAR and untransduced T-cells. MACS-enriched CAR and untransduced T-cells were flow cytometrically analyzed for (A) helper (CD4), cytotoxic (CD8), (B) central memory (CM, CD62L+ CD45RO+) and effector memory (EM, CD62L-CD45RO+) phenotype. Percentage values are represented as mean ± SEM.



Supplementary Figure S2. Validation experiments for experimental setup. (A) To determine the assay of choice for measuring CAR T-cell induced cytotoxicity results of viability assay (left panel) were compared with flow cytometry measurements (right panel). Graphs depict the mean values (±SD) for percentage of lysed cells. Black rhombus represents untransduced control, white square symbols represent the values CD19 controls, orange circle values for EGFR CAR. (B) To check for any effects of residual ROM on depicted CAR T- cells, ROM pretreated RT112 were either washed (green triangle) or not washed (orange circle) prior to co-culture with CAR T-cells. DMSO treated controls were handled likewise (white square and black rhombus). Graphs depict the mean values (±SD) for percentage of lysed cells.

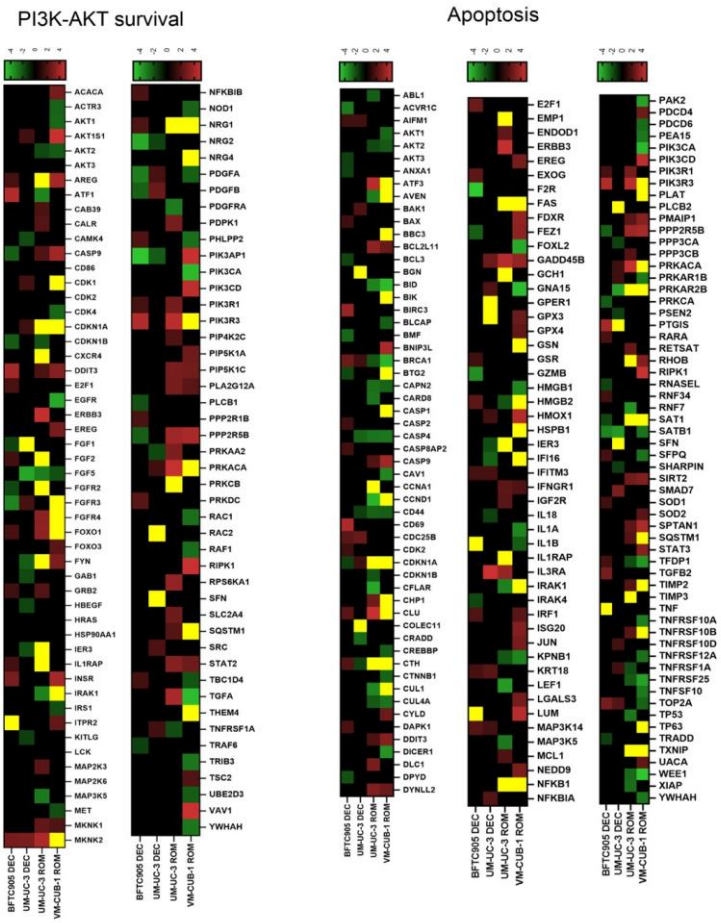


Supplementary Figure S3. CAR T-cell cytotoxicity and cytokine secretion against UM-UC-3 and BFTC905 spheroids. 100 nM DEC (black) and DSMO (grey) pretreated UM-UC-3 and BFTC905 cells were seeded in ultra-low attachment U-bottom 96-well plates. After overnight spheroid formation, CD19-, EGFR- or CD44v6 CAR T-cells or untransduced T-cells were added and cells were co-cultured for 16 h. Subsequently, (A) lysis was determined by CellTiter-Glo® 3D Viability assay and (B) Granzyme B, TNF-alpha and GM-CSF secretion of the 1:1 ratio by MACSPlex Cytotoxic T/NK cell assay. Graphs depict mean values ±SEM from at least three independent experiments.



Supplementary Figure S4. DEC induced differential gene expression determined by RNA sequencing. (A) Number of statistical significantly differentially expressed genes seven days after treatment with DEC compared to DMSO controls. As a cut-off we applied ≥ 1.5 fold-change and p ≤0.05 after Bonferroni adjustment. (B) Commonly altered genes between the two cell lines were identified by Venn diagram analysis using the online tool Venny 2.0. (C) RPKM expression of immune response related genes in untreated and DEC treated BFTC905 and UM-UC-3 cells displayed as

heatmap. Gene lists were downloaded from the Broad Institute GSEA gene sets database [https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=CP:KEGG]: GO:0070098~chemokine-mediated signaling pathway, GO:0002548~monocyte chemotaxis, GO:0071356~cellular response to tumor necrosis factor, GO:0048247~lymphocyte chemotaxis, GO:0071346~cellular response to interferon-gamma, GO:0034097~response to cytokine, GO:0042113~B cell activation, GO:0006935~chemotaxis, GO:0071347~cellular response to interleukin-1. Green colored boxes indicate low expression and red colored boxes indicate high expression. Genes with expression out of range are marked in yellow.



Supplementary Figure S5. Heatmap display of differential gene expression between untreated and epidrug treated UCC with regard to survival and apoptosis signaling. Differentially expressed genes after indicated treatment (fold-change ≥1.5) were checked for abundance in gene lists downloaded from GSEA gene set database associated with PI3K-AKT (A) and apoptosis signaling (B): HALLMARK APOPTOSIS, REACTOME EXTRINSIC PATHWAY FOR APOPTOSIS, INDUCTION OF APOPTOSIS BY EXTRACELLULAR SIGNALS, INDUCTION OF APOPTOSIS BY INTRACELLULAR SIGNALS, HALLMARK PI3K AKT MTOR SIGNALING. Fold change expression values were displayed as a heatmap with green colored boxes indicating low expression and red colored boxes indicating high expression. Genes with expression out of range are marked in yellow. Differentially expressed genes in UM-UC-3 and VM-CUB1 cells after treatment with 3 nM ROM for 72 h from our previous study (GEO accession GSE70120) were included for direct comparison.

Optimierung der Einzelketten Antikörperfragmente von Cetuximab für die CAR-T-Zelltherapie gegen HNSCC

Titel: Single-chain variable fragment of cetuximab engineered for CAR T-cell therapy against head and neck squamous cell carcinomas

Autoren: **Corinna Haist**, Zoe Poschinski, Arthur Bister, Katja Geipel, Michèle J. Hoffmann, Alexandra Hamacher, Matthias Kassack, Constanze Wiek, Kathrin Scheckenbach*, Helmut Hanenberg*

* Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen

Eigenleistung: 60 %

- Planung, Durchführung und Auswertung der Experimente, Schreiben und Korrektur des Manuskripts

Veröffentlicht in: Oral Oncology

Impact Factor: 5,337

Status: veröffentlicht

DOI: 10.1016/j.oraloncology.2022.105867

Oral Oncology 129 (2022) 105867

Contents lists available at ScienceDirect

Oral Oncology

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

Engineering a single-chain variable fragment of cetuximab for CAR T-cell therapy against head and neck squamous cell carcinomas

Corinna Haist [a,b], Zoe Poschinski [a], Arthur Bister [a,b], Michèle J. Hoffmann [c], Camilla M. Grunewald [c], Alexandra Hamacher [d], Matthias Kassack [d], Constanze Wiek [a], Kathrin Scheckenbach [a,1], Helmut Hanenberg [a,b,1,*]

[a] Department of Otorhinolaryngology, Head & Neck Surgery, Heinrich Heine University, Düsseldorf, Germany
[b] Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Essen, Germany
[c] Department of Urology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany
[d] Institute of Pharmaceutical and Medical Chemistry, Heinrich Heine University, Düsseldorf, Germany

ARTICLE INFO

Keywords:
Immunotherapy
Solid tumor
Chimeric antigen receptor
HNSCC
Cetuximab
EGF receptor

ABSTRACT

The monoclonal antibody cetuximab recognizes domain III of the epithelial growth factor receptor (EGFR) with high-affinity and is an important element in the treatment of several malignancies that overexpress non-mutated wild-type EGFR. In order to create an EGFR recognizing chimeric antigen receptor (CAR) for cellular immunotherapy of head and neck squamous cell carcinoma (HNSCC), we rationally designed single chain fragments of different lengths based on the cetuximab variable heavy and light chains. We then cloned the different cetuximab fragments into our second generation CAR construct, expressed CARs on primary human T-cells from healthy donors using mono- or biscistronic lentiviral vectors and tested the stability, functionality and specificity of the CARs. Our smallest CAR construct was most efficient with greatly improved vector production and T-cell transduction efficacy. Finally, we demonstrated that the new cetuximab CAR construct expressed on T-cells is highly reactive against EGFR-positive HNSCCs and also malignant cells from other solid cancer entities. In conclusion, we generated an optimized high-affinity EGFR CAR construct for the next steps in cancer immunotherapy, which need to focus on the development of armored CAR T-cells that will be more resistant and effective in the hostile microenvironment present in solid cancers.

Introduction

Head and neck squamous cell carcinoma (HNSCC) develops in the outer layer of the skin and in the mucous membranes and is the 8th most diagnosed solid cancer worldwide with more than 800,000 newly diagnosed patients per year [1,2]. 400,000 HNSCC patients still die annually worldwide, as no major improvement of the overall survival rate after surgery, platinum-based chemotherapy and radiation has been achieved within the last 50 years [3,4].

Approximately 80–90% of all HNSCCs overexpress the Epidermal Growth Factor Receptor (EGFR), a tyrosine kinase receptor with four extracellular domains [5], that plays a key role in cell survival, proliferation, migration and differentiation of epithelial and other cells [6]. About 95% of the free/non-ligand bound EGFR on cells is present in an auto-inhibited conformation, which is characterized by an interaction between domains I and IV. In the active conformation, EGFR binds one of its seven known ligands in the pocket formed between domains I and III [7,8]. The binding of a ligand induces homodimerization of two receptor proteins and thereby activates the intracellular kinase domains to trans-phosphorylate each other [5]. Through this signaling, EGFR controls normal cell growth and proliferation. However, when overexpressed on malignant cells, the increased EGFR signaling leads to uncontrolled cell proliferation and thereby promotes the development of aggressive cancers [9]. Therefore, patients with highly EGFR-positive tumors, mainly epithelial cancers of head and neck, breast, colon, lung and pancreas, often face a poor prognosis due to limited efficacy of the multimodal treatment regimens with decreased overall survival (OS) [10].

* Corresponding author.at: Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Hufelandstraße 55, 45122 Essen, Germany.
E-mail address: Helmut.Hanenberg@uk-essen.de (H. Hanenberg).
[1] These authors have contributed equally.

https://doi.org/10.1016/j.oraloncology.2022.105867
Received 28 August 2021; Received in revised form 7 April 2022; Accepted 12 April 2022
Available online 22 April 2022

To inhibit the rapid cell proliferation of EGFR-positive malignant cells, monoclonal antibodies such as the immunoglobulin G1 subclass mouse-human chimeric antibody cetuximab (Erbitux®) were developed to block EGFR signaling [11]. Cetuximab (Ctx) binds to domain III of EGFR with a 5- to 10-fold higher affinity than any of its natural ligands, thereby preventing ligand binding and switching of the receptor to an active conformation with dimerization and downstream signaling [11,12]. So far, cetuximab is the only FDA- and EMA-approved monoclonal antibody for the treatment of both, locoregionally advanced and recurrent/metastatic HNSCC, and usually combined with chemo- and/or radiotherapy, resulting in improved local disease control but only marginally increased OS [9]. Other antibody-based EGFR inhibitors (nimotuzumab, panitumumab, zalutumumab) and small molecule tyrosine kinase inhibitors (erlotinib, gefitinib, lapatinib, afatinib, dacomitinib) have also shown efficacy in clinical trials, but in comparison to cetuximab failed to demonstrate equal or improved benefits for the OS of patients [13–16].

As EGFR is also strongly expressed on the basal layer of the epidermis, treatment with EGFR monoclonal antibodies is associated with typical skin toxicities due to *off-tumor on-target* binding of the antibodies [12]. Mechanistically, binding of the IgG1 antibodies will direct the antibody-dependent cellular cytotoxicity of the immune system, mainly by CD16-positive NK-cells and indirectly also cytotoxic T-cells (via activation of dendritic cells and macrophages) to both, normal as well as malignant EGFR expressing cells [17]. Cetuximab has been widely studied in the treatment of various cancers, thus the side effects are well established and the therapy is known to be tolerated by the majority of patients [18]. However, treatment with cetuximab is also associated with an increased incidence of severe adverse reactions [19–21], while clinical studies with lower affinity EGFR monoclonal antibodies such as nimotuzumab revealed less side effects, but also less anti-tumor cell activity [13].

Chimeric antigen receptors (CARs) present a novel immunotherapeutic approach to direct autologous T-cells from patients against antigens expressed on the surface of malignant cells [22]. CARs combine a single chain fragment (scFv) from a monoclonal antibody recognizing a tumor-associated antigen with the cytoplasmic signaling domain of the CD3 ξ-chain and co-stimulatory motifs in a single molecule. T-cells genetically modified to express a CAR efficiently recognize the tumor-associated antigen and kill the malignant cell in a non-MHC restricted manner [23]. For targeting EGFR, Caruso *et al.* generated two CAR constructs with scFvs from cetuximab and nimotuzumab variable and constant regions, which both bind to overlapping epitopes of EGFR, and validated these constructs against EGFR-positive glioma cells *in vitro* and *in vivo* [24]. Our focus here was to develop a lentiviral vector for HNSCC CAR T-cell therapy by re-engineering the relatively long cetuximab scFv from Caruso *et al.* [24] for optimal vector production efficacy and cytotoxic activity.

Material and methods

Cell culture

Human embryonic kidney cells (HEK293T), different HNSCC cell lines and the human fibrosarcoma HT1080 cells were cultured as previously published [25]. RT-112, MCF-7, Cal27 and Kyse510 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A2780 and HEY cells were purchased from the European Collection of Cell Cultures (ECACC, UK) and TOV21G cells from the American Type Culture Collection (ATCC). All cell lines were grown as recommended. Normal human oral keratinocytes (HOK) were purchased from ScienCell Research Laboratories (Carlsbad, U.S.A) and dysplastic oral keratinocytes (DOK) were obtained from Sigma-Aldrich (Darmstadt, Germany).

Generation, expansion, transduction and selection of human CAR T-cells

Human primary T-cells were obtained from the peripheral blood of healthy adult donors after informed consent according to the protocols approved by the local ethics committee/IRB in Düsseldorf (study number: 4687) and activated as described previously [25]. 72 h after expansion and transduction, T-cells were selected and enriched by magnetic cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) as described in Bister *et al.* [26] according to the manufacturer's protocol.

Lentiviral vectors and transduction

The control CD19 and CD44v6 CAR constructs were previously published [25,27,28]. The original scFv of the cetuximab antibody containing both variable and constant regions (Ctx_{long}) was optimized for human codon usage and synthesized by GeneArt (Thermo Fisher Scientific, Regensburg, Germany) according to the description by Caruso *et al.* [24]. For construction of modified cetuximab V_H and V_L sequences (Ctx_{small}, $Ctx_{smallVL}$, $Ctx_{smallVH}$), overlap extension polymerase chain reaction was used. For the production of EGFR knockout cells, DNA oligonucleotides for two gRNAs were designed using the chop-chop algorithm [29], synthesized by Eurofins Genomics (Ebersberg, Germany) and cloned into the lentiviral expressing CRISPR/Cas9 vector Lenti-CRISPRv2 as described [30].

Vesicular stomatitis virus-G pseudotyped replication-deficient lentiviral particles were produced after transfection into HEK293T cells as described previously [25]. For the production of EGFR knockout clones, $1x10^6$ cells were transduced in limited dilutions of the lentiviral CRISPR/Cas9 vectors, selected with puromycin and then single-cell cloned [25]. Primary human T-cells were transduced on the fibronectin fragment CH296 (Takara Bio Inc., Otsu, Japan) as previously described [27,31]. To determine vector titers, serial dilutions (10^{-1} to 10^{-6}) of viral supernatant were added to $3.5x10^4$ HT1080 cells which had been seeded in six-well plates the day before. The lentiviral vector titers were determined by flow cytometric analysis using the linear range between viral concentration and EGFP-positive cells. All transductions were performed in the presence of 10 μg ml^{-1} protamine phosphate (Sigma-Aldrich, Darmstadt, Germany) as previously described [27,31].

Normal human oral keratinocytes (HOK) were immortalized with a VSV-G-pseudotyped lentiviral vector that expresses the SV40 large T-antigen (HOK-T) as previously described [32].

Flow cytometry analysis

The expression profiles of EGFR were analyzed by staining $1x10^6$ cells with an EGFR-PE antibody (REA688, Miltenyi Biotec) according to the manufacturer's specifications and measured on a MACSQuant Analyzer X (Miltenyi Biotec) or a BD Biosciences FACSCalibur (Heidelberg, Germany).

To verify the enrichment and selection of CAR T-cells, different MACS fractions were stained with the CD34-PE antibody (Clone: QBEND/10; Thermo Fisher) as described in Bister *et al.* [26]. HT1080 cells transduced with different dilutions of virus-containing supernatants were also stained with the CD34-PE antibody and analyzed by flow cytometry.

Cytotoxicity assay

The cytotoxicity of CAR T-cells was determined by the CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega, Walldorf, Germany) as described previously [25].

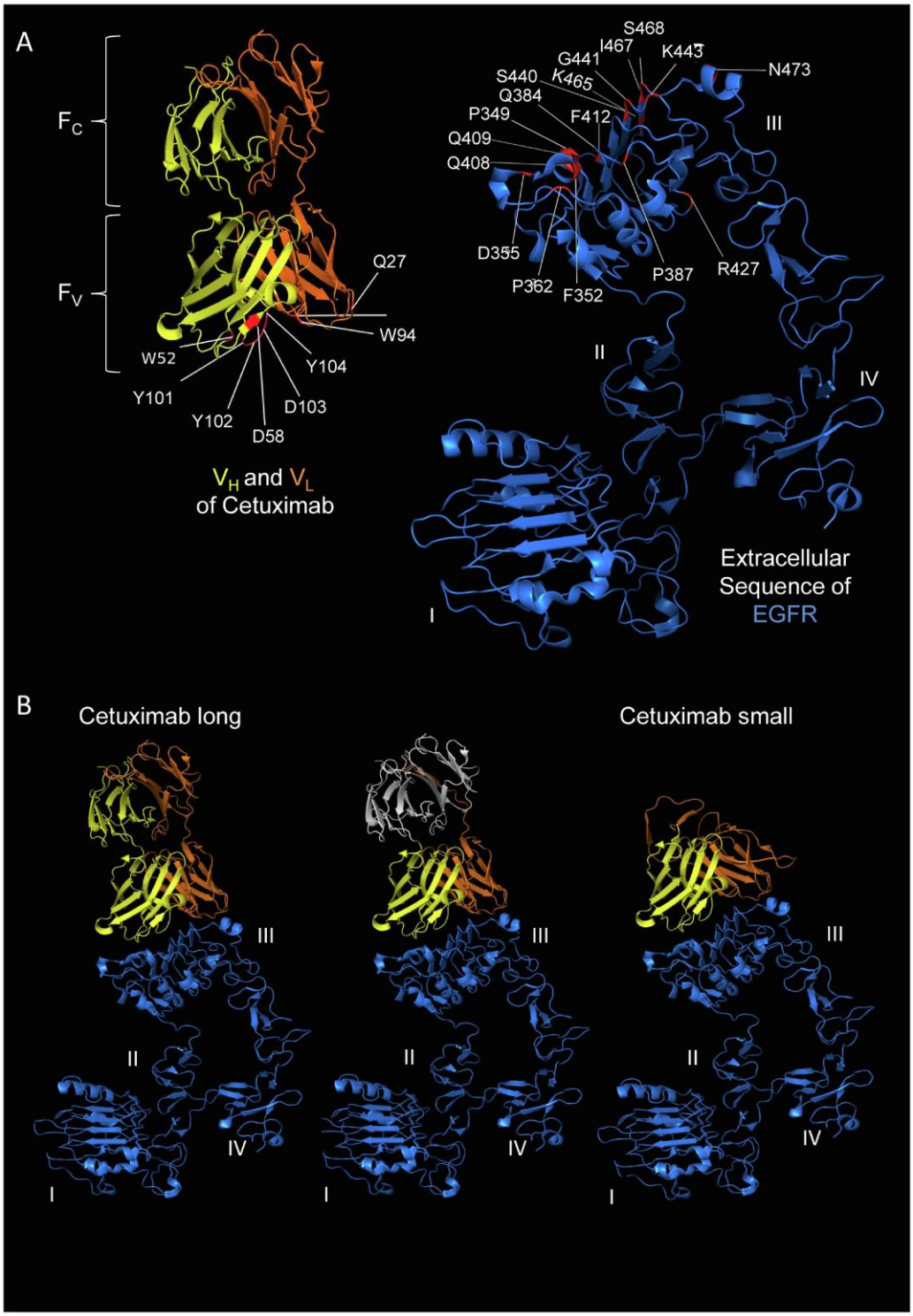


Fig. 1. Interaction of EGFR and cetuximab (Cartoon view). A) Amino acids mutated in cetuximab-resistant tumors or critical for cetuximab binding are shown in red [10,32–34] within the extracellular domains I-IV of EGFR pictured in blue (PDB 1NQL) [62]. Amino acids of the heavy (yellow) and light (orange) chains of cetuximab (PDB 1YY8) [10] involved in EGFR binding are depicted in red. B) Structure of the extracellular domain of EGFR (blue) in complex with heavy (yellow) and light (orange) chain of cetuximab (PDB 1YY9) [10] in its unmodified form (cetuximab long = Ctx_{long}, left). Amino acids, which were removed for generating an optimized cetuximab scFV, are shown in grey (middle). Potential structure of the shortened cetuximab scFv (cetuximab small = Ctx_{small}, right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

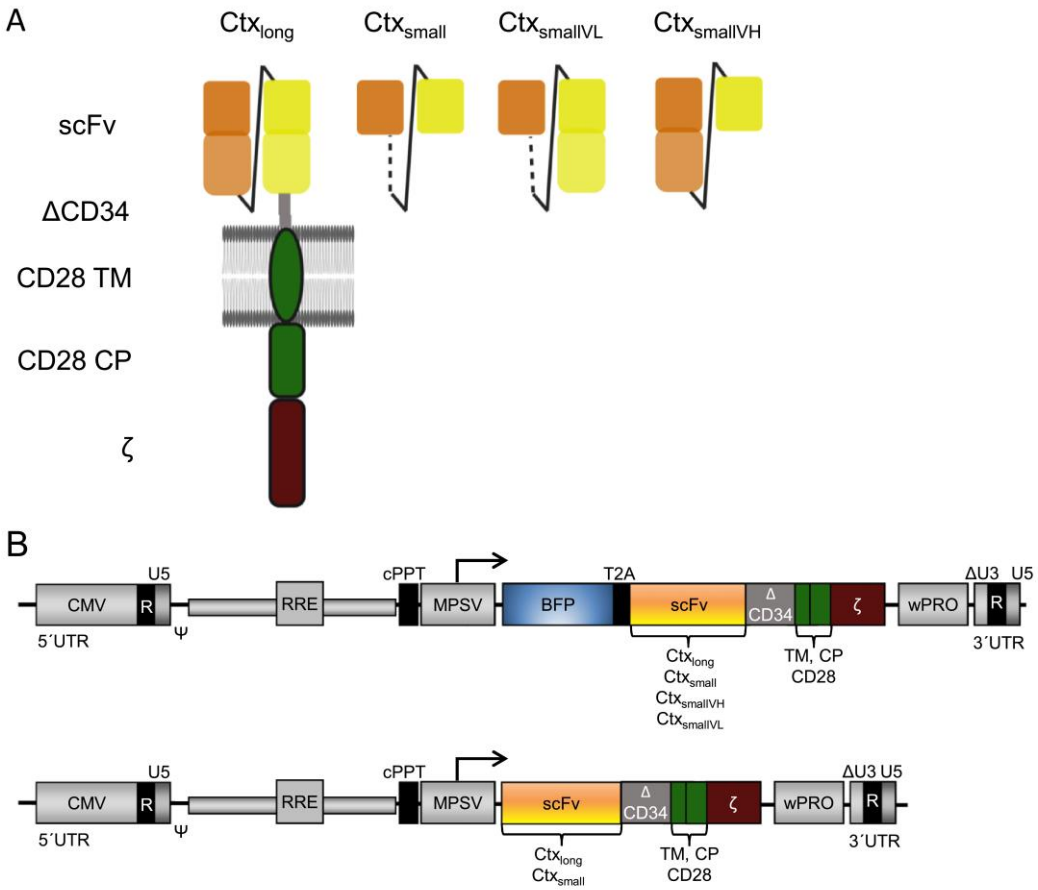


Fig. 2. Design of different cetuximab-based CARs. A) Schematic structure of the different CARs. The constructs contained the four variants of the cetuximab scFv followed by a ΔCD34 hinge domain, a CD28 transmembrane and intracellular domain and the CD3ζ cytosolic domain. Ctx_{long} (original VH and VL), Ctx_{small} (short VH and short VL), $Ctx_{smallVH}$ (original VL, short VH), $Ctx_{smallVL}$ (short VL, original VH). B) Schematic structures of the mono- and bicistronic lentiviral vectors for expression of CARs which we published previously [27].

controls (Fig. S2).

Ctx_{small} CAR T-cells kill normal and dysplastic EGFR-positive oral keratinocytes

In order to assess the cytotoxicity of Ctx_{small} CAR T-cells for non-malignant normal tissues, we bought normal human oral keratinocytes (HOK) and also dysplastic oral keratinocytes (DOK) described by others [44,45]. However, as the HOK cells grew quite poorly and we did not want to extend the culture period for more than 8 days, we also used our lentiviral SV40 large T expression vector [32] to generate immortalized HOK-T cells. Flow cytometry analysis revealed that EGFR was expressed at different levels on these three cell types (Fig. 6A). Co-culture of HOK, HOK-T and DOK cells with our Ctx_{small} CAR T-cells was associated with significant toxicity (Fig. 6A) that did not directly correlate with the EGFR expression levels on the cells.

Ctx_{small} CAR T-cells are effective against malignant cells of several entities

Finally, after proving the high sensitivity (Fig. 4) and specificity (Fig. 5) of the Ctx_{small} CAR for EGFR-expressing HNSCC cell lines and also non-malignant keratinocytes, we validated our new Ctx CAR construct for the cytotoxic activity against cancer cell lines of different origin. Cells of the EGFR-positive OSCC cancer cell line CAL 27 (MFI 2.25) and the $EGFR^{low}$ expressing bladder cancer RT-112 cell line [46] (MFI 2.16) were efficiently eradicated by the Ctx_{small} CAR T-cells (Fig. 6B). The killing pattern for the low EGFR expressing cells (MFI 1.44) of ESCC cancer cell line Kyse-510 (Fig. 6B) [47] confirmed the high sensitivity of the Ctx_{small} CAR. Also EGFR-positive cells from ovarian cancer, HEY and TOV21G, were efficiently eliminated (between 96.2 and 89.2% at 3:1 ratios) by the Ctx_{small} CAR (Fig. 6B), independent of their EGFR expression levels (HEY MFI 6.7 and TOV21G MFI 1.84). Importantly, cells from the EGFR negative ovarian cancer cell line A2780 were not killed by Ctx_{small} CAR T-cells (Fig. 6B).

Discussion

Immunotherapy using autologous T-cells genetically modified to express CAR constructs has yielded high response and cure rates in patients with B-cell malignancies, especially with CD19 CAR T-cells for B-cell acute lymphoblastic leukemia [22]. In contrast, administration of CAR T-cells to treat solid tumors achieved very limited successes so far [23], due to a unique set of challenges, including the lack of tumor-specific antigens that are homogeneously expressed on all malignant cells, and the presence of an immune-suppressive tumor-tolerant and metabolically challenging microenvironment [48]. This hypoxic tumor microenvironment (TME) with altered nutrient availability also contains stroma cells, fibroblasts and regulatory immune cells that all can produce immunosuppressive cytokines and chemokines. Importantly, this hostile microenvironment attenuates CAR T-cell infiltration, survival

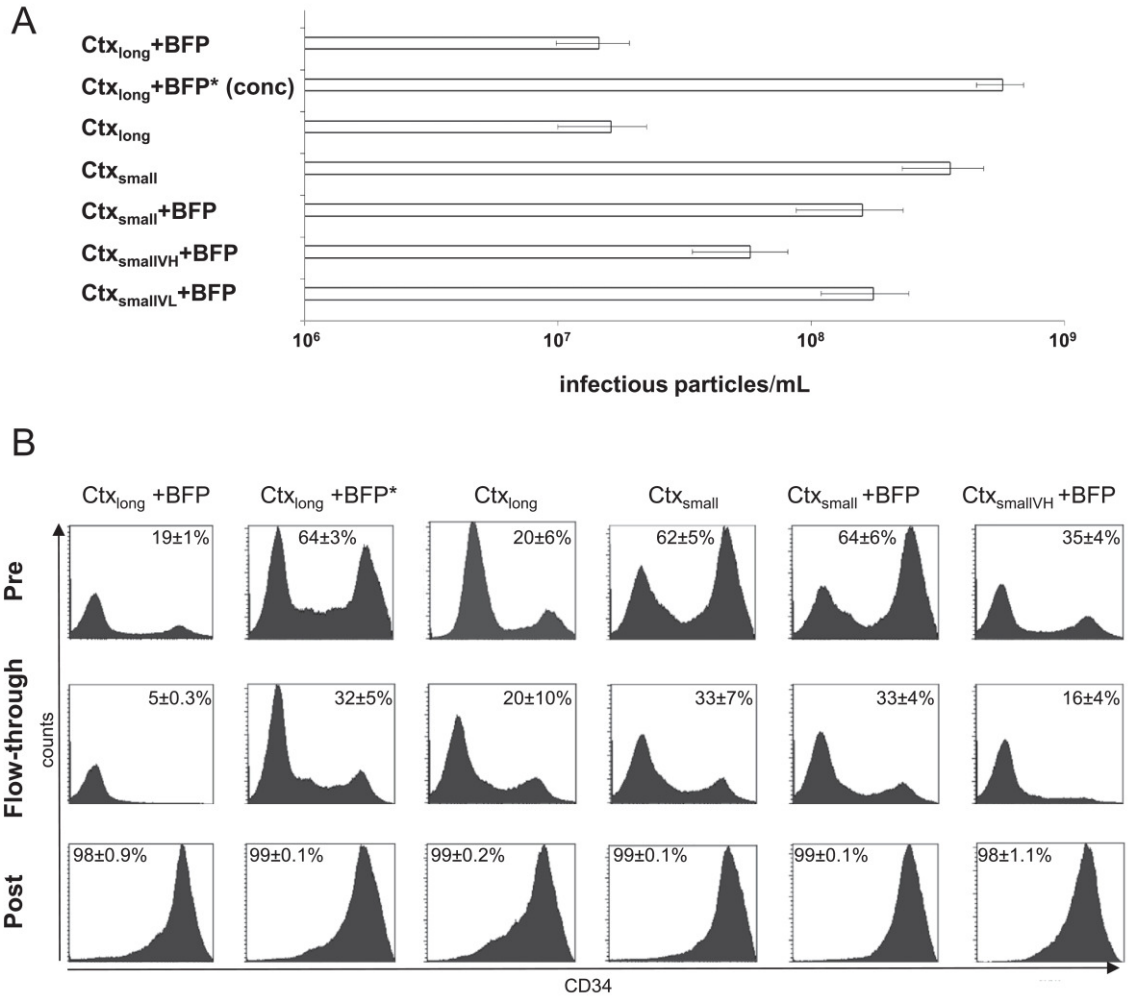


Fig. 3. Shortening of the cetuximab-derived scFv improves lentiviral and titer and transduction efficiency. A) HT1080 cells were lentivirally transduced with serially diluted vectors encoding cetuximab CAR constructs with or without BFP (Ctx$_{long}$, transduction with 15-fold virus supernatant (*(conc)), Ctx$_{small}$, Ctx$_{smallVH}$, Ctx$_{smallVL}$). Cells were stained with the CD34-PE antibody and CAR expression was flow cytometrically analyzed to calculate the viral vector titers. B) Primary human T-cells were transduced with lentiviral vectors encoding the cetuximab CAR constructs with or without BFP. CAR T-cells were enriched via CD34-mediated MACS and the three fractions (Pre, Flow-through, Post) were flow cytometrically analyzed for CAR expression after staining with CD34-PE antibody. CAR expression (x-axis) was plotted against the absolute cell count (y-axis). The numbers indicated the percentages of CAR-positive T-cells and representative histograms were used. The graph showed the CAR-positive cells in % (grey bars) and the mean fluorescence intensity (white bars) of PreMACS fractions. Data were depicted as mean ± SEM for three different experiments/biological replicates.

and anti-tumor activity.

To improve the efficacy of CAR T-cells, our aim here was to develop a small cetuximab-based CAR for inclusion in bi- or multicistronic lentiviral vectors that can also express additional transgenes to armor the CAR T-cells against immunosuppressive signalling in the TME. By optimizing the scFv design, we were able to improve the lentiviral vector production and the transduction efficiency of human primary T-cells compared to a previously established cetuximab-based CAR [24], thereby potentially reducing the costs for large-scale productions of multigene lentiviral vectors in the future. Particularly attractive here is that the Ctx$_{small}$ CAR is based on an already clinically approved and widely used therapeutic antibody, for whom the *off-tumor on-target* toxicity is well established [18]. Even MCF-7 cells with very little or no EGFR on their surface [40–42] were efficiently killed by the Ctx CAR T-cells. The ultimate proof for the specificity of our Ctx$_{small}$ CAR construct was the observation that EGFR knockout cells generated by the CRISPR/Cas9 technology in strongly EGFR-expressing HNSCC cell lines were not killed at all. Importantly, the high sensitivity of the Ctx$_{small}$ CAR construct increases the risk of *on-target off-tumor* toxicity in EGFR expressing tissues like skin, gastrointestinal system and kidney [24]. We confirmed experimentally that Ctx$_{small}$ CAR T-cells exhibit significant cytotoxicity against human normal (HOK) as wells as dysplastic oral keratinocytes (DOK) and also against SV40 large T-immortalized HOK-T cells. This cytotoxicity did not directly correlate with the EGFR expression levels on the cells, as we also demonstrated for malignant cells of different cancer entities. It therefore has been suggested that EGFR CARs with a lower affinity for their target antigens would suffice to target HNSCC cells due to the strong EGFR overexpression in up to 90% of all HNSCC tumor tissues [9,12,49], thus resulting in a reduced risk of undesired *off-tumor* toxicity. Consequently, Caruso *et al.* compared the killing characteristics of a high-affinity cetuximab-based CAR with a lower affinity nimotuzumab-derived CAR construct [24]. Both EGFR CAR T-cell constructs efficaciously killed EGFR strongly expressing HNSCC cell lines, but the nimotuzumab-based CAR spared

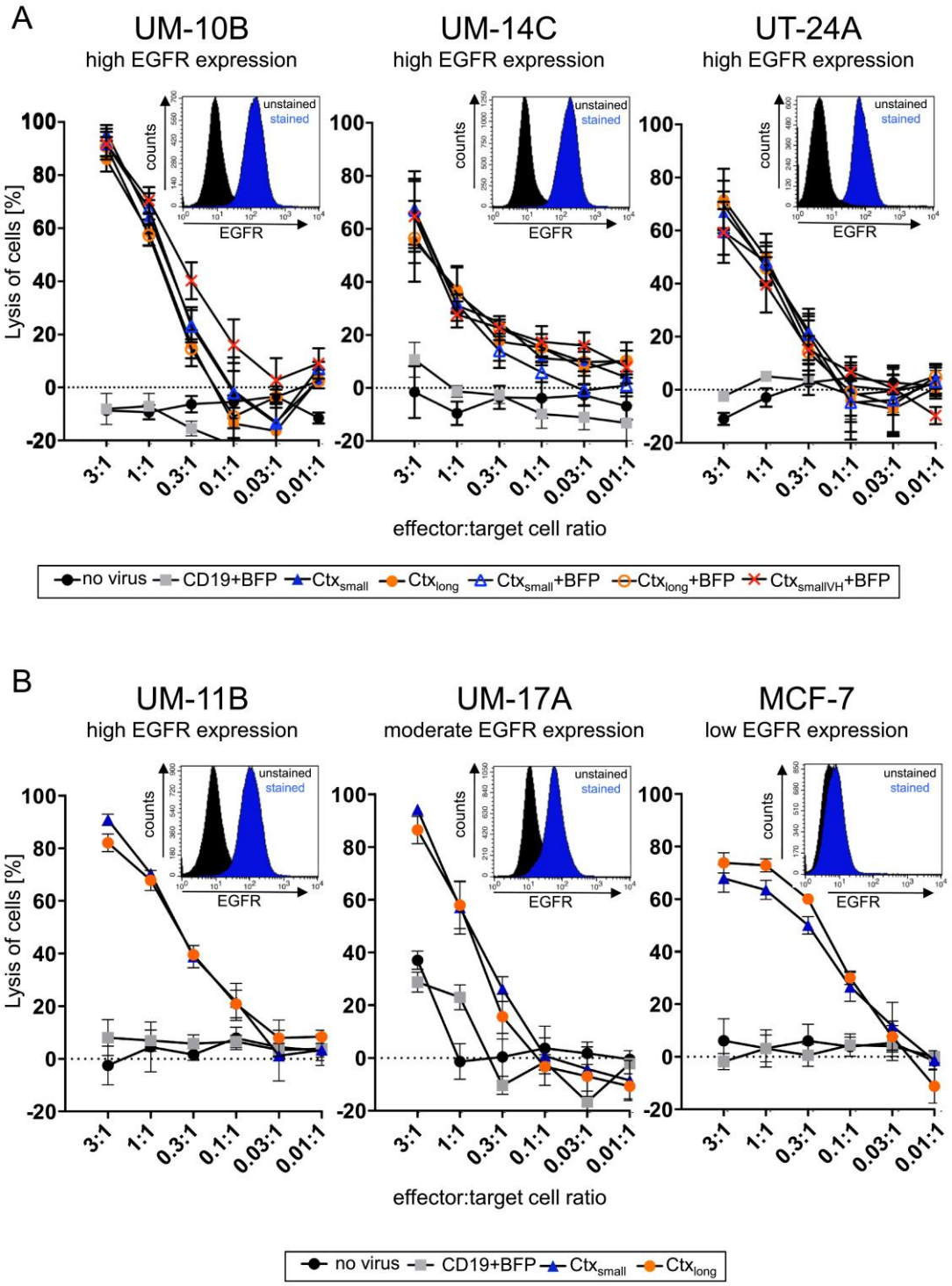


Fig. 4. Ctx-derived CARs eradicated HNSCC cells with high efficacy. EGFR expression was determined by staining with an EGFR-PE antibody. Antigen expression (x-axis) was plotted against the absolute cell count (y-axis) for unstained control cells (black) and stained cells (blue). Primary human T-cells were lentivirally transduced with VSVG-pseudotyped CAR vectors. After three days, the CAR-positive T-cells were enriched by MACS and co-cultured with **A**) the high EGFR expressing HNSCC cell lines UM-10B, UM-14C and UT-24A or **B**) cell lines with distinct EGFR expression levels, UM-11B^{high}, UM-17B^{moderate} and MCF-7^{low}, at different effector to target cell ratios. After 16 h, the lysis of target cells was determined via CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega). Data were depicted as mean ± SEM for three different experiments.

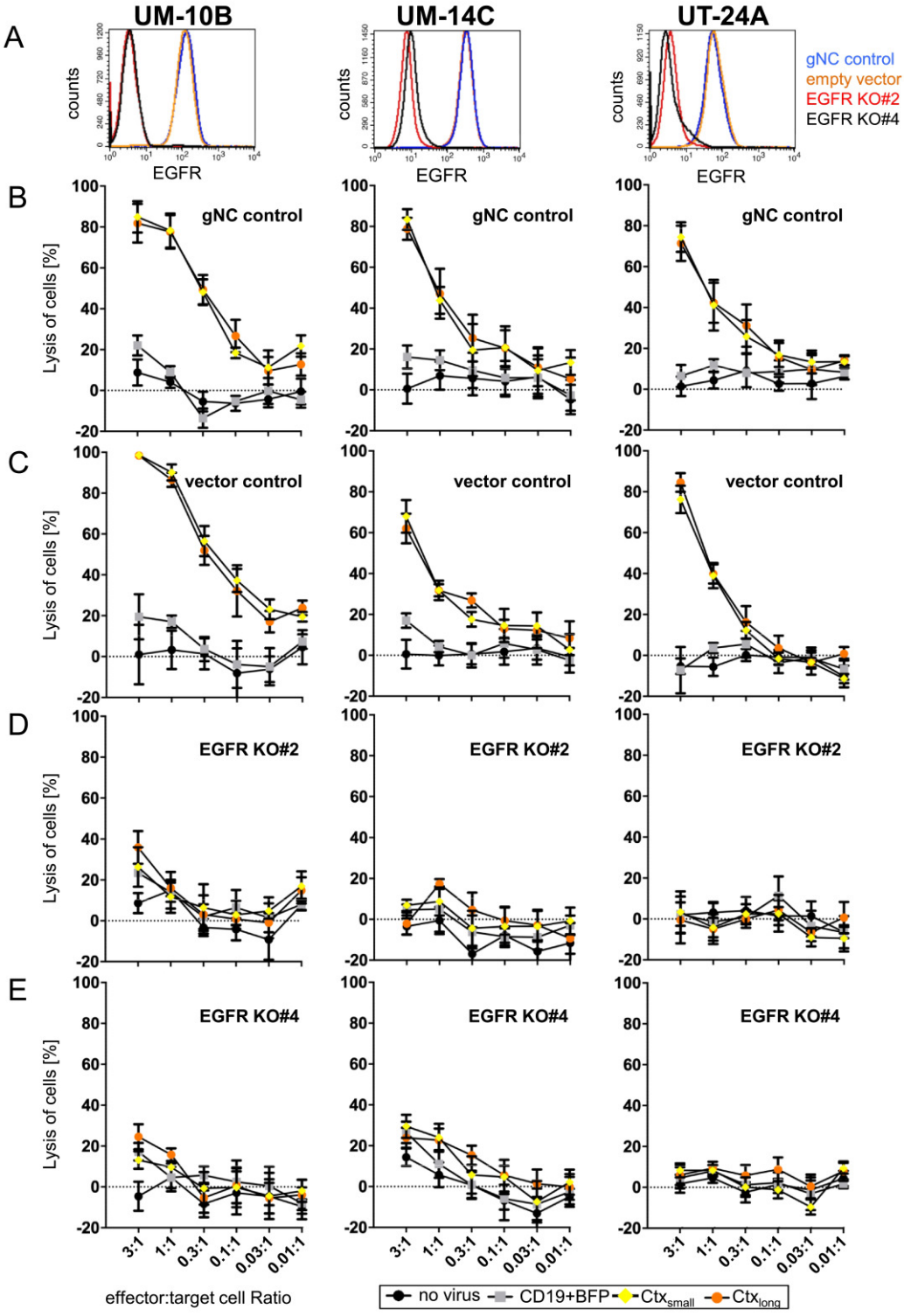


Fig. 5. Ctx$_{long}$ and Ctx$_{small}$ CAR T-cells eradicated HNSCC cells with high efficacy. A) EGFR expression of UM-10B, UM-14C and UT-24A cells transduced with a CRISPR/Cas9 negative control vector (blue), the empty vector (orange) or the two CRISPR/Cas9 gRNA vectors (KO#2 red, KO#4 black). EGFR expression levels were determined by staining with EGFR-PE antibody. Antigen expression (x-axis) was plotted against the absolute cell counts (y-axis). Human T-cells were transduced with VSVG-pseudotyped BFP/CD19, Ctx$_{long}$ or Ctx$_{small}$ CAR lentiviral vectors. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with the target cells. B) gNC control cells, C) vector control cells, D) EGFR knockout #2 cells, E) EGFR knockout #4 cells. After 16 h, the lysis of target cells was determined via CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Data were depicted as mean ± SEM for three experiments.

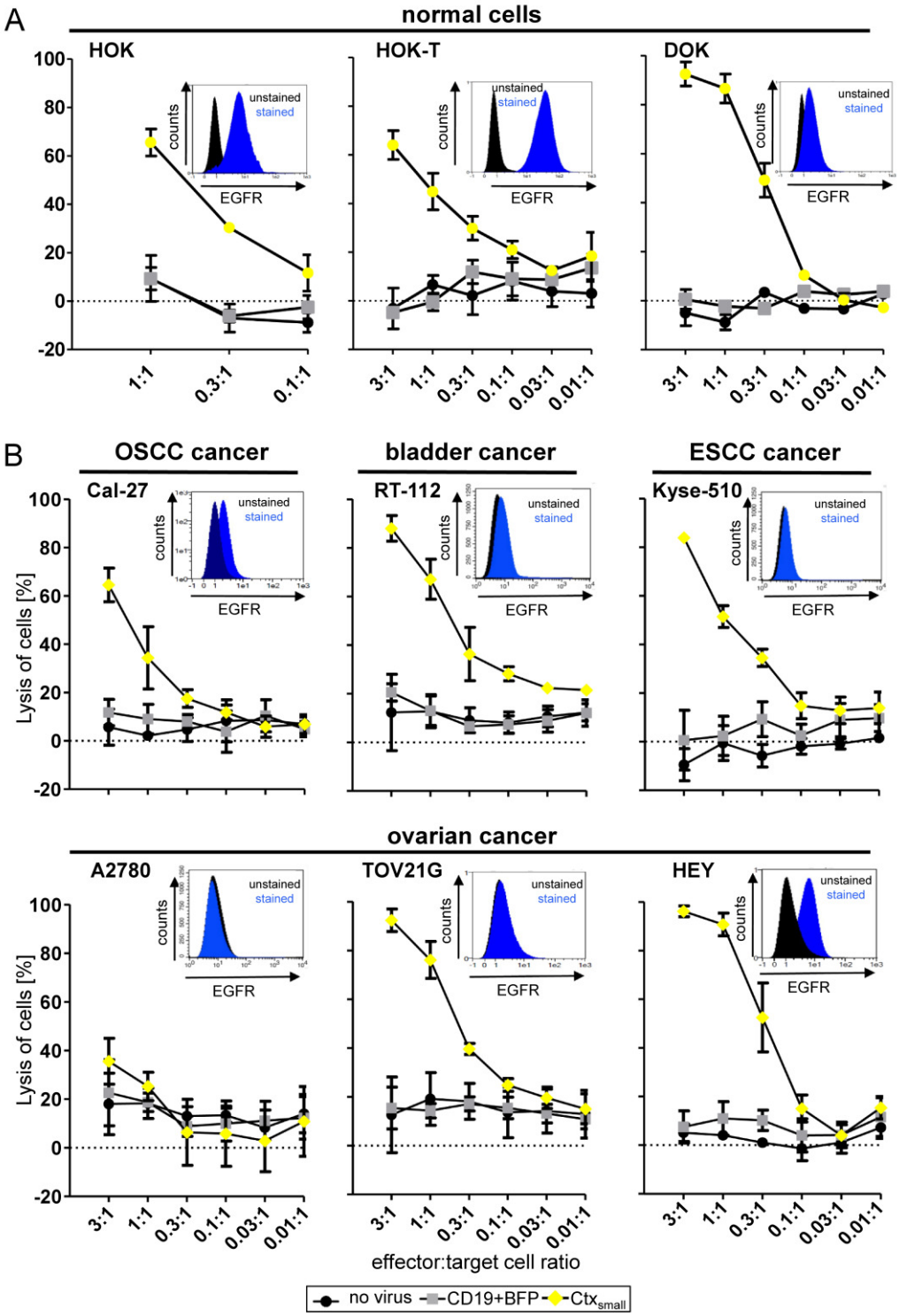


Fig. 6. Ctx$_{small}$ CAR T-cells specifically eradicated EGFR-positive cells of different origin. EGFR expression was assessed by staining with EGFR-PE antibody. Antigen expression (x-axis) was plotted against the absolute cell count (y-axis) for unstained control cells (black) and stained cells (blue). Primary human T-cells were transduced with VSVG-pseudotyped BFP/CD19 or Ctx$_{small}$ CARs. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with (**A**) human healthy/normal oral keratinocytes, both as native (HOK) as well as large SV40 T-immortalized cells (HOK-T), and unmodified dysplatic oral keratinocytes (DOK) and (**B**) tumor cells of different entities: Cal-27, RT-112, Kyse-510, A2780, TOV21G, HEY. Data were depicted as mean ± SEM for three different experiments.

tissues/cells with lower EGFR expression [24]. Similarly, other groups adjusted the affinities of their scFvs for CARs by mutating parts of the sequences involved in binding of the target antigen [50] or by using hybridoma technology for the generation of low-affinity CARs [51,52], which then enabled the CAR T-cells to better discriminate between malignant and normal tissues. However, as the target expression on solid tumor cells within the same patients is highly heterogeneous and persistence/relapse of target antigen negative cells is the main reason for the failure of CAR T-cells in glioblastoma clinical trials [23,53], we believe that, if the *off-tumor* toxicity can be managed, high-affinity CAR constructs are better candidates for adoptive cellular immunotherapy of solid cancers.

In general, *off-tumor on-target* toxicities against normal tissues can be associated with severe safety problems that are controllable by modifying the design of the CAR vector, e.g. by including a safety switch or suicide genes or by using inducible promoters in more complex vectors [22,23,27,54]. However, we favour the concept of directly injecting the CAR T-cells into the solid tumor tissues. Several clinical studies in glioblastoma demonstrated that the repeated injections of CAR T-cells into the tumors are feasible and well tolerated and not associated with toxicities of the immune effector cells in other organs [53,55]. As the residual/recurrent tumors in glioblastoma often show a decrease or complete loss of the target antigen, the next generation of CAR T-cells in this tumor entity needs to target at least two antigens simultaneously [53]. Recently, a phase I/II dose-escalation trial for locally recurrent or metastatic HNSCC (NCT01818323)[2] reported the outcome of using 2nd generation CAR T-cells with an ErbB-ligand binding moiety recognizing several receptors of the ErbB family for direct injection into the tumor site [56]. Stable disease control could be achieved in 9 out of 13 patients after 6 weeks and one patient achieved a rapid and complete clinical response, albeit in combination with PD1 check point inhibition and additional oncolytic therapy [56]. Importantly, no severe adverse events were noted. In a recent short update, the group in London reported that up to 1 billion CAR T-cells can be injected safely into the tumor tissues and that no dose-limiting toxicities occurred, including no clinically relevant leakage of CAR T-cells into the circulation [57].

With the Ctx_{small} CAR construct described here, we developed a small and high-affinity EGFR CAR that can be readily integrated in multi-transgene lentiviral vectors. Whether the best approach to limit tumor cell escape is to express two CARs simultaneously via a 2A site or to use one CAR with two scFvs needs to be tested in preclinical models and clinical trials [23,58]. Other factors to be addressed in the design of multi-transgene vectors are the necessity to neutralize immunosuppressive factors like TGFβ or adenosine, e.g. by shRNA-mediated down-regulation of their cognitive receptors or by expression of cDNAs for dominant negative or switch receptors [59]. In addition, secretion of proteins that neutralize immunosuppressive tumor cell antigens (e.g. PD-L1) or bispecific antibodies that recruit the immunosuppressive Treg-cells for killing of the malignant cells are other exciting strategies [60,61] that can readily be combined with the EGFR CAR construct developed here.

Funding

This work was supported, in part, by funding from the Medical Research School Düsseldorf, DSO, Heinrich Heine University, Düsseldorf, and within the framework of the iCAN33 project, funded by the European Regional 470 Development Fund NRW (ERDF, German EFRE) 2014-2020. This work was supported, in part, by the Forschungskommission of the Medical Faculty of the Heinrich Heine University Duesseldorf to C.G.

[2] https://clinicaltrials.gov/ct2/show/NCT01818323.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully acknowledge the healthy donors that provided peripheral blood for the cytotoxicity studies. We would like to thank Jörg Schipper, MD, director of ENT clinic, for his support of this research project.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2022.105867.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68(6):394–424. https://doi.org/10.3322/caac.21492.

[2] Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE, Grandis JR. Head and neck squamous cell carcinoma. Nat Rev Dis Prim 2020;6(1). https://doi.org/10.1038/s41572-020-00224-3.

[3] Devaraja K. Current prospects of molecular therapeutics in head and neck squamous cell carcinoma. Pharmaceut Med 2019;33(4):269–89. https://doi.org/10.1007/s40290-019-00288-x.

[4] Mehra R, Ang KK, Burtness B. Management of human papillomavirus-positive and human papillomavirus-negative head and neck cancer. Semin Radiat Oncol 2012;22(3):194–7.

[5] Maruyama I. Mechanisms of activation of receptor tyrosine kinases: monomers or dimers. Cells 2014;3:304–30. https://doi.org/10.3390/cells3020304.

[6] Martin P, Kelly CMA, Carney D. Epidermal growth factor receptor-targeted agents for lung cancer. Cancer Control 2006;13(2):129–40. https://doi.org/10.1177/107327480601300207.

[7] Singh B, Carpenter G, Coffey RJ. EGF receptor ligands: recent advances. F1000Res 2016;5:2270. https://doi.org/10.12688/f1000research.9025.1.

[8] Talavera A, Friemann R, Gómez-Puerta S, Martinez-Fleites C, Garrido G, Rabasa A, et al. Nimotuzumab, an antitumor antibody that targets the epidermal growth factor receptor, blocks ligand binding while permitting the active receptor conformation. Cancer Res 2009;69(14):5851–9. https://doi.org/10.1158/0008-5472.CAN-08-4518.

[9] Concu R, Cordeiro MNDS. Cetuximab and the head and neck squamous cell cancer. Curr Top Med Chem 2018;18(3):192–8. https://doi.org/10.2174/1568026618666180112162412.

[10] Li S, Schmitz KR, Jeffrey PD, Wiltzius JJW, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 2005;7(4):301–11. https://doi.org/10.1016/j.ccr.2005.03.003.

[11] Specenier P, Vermorken JB. Cetuximab in the treatment of squamous cell carcinoma of the head and neck. Expert Rev Anticancer Ther 2011;11(4):511–24. https://doi.org/10.1586/era.11.20.

[12] Mehra R, Cohen RB, Burtness BA. The role of cetuximab for the treatment of squamous cell carcinoma of the head and neck. Clin Adv Hematol Oncol 2008;6:742–50.

[13] Crombet Ramos T, Mestre Fernández B, Mazorra Herrera Z, Iznaga Escobar NE. Nimotuzumab for patients with inoperable cancer of the head and neck. Front Oncol 2020;10:817. https://doi.org/10.3389/fonc.2020.00817.

[14] Vermorken JB, Stöhlmacher-Williams J, Davidenko I, Licitra L, Winquist E, Villanueva C, et al. Cisplatin and fluorouracil with or without panitumumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck (SPECTRUM): an open-label phase 3 randomised trial. Lancet Oncol 2013;14(8):697–710. https://doi.org/10.1016/S1470-2045(13)70181-5.

[15] Machiels J-P, Subramanian S, Ruzsa A, Repassy G, Lifirenko I, Flygare A, et al. Zalutumumab plus best supportive care versus best supportive care alone in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy: an open-label, randomised phase 3 trial. Lancet Oncol 2011;12(4):333–43. https://doi.org/10.1016/S1470-2045(11)70034-1.

[16] Patel B, Saba NF. Current aspects and future considerations of EGFR inhibition in locally advanced and recurrent metastatic squamous cell carcinoma of the head and neck. Cancers (Basel) 2021;13:3545. https://doi.org/10.3390/cancers13143545.

[17] Ferris RL, Lenz H-J, Trotta AM, García-Foncillas J, Schulten J, Audhuy F, et al. Rationale for combination of therapeutic antibodies targeting tumor cells and immune checkpoint receptors: Harnessing innate and adaptive immunity through IgG1 isotype immune effector stimulation. Cancer Treat Rev 2018;63:48–60. https://doi.org/10.1016/j.ctrv.2017.11.008.

[18] Abdel-Rahman O, Fouad M. Correlation of cetuximab-induced skin rash and outcomes of solid tumor patients treated with cetuximab: a systematic review and meta-analysis. Crit Rev Oncol Hematol 2015;93(2):127–35. https://doi.org/10.1016/j.critrevonc.2014.07.005.

[19] Shablak A, Conn A. A case of fatal cetuximab-induced interstitial lung disease during the first weeks of treatment. Target Oncol 2014;9(2):177–80. https://doi.org/10.1007/s11523-013-0295-4.

[20] Chua W, Peters M, Loneragan R, Clarke S. Cetuximab-associated pulmonary toxicity. Clin Colorectal Cancer 2009;8(2):118–20. https://doi.org/10.3816/CCC.2009.n.019.

[21] Hoag JB, Azizi A, Doherty TJ, Lu J, Willis RE, Lund ME. Association of cetuximab with adverse pulmonary events in cancer patients: a comprehensive review. J Exp Clin Cancer Res 2009;28:113. https://doi.org/10.1186/1756-9966-28-113.

[22] June CH, Sadelain M. Chimeric Antigen Receptor Therapy. N Engl J Med 2018;379(1):64–73. https://doi.org/10.1056/NEJMra1706169.

[23] Larson RC, Maus MV. Recent advances and discoveries in the mechanisms and functions of CAR T cells. Nat Rev Cancer 2021;21(3):145–61. https://doi.org/10.1038/s41568-020-00323-z.

[24] Caruso HG, Hurton LV, Najjar A, Rushworth D, Ang S, Olivares S, et al. Tuning sensitivity of CAR to EGFR density limits recognition of normal tissue while maintaining potent antitumor activity. Cancer Res 2015;75(17):3505–18. https://doi.org/10.1158/0008-5472.CAN-15-0139.

[25] Haist C, Schulte E, Bartels N, Bister A, Poschinski Z, Ibach TC, et al. CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells. Oral Oncol 2021;116:105259. https://doi.org/10.1016/j.oraloncology.2021.105259.

[26] Bister A, Ibach T, Haist C, Smorra D, Roellecke K, Wagenmann M, et al. A novel CD34-derived hinge for rapid and efficient detection and enrichment of CAR T cells. Mol Ther - Oncolytics 2021;23:534–46. https://doi.org/10.1016/j.omto.2021.11.003.

[27] Roellecke K, Virts EL, Einholz R, Edson KZ, Altvater B, Rossig C, et al. Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies. Gene Ther 2016;23(7):615–26. https://doi.org/10.1038/gt.2016.38.

[28] Roessig C, Scherer SP, Baer A, Vormoor J, Rooney CM, Brenner MK, et al. Targeting CD19 with genetically modified EBV-specific human T lymphocytes. Ann Hematol 2002;81(Suppl 2):S42–3.

[29] Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H, Valen E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Res 2019;47:W171–4. https://doi.org/10.1093/nar/gkz365.

[30] Virts EL, Jankowska A, Mackay C, Glaas MF, Wiek C, Kelich SL, et al. AluY-mediated germline deletion, duplication and somatic stem cell reversion in UBE2T defines a new subtype of Fanconi anemia. Hum Mol Genet 2015;24(18):5093–108. https://doi.org/10.1093/hmg/ddv227.

[31] Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 1996;2(8):876–82. https://doi.org/10.1038/nm0896-876.

[32] Gu D, Liu H, Su GH, Zhang X, Chin-Sinex H, Hanenberg H, et al. Combining hedgehog signaling inhibition with focal irradiation on reduction of pancreatic cancer metastasis. Mol Cancer Ther 2013;12(6):1038–48. https://doi.org/10.1158/1535-7163.MCT-12-1030.

[33] Fujiwara K, Masutani M, Tachibana M, Okada N. Impact of scFv structure in chimeric antigen receptor on receptor expression efficiency and antigen recognition properties. Biochem Biophys Res Commun 2020;527(2):350–7. https://doi.org/10.1016/j.bbrc.2020.03.071.

[34] Voigt M, Braig F, Göthel M, Schulte A, Lamszus K, Bokemeyer C, et al. Functional dissection of the epidermal growth factor receptor epitopes targeted by panitumumab and cetuximab. Neoplasia 2012;14(11):1023–IN3. https://doi.org/10.1593/neo.121242.

[35] García-Foncillas J, Sunakawa Y, Aderka D, Wainberg Z, Ronga P, Witzler P, et al. Distinguishing features of cetuximab and panitumumab in colorectal cancer and other solid tumors. Front Oncol 2019;9:849. https://doi.org/10.3389/fonc.2019.00849.

[36] Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, et al. Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. Nat Med 2012;18(2):221–3. https://doi.org/10.1038/nm.2609.

[37] Arena S, Bellosillo B, Siravegna G, Martínez A, Cañadas I, Lazzari L, et al. Emergence of multiple EGFR extracellular mutations during cetuximab treatment in colorectal cancer. Clin Cancer Res 2015;21(9):2157–66. https://doi.org/10.1158/1078-0432.CCR-14-2821.

[38] Braig F, März M, Schieferdecker A, Schulte A, Voigt M, Stein A, et al. Epidermal growth factor receptor mutation mediates cross-resistance to panitumumab and cetuximab in gastrointestinal cancer. Oncotarget 2015;6(14):12035–47.

[39] Bertotti A, Papp E, Jones S, Adleff V, Anagnostou V, Lupo B, et al. The genomic landscape of response to EGFR blockade in colorectal cancer. Nature 2015;526(7572):263–7. https://doi.org/10.1038/nature14969.

[40] Wang L, Wang ZY. The Wilms' tumor suppressor WT1 induces estrogen-independent growth and anti-estrogen insensitivity in ER-positive breast cancer MCF7 cells. Oncol Rep 2010;23:1109–17. https://doi.org/10.3892/or_00000739.

[41] Ohnaga T, Takei Y, Nagata T, Shimada Y. Highly efficient capture of cancer cells expressing EGFR by microfluidic methods based on antigen-antibody association. Sci Rep 2018;8:12005. https://doi.org/10.1038/s41598-018-30511-9.

[42] Davidson NE, Gelmann EP, Lippman ME, Dickson RB. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinol 1987;1(3):216–23. https://doi.org/10.1210/mend-1-3-216.

[43] Kearns NA, Genga RMJ, Enuameh MS, Garber M, Wolfe SA, Maehr R. Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. Development 2014;141:219–23. https://doi.org/10.1242/dev.103341.

[44] Li J, Zhou J, Zhang D, Song Y, She J, Bai C. Bone marrow-derived mesenchymal stem cells enhance autophagy via PI3K/AKT signalling to reduce the severity of ischaemia/reperfusion-induced lung injury. J Cell Mol Med 2015;19:2341–51. https://doi.org/10.1111/jcmm.12638.

[45] Chang SE, Foster S, Betts D, Marnock WE. DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. Int J Cancer 1992;52(6):896–902. https://doi.org/10.1002/ijc.2910520612.

[46] Dominguez-Escrig JL, Kelly JD, Neal DE, King SM, Davies BR. Evaluation of the therapeutic potential of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib in preclinical models of bladder cancer. Clin Cancer Res 2004;10(14):4874–84. https://doi.org/10.1158/1078-0432.CCR-04-0034.

[47] Yang Y, Tian Z, Ding Y, Li X, Zhang Z, Yang L, et al. EGFR-targeted immunotoxin exerts antitumor effects on esophageal cancers by increasing ROS accumulation and inducing apoptosis via inhibition of the Nrf2-Keap1 pathway. J Immunol Res 2018;2018:1–10. https://doi.org/10.1155/2018/1090287.

[48] Economopoulou P, Kotsantis I, Psyrri A. Tumor microenvironment and immunotherapy response in head and neck cancer. Cancers (Basel) 2020;12:3377. https://doi.org/10.3390/cancers12113377.

[49] Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 2006;24(17):2666–72. https://doi.org/10.1200/JCO.2005.04.8306.

[50] Liu X, Jiang S, Fang C, Yang S, Olalere D, Pequignot EC, et al. Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. Cancer Res 2015;75(17):3596–607. https://doi.org/10.1158/0008-5472.CAN-15-0159.

[51] Ghorashian S, Kramer AM, Onuoha S, Wright G, Bartram J, Richardson R, et al. Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. Nat Med 2019;25(9):1408–14. https://doi.org/10.1038/s41591-019-0549-5.

[52] Ghorashian S, Kramer AM, Albon SJ, Wright G, Castro F, Popova B, et al. A novel low affinity CD19CAR results in durable disease remissions and prolonged CAR T cell persistence without severe CRS or neurotoxicity in patients with paediatric ALL. Blood 2017;130(Suppl_1):806.

[53] Yu X, Curry WT, Gerstner ER, Cahill DP, Nahed BV, Maus MV, et al. Commentary: chimeric antigen receptor T-cell therapy: updates in glioblastoma treatment. Neurosurgery 2021;89(1):E68–9. https://doi.org/10.1093/neuros/nyab099.

[54] Uchibori R, Teruya T, Ido H, Ohmine K, Sehara Y, Urabe M, et al. Functional analysis of an inducible promoter driven by activation signals from a chimeric antigen. Receptor 2019;12:16–25. https://doi.org/10.1016/j.omto.2018.11.003.

[55] Vitanza NA, Johnson AJ, Wilson AL, Brown C, Yokoyama JK, Künkele A, et al. Locoregional infusion of HER2-specific CAR T cells in children and young adults with recurrent or refractory CNS tumors: an interim analysis. Nat Med 2021;27(9):1544–52. https://doi.org/10.1038/s41591-021-01404-8.

[56] Papa S, Adami A, Metoudi M, Achkova D, van Schalkwyk M, Parente Pereira A, et al. A phase I trial of T4 CAR T-cell immunotherapy in head and neck squamous cancer (HNSCC). J Clin Oncol 2018;36(15_suppl):3046.

[57] Larcombe-Young D, Papa S, Maher J. PanErbB-targeted CAR T-cell immunotherapy of head and neck cancer. Expert Opin Biol Ther 2020;20(9):965–70. https://doi.org/10.1080/14712598.2020.1786531.

[58] Fernández de Larrea C, Staehr M, Lopez AV, Ng KY, Chen Y, Godfrey WD, et al. Defining an optimal dual-targeted CAR T-cell therapy approach simultaneously targeting BCMA and GPRC5D to prevent BCMA escape-driven relapse in multiple myeloma. Blood Cancer Discov 2020;1(2):146–54.

[59] Kloss CC, Lee J, Zhang A, Chen F, Melenhorst JJ, Lacey SF, et al. Dominant-negative TGF-β receptor enhances PSMA-targeted human CAR T cell proliferation and augments prostate cancer eradication. Mol Ther 2018;26(7):1855–66. https://doi.org/10.1016/j.ymthe.2018.05.003.

[60] Suarez ER, Chang D-K, Sun J, Sui J, Freeman GJ, Signoretti S, et al. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. Oncotarget 2016;7(23):34341–55.

[61] Dees S, Ganesan R, Singh S, Grewal IS. Regulatory T cell targeting in cancer: emerging strategies in immunotherapy. Eur J Immunol 2021;51(2):280–91. https://doi.org/10.1002/eji.202048992.

[62] Ferguson KM, Berger MB, Mendrola JM, Cho H-S, Leahy DJ, Lemmon MA. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell 2003;11(2):507–17. https://doi.org/10.1016/S1097-2765(03)00047-9.

EINE NEUE AUF CD34-BASIERENDE *HINGE* ZUR SCHNELLEN UND EFFIZIENTEN ANREICHERUNG UND DETEKTION VON CAR-T-ZELLEN

Titel: A novel CD34-derived hinge for rapid and efficient enrichment and detection of CAR T-cells

Autoren: Arthur Bister, Tabea C. Ibach, **Corinna Haist**, Denise Smorra, Katharina Roellecke, Martin Wagenmann, Kathrin Scheckenbach, Norbert Gattermann, Constanze Wiek*, Helmut Hanenberg*

* Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen

Eigenleistung: 10 %

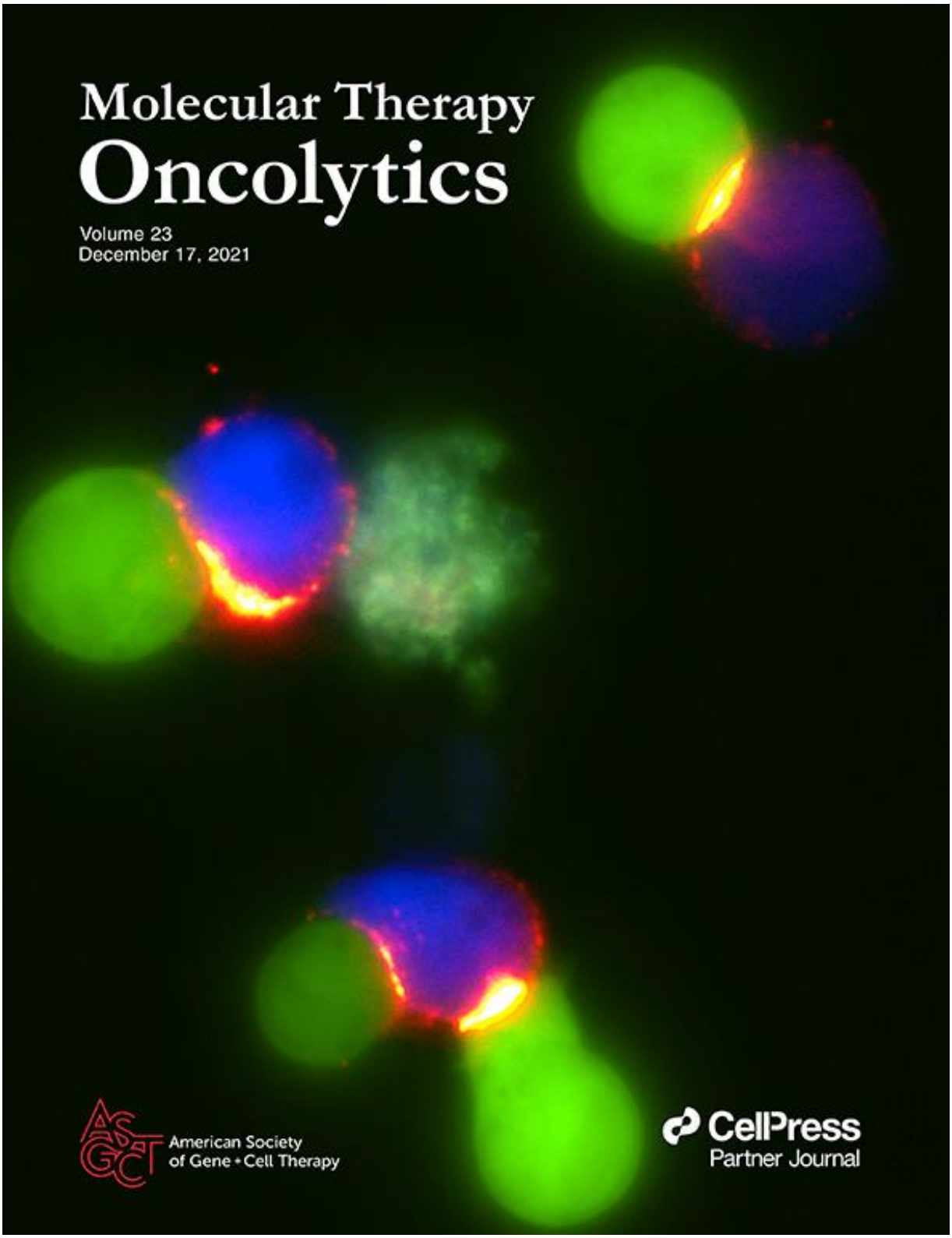
- Hilfestellung bei der Durchführung der Experimente, Schreiben und Korrektur des Manuskripts

Veröffentlicht in: Molecular Therapy – Oncolytics

Impact Factor: 7,200

Status: veröffentlicht

DOI: 10.1016/j.omto.2021.11.003



On the Cover: Formation of immunological synapses between CD19 CAR T-cells and CD19+ REH ALL cells. Three genetically modified T-cells (blue cells) attack via their CD19 CAR construct (red membrane staining) either a single (top), two (middle) or three (bottom) REH cells (green cells). Untransduced T-cells in the cultures do not express any fluorescence (see bright field image).

Molecular Therapy
Oncolytics
Original Article

A novel CD34-derived hinge for rapid and efficient detection and enrichment of CAR T cells

Arthur Bister,[1,2] Tabea Ibach,[1] Corinna Haist,[1,2] Denise Smorra,[2] Katharina Roellecke,[1] Martin Wagenmann,[1] Kathrin Scheckenbach,[1] Norbert Gattermann,[3] Constanze Wiek,[1,4] and Helmut Hanenberg[1,2,4]

[1]Department of Otorhinolaryngology, Head & Neck Surgery, Heinrich Heine University, 40225 Düsseldorf, Germany; [2]Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Hufelandstrasse 55, 45147 Essen, Germany; [3]Department of Hematology, Heinrich Heine University, 40225 Düsseldorf, Germany

Immunotherapy including chimeric antigen receptor (CAR) T cell therapy has revolutionized modern cancer therapy and has achieved remarkable remission and survival rates for several malignancies with historically dismal outcomes. The hinge of the CAR connects the antigen binding to the transmembrane domain and can be exploited to confer features to CAR T cells including additional stimulation, targeted elimination or detection and enrichment of the genetically modified cells. For establishing a novel hinge derived from human CD34, we systematically tested CD34 fragments of different lengths, all containing the binding site of the QBend-10 monoclonal antibody, in a FMC63-based CD19 CAR lentiviral construct. A final construct of 99 amino acids called C6 proved to be the best candidate for flow cytometry-based detection of CAR T cells and >95% enrichment of genetically modified T cells on MACS columns. The C6 hinge was functionally indistinguishable from the commonly used CD8α hinge *in vitro* as well as in *in vivo* experiments in NSG mice. We also showed that the C6 hinge can be used for a variety of different CARs and mediates high killing efficacy without unspecific activation by target antigen-negative cells, thus making C6 ideally suited as a universal hinge for CARs for clinical applications.

INTRODUCTION

Chimeric antigen receptors (CARs) are part of a novel immunotherapeutic approach potentially suitable for a wide range of malignancies.[1,2] In CAR constructs, the single-chain variable fragment (scFv) of a monoclonal antibody (mAb) is linked with a hinge to a transmembrane region and at least one intracellular T cell activation motif, thereby combining antigen recognition and T cell activation in a single molecule.[1,2] Consequently, autologous T cells equipped with a CAR construct can detect and eliminate the target antigen-expressing tumor cells in a major histocompatibility complex-independent fashion.[1,2] In the last decade, major clinical breakthroughs have been achieved with CARs targeting CD19 and other antigens on B cell lineage-derived leukemia and lymphoma cells, which led to several CAR T cell therapies being approved in the United States and Europe for hematological malignancies.[1–3]

In CAR constructs, not only the scFv and the cytoplasmic signaling domains but also the hinge domain can greatly influence expression, stability and flexibility of the CAR and can enhance expansion as well as persistence of T cells.[4–7] Moreover, its length and flexibility are crucial for optimal recognition and binding to the antigen epitope by the scFv. Longer hinges are needed for recognizing target motifs, where the epitope is located proximal to the membrane of target cells or embedded within heavily glycosylated structures; shorter hinges are preferred when the epitope is located distal to the membrane and easily accessible.[8–12] Importantly, the hinge can also bind ligands itself. For example, constant heavy domains from human immunoglobulin G (IgG) have been used as hinges in CARs, but caused off-target toxicities due to binding to its natural receptors, FcγRI, FcγRII and FcγRIII, on immune cells, which led to unspecific T cell activation, exhaustion and activation-induced cell death, ultimately resulting in insufficient disease control *in vivo* in animals.[11,13] Introducing mutations or truncations in the FcγR binding domains can abrogate the off-target binding/activation and improved CAR function and efficacy.[4,11,14] Of note, the hinge in lisocabtagene maraleucel/JCAR017/Breyanzi, the US Food and Drug Administration (FDA)-approved CAR T cell therapy of Juno Therapeutics/Bristol Meyers Squibbs for $CD19^+$ B cell lymphomas, harbors a 12-amino acid (aa) IgG4 hinge without the CH_2CH_3 sequence,[11,15] while the hinges in the other FDA-approved CAR T cell products are derived from CD28 (Yescarta, Tecartus) or from CD8α (Kymriah).[16] The latter two antigens are both naturally expressed on T cells and thus can be considered safer for clinical use than the artificial expression of immunoglobulin protein sequences on T cells.

The hinge in CARs can facilitate elimination of the genetically modified T cells *in vivo* by including epitopes that can be specifically targeted, e.g., by administering mAbs such as rituximab[17] or by using CAR T cells that are redirected against the specific epitope in the hinge.[18] Other groups modified the hinge to include epitopes derived from CD34,[17] NGFR[19] or artificial sequences such as Strep-Taq II[20] to

Received 4 August 2021; accepted 8 November 2021;
https://doi.org/10.1016/j.omto.2021.11.003.

[4]These authors contributed equally

Correspondence: Helmut Hanenberg, MD, Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany.
E-mail: helmut.hanenberg@uk-essen.de

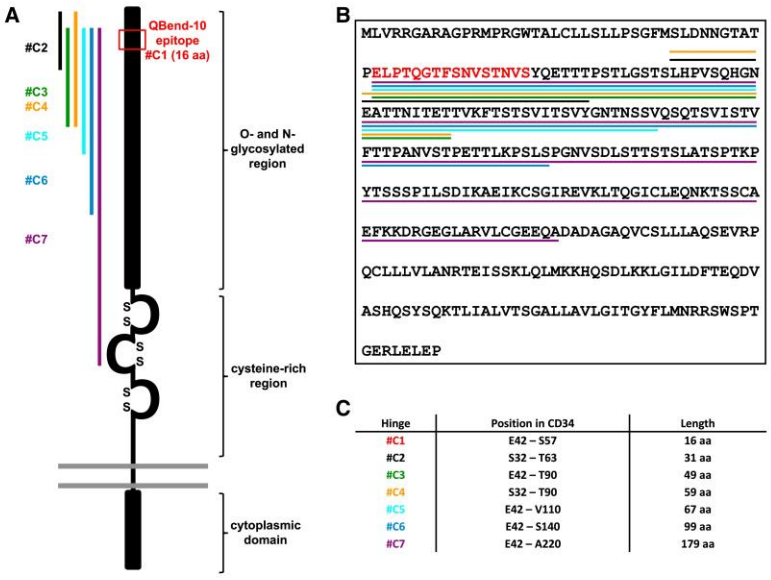


Figure 1. Structure and amino acid sequence of human CD34

(A) Human CD34 consists of a heavily *O*- and *N*-glycosylated region followed by a six-cysteine rich domain with three IgG-like domains and a cytoplasmic domain. The 16 aa QBend-10 epitope (#C1) is located close to the N terminus and the newly derived hinges #C2–#C7 are designed around this epitope. (B) Full amino acid sequence of human CD34. The critical 16 aa stretch is marked in red. Amino acid sequences of hinges #C2–#C7 are underlined in the corresponding color. (C) Overview of the length and position within the CD34 molecule of #C1–#C7.

enable enrichment as well as flow-cytometric detection of CAR T cells. Flow cytometry, especially in research settings, is optimally suited for determining the immunological phenotypes of CAR-expressing cells and also for comparing the expression patterns of different CAR constructs on the transgenic immune effector cells.[14,19] In clinical settings, these analyses are often much more laborious and rely on qRT-PCR[21,22] droplet digital PCR,[23] RNA sequencing,[24] positron emission topography,[25] antibodies against the scFv[26,27] linkers between heavy and light chain,[28] Fc-tagged antigens[23,29] or the expression of separate cell-surface marker genes co-expressed in the CAR lentiviral constructs, e.g., truncated epidermal growth factor receptor.[15,30]

When designing a new hinge for potential clinical purposes, we considered the human CD34 adhesion molecule to be ideally suited as candidate, as CD34 is of human origin and not expressed on mature immune effector cells, including human T and natural killer (NK) cells; also the natural ligands for CD34, CD62L, CD62E and CD62P, are well known.[31] GMP-grade immunomagnetic CD34 enrichment reagents (MACS; Miltenyi Biotec) are commercially available and have been used for more than 15 years to enrich $CD34^+$ hematopoietic stem cells from different source materials for human stem cell transplantation without the need to remove the microbeads from the infused products.[32,33] The aim of this work was to systematically establish a human CD34-derived hinge for CAR constructs, which facilitates to routinely enrich CAR T cells to high purities and which functions well in a variety of CAR constructs *in vitro* and *in vivo* comparably to a clinically used human CD8-derived hinge.

RESULTS

Vector, CAR and hinge design

The CD34 MACS enrichment system from Miltenyi Biotec is based on the CD34 antibody clone QBend-10 recognizing a well-defined 16-aa sequence, ELPTQGTFSNVSTNVS,[34] which we named C1 and that is located in a heavily *O*- and *N*-glycosylated region of the protein (Figure 1A). We first designed six hinges of different lengths by adding amino acid stretches from the wild-type protein up- and/or downstream of the QBend-10 recognition site in CD34, labeled as C2 (31 aa), C3 (49 aa), C4 (59 aa), C5 (67 aa), C6 (99 aa) and C7 (179 aa), shown in Figures 1B and 1C.

Expression and enrichment of CD34-hinged CARs in Jurkat cells

The CD34 fragments C2–C7 were cloned as hinges into our previously published CD19 CAR lentiviral vector, which co-expresses the enhanced green fluorescent protein (EGFP) and a human codon usage-optimized FMC63-based second-generation CAR construct with the transmembrane and cytoplasmic region of human CD28 via a T2A site under the control of the MPSV promoter (Figure 2A).[35] To test whether cells expressing CD34-hinged CARs can be detected by antibody staining and can be enriched by CD34 microbeads, Jurkat cells were transduced with the six lentiviral CD19 CAR constructs harboring C2–C7 as hinges. Flow-cytometric analysis of the transduced Jurkat cells stained with the QBend-10 CD34-PE antibody revealed that EGFP expression for all six constructs strongly correlated with the CAR expression levels (Figure 2B). Next, batches of transduced Jurkat cells were incubated with CD34 microbeads and subjected to one round of enrichment on MACS columns. Prior to the enrichments, the transduction efficiencies for C2-, C3-, C4-, and C5-hinged CARs were very similar (61.6% ± 2.7% to 68.7% ± 2.7%), while C6-hinged CARs showed the lowest, 50.7% ± 10.5%, and C7-hinged onstructs the highest transduction efficiency of 78.4% ± 3.2% (Figure 2C; preMACS). For all hinges, the enrichment with CD34 microbeads led to $EGFP^+$ populations well above 90%, with C4, C5 and C7 having the purest postMACS populations, 97.8%, 97.1%, and 98.8%, respectively (Figure 2C; postMACS). However, compared with the percentages of $EGFP^+$ cells prior to MACS

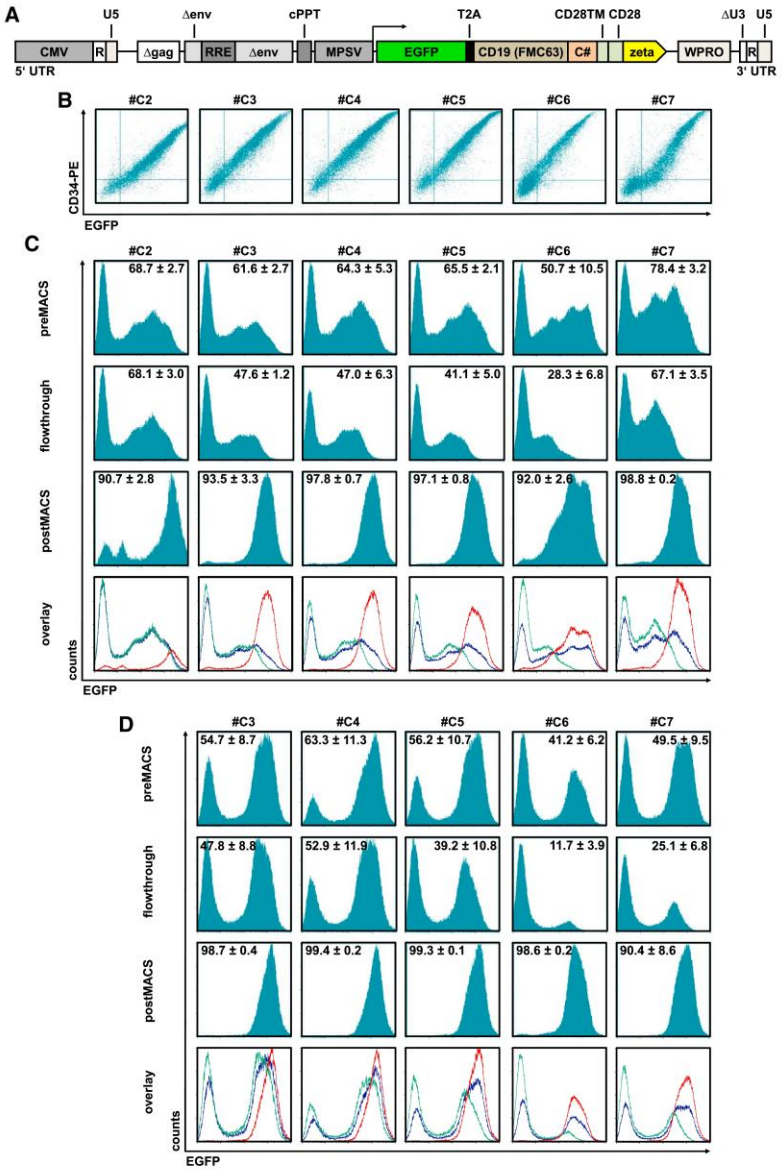


Figure 2. CD34-hinged CAR expression and enrichment

Jurkat or primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding EGFP in *cis* with ΔCD34-hinged CD19 CARs (vector depicted schematically in A). (B) Three days after transduction, Jurkat cells were flow-cytometrically analyzed for CAR (via CD34-PE) and EGFP expression. ΔCD34-hinged CD19 CAR (C) Jurkat or (D) primary human T cells were enriched via magnetic cell sorting using CD34 microbeads, and the three fractions (preMACS, flowthrough, and postMACS) were analyzed for EGFP expression by flow cytometry. In the overlay, preMACS is depicted as a blue line, the flowthrough as a green line, and postMACS as a red line. Representative blots were used. Values indicate the percentages of CAR-positive cells and are shown as means ± SEM from at least three experiments.

selection, the enrichments of C2–C5 hinged CARs were quite inefficient, as the majority of the CAR-expressing cells was lost in the flowthrough (Figure 2C; flowthrough). Here, the C6 hinge proved to be most efficient for selection purposes, as only 28.3% out of 50.7% (55.8%) mostly low EGFP-expressing CAR cells were present in the flowthrough of the columns and 92.0% transduced cells could be eluted from the columns. Due to the high inefficiency for selection on MACS columns, the C2-hinge construct was not further analyzed.

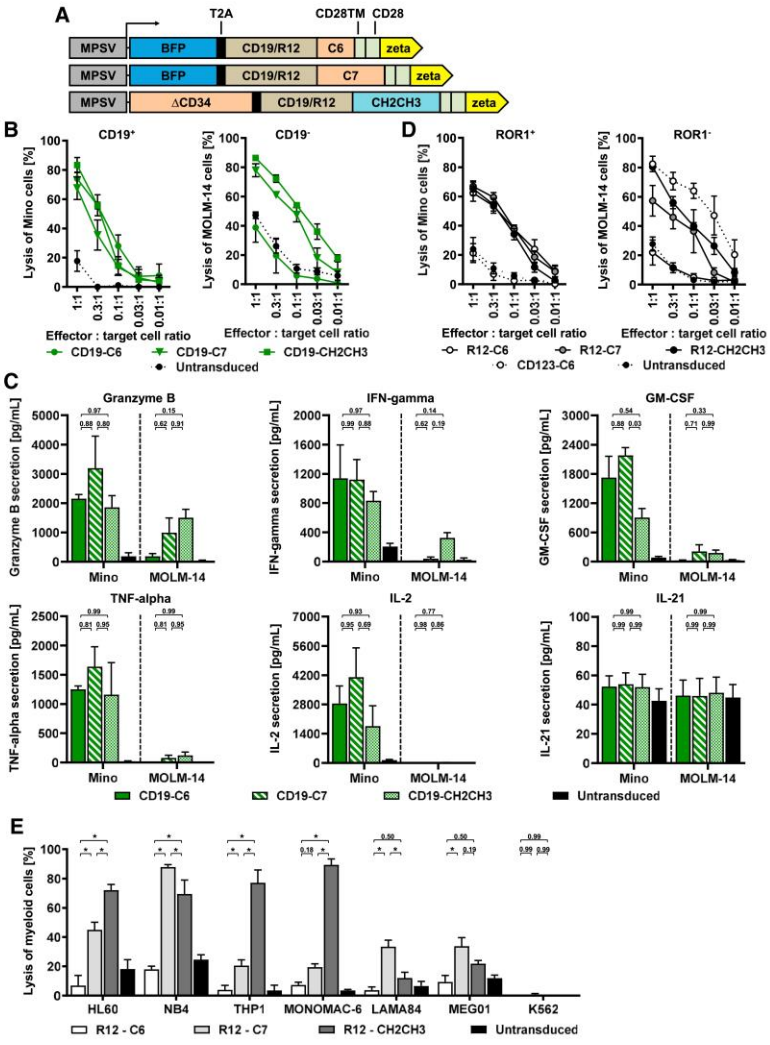


Figure 3. Comparison of C6- and C7-hinged CD19 and R12 CARs with CH_2CH_3-hinged counterparts

Primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP in *cis* with C6- or C7-hinged or ΔCD34 in *cis* with CH_2CH_3-hinged CD19 or R12 CARs (vectors depicted schematically in A). Three days after transduction, the CAR T cells were enriched via MACS and subsequently co-cultured with (B and D) EGFP-expressing Mino or MOLM-14 cells at decreasing effector-to-target cell ratios or (E) with various myeloid cell lines at an effector-to-target cell ratio of 1:1. After 16 h, the target cell lysis was assessed by flow cytometry and (C) supernatants were harvested and analyzed for granzyme B, IFN-γ, GM-CSF, IL-2, TNF-α and IL-21 secretion via the cytotoxic T and NK cell MACSPlex cytokine assay. P values were calculated by two-way ANOVA (B, D, and E) or one-way ANOVA (C) followed by Tukey's or Dunnett's multiple comparison testing, respectively; asterisks indicate p values of <0.05; the p values of the lysis curves are summarized in Table S1. Data are presented as means ± SEM of at least three biological replicates.s

Expression and enrichment of CD34-hinged CARs in primary T cells

Next, primary human T cells were transduced with the C3–C7-hinged CD19 CAR lentiviral constructs. Three days later, the T cells were harvested, stained with CD34 microbeads and subjected to enrichment for CAR-expressing cells on MACS columns. Flow-cytometric analysis of the T cell cultures prior to selection demonstrated that the transduction efficiencies ranged between 41.2% ± 6.2% for the C6 construct and 63.3% ± 11.3% for C4 (Figure 2D: preMACS). One run over the MACS columns was sufficient to enrich the CAR-positive T cells to high purities between 90.4% ± 8.6% for C7 and 99.4% ± 0.2% for C4 (Figure 2D; postMACS). Similar to the experiments with Jurkat, the use of the shorter hinges C3–C5 was associated with inefficient enrichment of transduced cells, as the majority of CAR T cells was lost in the flowthrough of the columns (Figure 2D; flowthrough). The C6 hinge with 99 aa proved to be highly efficient to retain the majority of CAR T cells on the MACS column, as only 11.7% out of 41.2% (28.4%) of T cells with low expression of EGFP/CAR were detectable in the flowthrough (Figure 2D). With respect to the ability to retain the CAR T cells on the column, the C7 construct came in as the second best with slightly lower purity (90.4%).

Killing efficacy and specificity of CD19 CARs with C6, C7, or CH_2CH_3 hinges

As the CH_2CH_3 fragments of human IgG1 or IgG4 have been commonly used as hinges for CARs,[11,14] we next compared the cytotoxic efficacy and specificity of CD19 CAR T cells with the C6 or C7 hinges against a corresponding CH_2CH_3-hinged CAR construct. To enable enrichment of the transduced T cells on MACS columns in this set of experiments, we transduced primary human T cells with vectors encoding blue fluorescent protein (BFP) in *cis* with C6- or C7-hinged or the previously described ΔCD34,[35] which is truncated after the transmembrane domain and thus lacks the signaling domains, in *cis* with CH_2CH_3-hinged CD19 CARs (Figure 3A). The CAR T cells were enriched via CD34 MACS to >90% and then co-cultured with the $CD19^+$ $ROR1^+$ mantle cell lymphoma (MCL) cell line Mino or the $CD19^-$ $ROR1^-$ acute myeloid leukemia (AML) cell line MOLM-14 (Figure S1) for 16 h with subsequent cytotoxicity and cytokine secretion measurements. We also included a CD123 CAR[36] with the C6 hinge in these experiments, which served as a negative control for Mino and a positive control for MOLM-14 cells.

All three CD19 CAR constructs showed comparable cytotoxicity when expressed on T cells (Figure 3B) and also comparable secretion of granzyme B, interferon-γ (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α) and interleukin-2 (IL-2) against Mino cells (Figure 3C). Similarly, T cells expressing the C6-, C7- and CH_2CH_3-hinged ROR1 CARs based on the mAb clone R12[37] equally efficaciously eliminated Mino cells (Figure 3D). In contrast, when using a CAR construct based on the ROR1 mAb clone R11, which targets a membrane-proximal epitope,[37] only the long CH_2CH_3 hinge mediated recognition and killing of Mino cells (Figure S2A).

The $CD19^-$ and $ROR1^-$ MOLM-14 cell line was nonspecifically lysed by C7- and CH_2CH_3-hinged CARs, including the R11-C7 CAR, but not by C6-hinged CD19 or ROR1 CARs (Figures 3B and 3D; Figure S2A). Importantly, the off-target toxicity of C7- and CH_2CH_3-hinged CARs was not restricted to MOLM-14 cells, but also occurred when the R12-C7 (but not R12-C6) CAR T cells were incubated with other $ROR1^-$ acute and chronic myeloid leukemia cell lines (Figure 3E). Consistent with the lysis data, CD19-C7 and CD19-CH_2CH_3, but not CD19-C6 CAR T cells secreted granzyme B and also low levels of IFN-γ, GM-CSF, and TNF-α after co-culture with MOLM-14 cells (Figure 3C). While the CH_2CH_3-mediated lysis of MOLM-14 was presumably caused by the binding of the CH_2CH_3 hinge to Fc receptors on MOLM-14,[14] the reason for C7-mediated CAR T cell activation was unknown. Nevertheless, we were able to exclude CD34 microbeads stuck to the C7 hinge as an inducer of the off-target toxicity, as the unspecific activation of C7-hinged CD19 CAR T cells also occurred when MOLM-14 cells were co-cultured with non-enriched CAR T cells (Figure S2B).

CD34 glycosylation plays no role for the activation of C7-hinged CARs by AML blasts

Post-translational modifications of the adhesion molecule CD34 and its main ligand on leukocytes, L-selectin, are essential for homing of leukocytes to several organ systems and occur by *N*-linked or *O*-linked glycosylation of CD34.[38,39] Importantly, for the adhesion mediated by L-selectin, CD34 needs to carry sulfated sialyl Lexis X (6-sulfo SLe^X) epitopes on either core-1 or core-2 sugar structures.[38] As *N*-linked glycans seem to play no major role for the binding to L-selectin,[39] we focused our efforts here on the 70 potential extracellular *O*-linked glycosylation sites present in CD34. According to the predictions from the NetOGlyc server (www.cbs.dtu.dk), the attachment of a sugar molecule to the oxygen of serine or threonine occurs at approximately 30 of these sites[39] and as many as fifteen are located in the fragment of human CD34 that is present in C7 but not in C6.

Combining three separate *O*-glycosylation prediction algorithms,[40,41] we mutated the three top hits (S109, T110, S123) in the R12 CAR C7 construct, either singly or in combination, to glutamine, giving rise to seven altered C7 hinges (S109Q, T110Q, S123Q, S109Q + T110Q, S109Q + S123Q, T110Q + S123Q, and S109Q + T110Q + S123Q). When expressed on primary T cells, R12 CAR constructs with altered C7 hinges had transduction and MACS enrichment efficiencies comparable with those of their unaltered counterparts (data not shown). When co-cultured with $ROR1^+$ Mino cells, the R12 CARs with altered C7 hinges showed the same lytic capabilities as the original C7-hinged CARs, demonstrating that the S/T > Q substitutions did not render the CAR ineffective. However, when co-cultured with $ROR1^-$ MOLM-14 cells, the cells were still nonspecifically eliminated by ROR1 CARs with mutated C7 hinges, while the $ROR1^-$ Granta519 cells were not killed at all (Figure 4A).

To exclude that our prediction had missed a crucial *O*-glycosylation site, we mutated all 15 serine and threonine residues in C7, which were not present in C6, to glutamine. When introduced into the R12 CAR construct, the C7 15xQ hinge showed the same transduction efficiency, MACS enrichment efficacy and purity of the eluted fraction as the wild-type C7 hinge (data not shown). The lysis against $ROR1^+$ Mino cells was comparable to the lysis of C6- and C7-hinged R12 CARs, hence the cytotoxic capabilities were not altered by the 15 S/T > Q substitutions (Figure 4B). However, the unspecific lysis against $ROR1^-$ MOLM-14 cells still occurred, suggesting that the binding of the C7 hinge to its unknown target structure was not dependent on O-linked glycolysation of CD34. As the two MCL cell lines express L-selectin and also showed weak staining with an E-selectin mAb by flow cytometry and MOLM-14 cells analyzed in parallel stained negative (Figure 4C), we can exclude that selectins are the unknown structure(s) on MOLM-14 and other myeloid cells, which bind the C7 hinge in our CAR constructs.

Comparison of C6-hinged CD19 and CD33 CARs with their CD8-hinged counterparts

Some CAR constructs currently tested in clinical trials use a short hinge derived from the human CD8α chain.[2,42] We therefore compared the killing efficacy and specificity of CD19 and CD33 CAR constructs with a C6 hinge to analogous constructs with a CD8 hinge. To enable selection of the CD8-hinged constructs with CD34 microbeads, primary human T cells were transduced with lentiviral vectors encoding BFP or ΔCD34 in *cis* with C6- or CD8-hinged CD19 or CD33 CARs (Figure 5A), enriched via CD34 microbeads on MACS columns and then co-cultured with Mino, REH and MOLM-14 cells for 16 h. Mino cells, which express CD19 but not CD33 (Figure S1), were eliminated by C6- and CD8-hinged CD19 CARs, but not by CD33 CAR T cells (Figure 5B). Similarly, REH cells ($CD19^+$ $CD33^-$, Figure S1) were efficaciously killed by both CD19 CAR T cells, however also experienced nonspecific toxicity of CD33-CD8h CAR T cells from two out of four healthy donors. A similar observation was not made with the corresponding C6-hinged CD33 CAR construct (Figure 5B). Finally, MOLM-14 cells, characterized by the absence of CD19 and high CD33 expression (Figure S1), were comparably lysed by both CD33 CARs but not by the CD19 CARs (Figure 5B).

When analyzing the culture supernatants, CD19 CAR T cells only secreted granzyme B, IFN-γ, GM-CSF, TNF-α and IL-2 when cultured with the $CD19^+$ Mino and REH cells (Figure 5C).

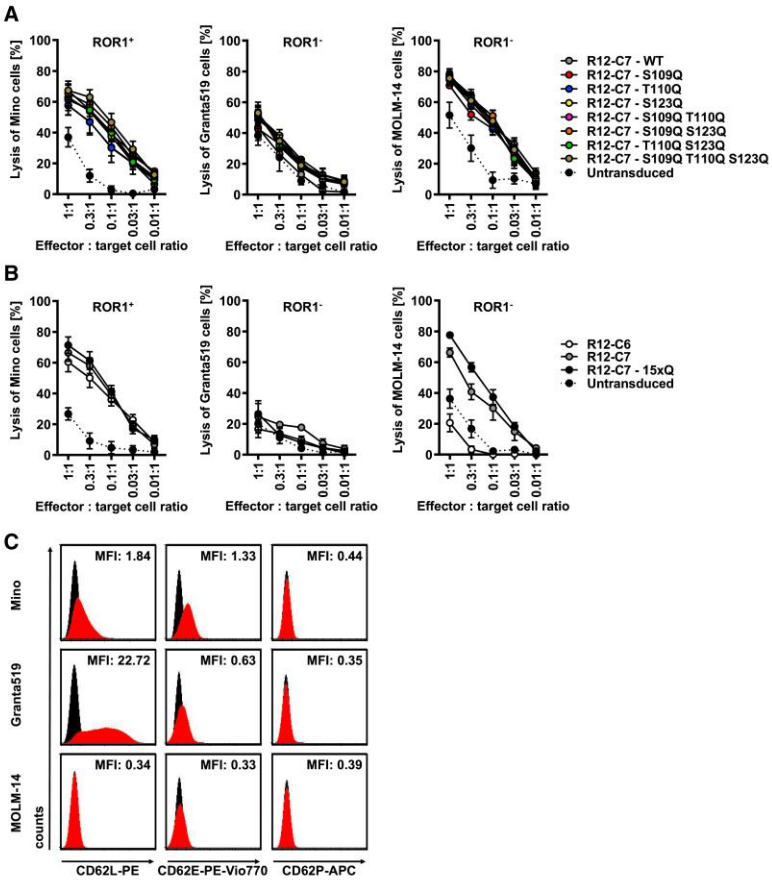


Figure 4. Unspecific lysis by C7-hinged ROR1 CARs does not depend on *O*-glycosylation

Primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP in *cis* with C6-, C7- or mutated C7-hinged ROR1 CARs (clone R12). (A and B) Three days after transduction, the CAR T cells were enriched via MACS and subsequently co-cultured with EGFP-transduced Mino, Granta519 or MOLM-14 cells at decreasing effector-to-target cell ratios. After 16 h, the target cell lysis was assessed by flow cytometry. (C) Mino, Granta519 and MOLM-14 cells were stained with CD62L-PE, CD62E-PE-Vio770 and CD62P-APC and the antigen expression profiles were assessed by flow cytometry. P values were calculated by two-way ANOVA followed by Tukey's multiple comparison testing (A and B) and are summarized in Table S1. Data in (A) and (B) are presented as means ± SEM of at least three biological replicates.

Interestingly, although Mino induced higher levels of granzyme B, IFN-γ and GM-CSF, REH cells were more efficaciously killed. Although the CD33-CD8h CAR T cells eliminated REH cells at lower efficiencies (Figure 5B), this non-specific killing was not reflected in the cytokine secretion profiles (Figure 5C). In line with the cytotoxicity data, CD33 CAR T cells secreted, when co-cultured with MOLM-14 cells, granzyme B, IFN-γ, GM-CSF and TNF-α. Interestingly only minimal amounts of IL-2 were detected here, when compared with the CD19 CAR T cells co-cultured with Mino or REH cells (Figure 5C). In summary, the C6 hinge proved to be as specific and efficacious as the CD8-derived hinge *in vitro*.

The C6 hinge can be used for a wide variety of CARs

Subsequently, we tested the usability of the C6 hinge for several CARs based on single scFvs against ROR1, CD5, CD19, CD33 and CD123. Primary human T cells were transduced with vectors encoding BFP in *cis* with C6-hinged CARs (Figure 5A), enriched via MACS, and co-cultured with Mino, JeKo-1, Granta519, MOLM-14, REH or Jurkat cells (see Figure S1 for the CD19, CD5, ROR1, CD33 and CD123 cell-surface antigen expression profiles). When targeting ROR1, CAR T cells only eliminated cells of the ROR1$^+$ cell lines Mino and JeKo-1. CD5 CAR T cells efficaciously eliminated Mino and Jurkat cells and to a lesser extent JeKo-1 cells, which are only partially CD5$^+$. CD19 CARs were effective in killing the CD19-expressing cell lines Mino, JeKo-1, Granta519 and REH, but not kill cells of the CD19$^-$ cell lines MOLM-14 and Jurkat. CD33 CAR T cells specifically eradicated MOLM-14 cells and CD123 CAR T cells eliminated Granta519 and MOLM-14 cells (Figure 6). Thus, all tested CARs were functional with our newly established C6 hinge and the killing characteristics of the various C6-hinged CAR T cells against established hematopoietic cell lines strongly correlated with the antigen expression levels of the target antigens.

C6- and CD8-hinged CAR constructs mediate equal leukemia control *in vivo*

Finally, we explored the performance of the C6 hinge in xenotransplantation studies *in vivo*. Therefore, 8- to 10-week-old female NOD-SCID gamma (NSG) mice were xenografted with 3×10^6 CD19$^+$ REH/ffluc-EGFP cells, expressing a fusion protein of human codon-optimized firefly luciferase and EGFP.[43] Seven days later, 3×10^6 primary human T cells expressing BFP/CD19-C6, ΔCD34/CD19-CD8h, BFP/CD33-C6 or ΔCD34/CD33-CD8h constructs (Figure 5A) were intravenously injected after MACS enrichment without any conditioning (Figure 7A). Mice were monitored for the persistence and growth of REH/ffluc-EGFP cells at days 6, 14, 20, 28, and 38 via luminescence imaging and blood sample analysis (Figure 7). Mice in the control group (Untreated) showed the characteristic clinical presentation of a pre-B cell leukemia and had to be sacrificed between days 19 and 23 due to high disease burden (Figures 7A–7C).

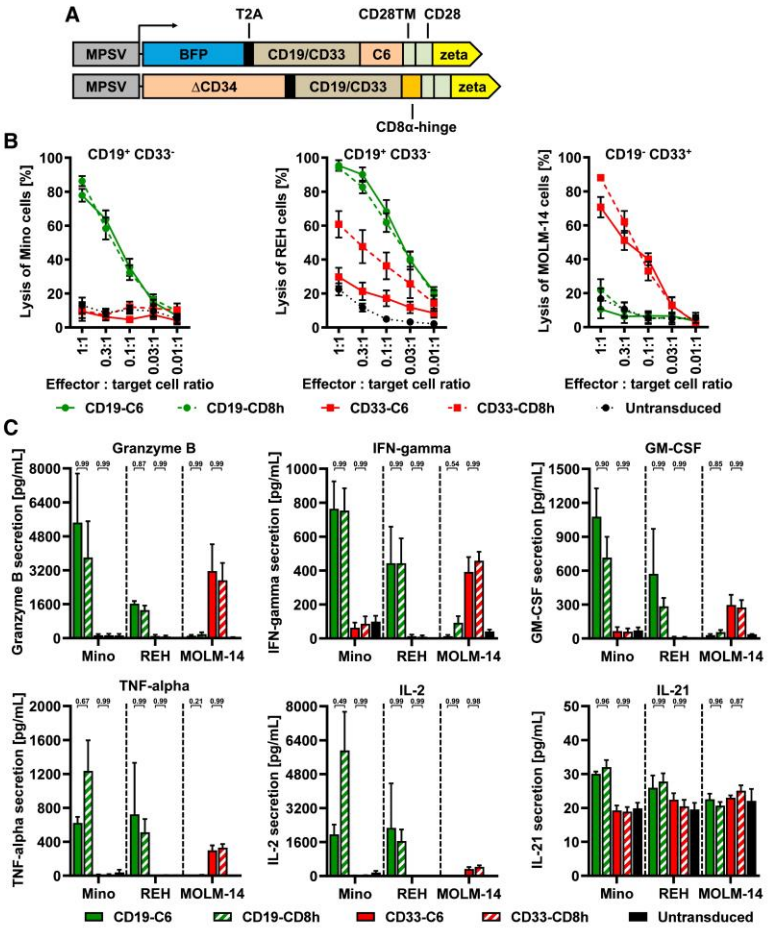


Figure 5. C6-hinged CD19 and CD33 CAR constructs on T cells are as efficient as their CD8-hinged counterparts *in vitro*

Primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP in *cis* with C6-hinged or ΔCD34 in *cis* with CD8-hinged CD19 or CD33 CARs (vectors depicted schematically in A). (B) Three days after transduction, the CAR T cells were enriched via MACS and subsequently co-cultured with EGFP-transduced Mino, REH and MOLM-14 cells at decreasing effector-to-target cell ratios. After 16 h, the target cell lysis was assessed by flow cytometry and (C) supernatants were harvested and analyzed for secretion of granzyme B, IFN-γ, GM-CSF, IL-2, TNF-α and IL-21 via the MACSPlex cytokine assay. p values were calculated by two-way ANOVA (B) or one-way ANOVA (C) followed by Tukey's or Dunnett's multiple comparison testing, respectively; asterisks indicate a p value of <0.05, and p values of lysis curves are summarized in Table S1. Data are presented as means ± SEM of at least three biological replicates.

Both CD33 CAR constructs (C6- or CD8-hinged) were not able to control the leukemia growth, and all animals in these two groups also had to be sacrificed between days 19 and 23, when the leukemia burden reached critical levels (Figures 7A–7C). For these three animal groups, we observed no CAR T cell persistence in the peripheral blood or bone marrow at sacrifice by flow cytometry, but detected a steadily increasing REH cell population in the blood (Figures 7D and 7E).

In contrast, mice that received CD19 CAR T cells showed a significantly prolonged survival and the persistence of REH/ffluc-EGFP cells was markedly lower or even abolished, as shown via luminescence imaging and blood sample analysis (Figures 7A–7D). Importantly, the CD19-C6 CAR construct proved, with the exception of one animal in which we had problems during the CAR T cell injection, to be as efficient as the CD8-hinged counterpart construct by days 14 and 20. At days 28 and 38, we noted a recurrence/persistence of low levels of ALL cells in some NSG mice in CD19-C6 as well as CD19-CD8h animals (Figure 7A). For both CD19 CAR constructs, it was possible to detect transduced T cells in the peripheral blood of animals by flow cytometry when staining with CD3-PerCP-Vio700, CD45-APC, and CD34-PE (QBend-10) antibodies. As a reflection of the *in vivo* expansion of the CD19 CAR T cells in reaction to the leukemia cell burden, mice initially even showed around 12% CD19-C6 and 10% CD19-CD8h CAR T cells in their peripheral blood, which decreased to around 2% and 0.5%, respectively, after 38 days (Figure 7E).

DISCUSSION

Compared with other immunotherapies, autologous CAR T cells have shown an unprecedented efficacy for relapsed and/or refractory liquid malignancies of the B cell lineage.[1] This level of efficacy has been achieved with relatively simple overexpression strategies/vectors in simple treatment protocols and by targeting tumor-associated antigens (TAAs), for which the loss of the normal antigen-positive cells is clinically tolerated.[1] However, the future goals for CAR therapies must include to make these treatments more affordable and also effective for other malignancies, including solid tumors, where antigen heterogeneity and an immunosuppressive environment are major challenges.[2] In addition, using alternative allogeneic immune effector cells will require increased safety measures to prevent adverse immunological reactions, e.g., by inclusion of a suicide gene.[44] Thus, developing CAR therapies further will require more complex vector constructs with elements that have specific functions. With our CD34-derived 99 aa hinge C6, we have established an important hinge element for CARs that is functionally indistinguishable from the commonly used CD8α hinge, but additionally facilitates antibody-based

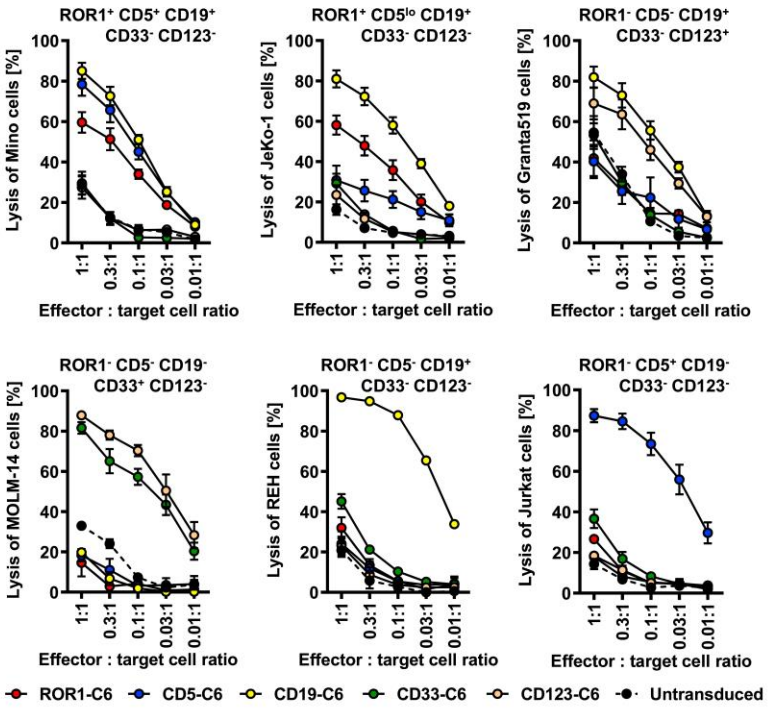


Figure 6. The C6 hinge can be used for a variety of CARs against hematological malignancies

Primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP in *cis* with C6-hinged ROR1, CD5, CD19, CD33 or CD123 CARs. Three days after transduction, the CAR T cells were enriched via MACS and subsequently co-cultured with EGFP-expressing Mino, JeKo-1, Granta519, MOLM-14, REH or Jurkat cells at decreasing effector-to-target cell ratios. After 16 h, the target cell lysis was assessed by flow cytometry. p values were calculated by two-way ANOVA followed by Tukey's multiple comparison testing and are summarized in Table S1. Data are presented as means ± SEM of at least three biological replicates.

detection of CARs on transduced cells and microbead-mediated enrichment of genetically modified CAR effector cells to high purity under GMP conditions.

Characteristics of the hinge

The majority of clinically tested and approved CAR products harbor hinges derived from CD8 or CD28,[42] which are, depending on which sequences are used, approximately 40–50 aa long.[7,45] Often, the epitopes in TAAs recognized by scFVs are located membrane-distal or are embedded within rather small antigen structures and therefore the CAr constructs do not need longer hinges. For example, the epitope for the high-affinity scFV of FMC63, the recognition unit for the most commonly studied CD19 CAR so far, is located in a membrane-distal area of CD19 and therefore readily accessible by CAR constructs with short as well as long hinges.[8,11] Still, certain scFVs exist where the length of the hinge is critical for efficient recognition of the TAA and killing of the target cells by CAR T cells. Hudecek et al.[11] demonstrated that ROR1 CARs derived from mAb clone R11, which binds an epitope of ROR1 in the Kringle domain close the cell membrane,[37] needs a full-length hinge (CH_2CH_3, 229 aa) to function properly. Reducing the hinge length to 119 or 12 aa completely abolishes the CAR-mediated cytotoxicity *in vitro* as well as *in vivo*.[11] These findings are in line with our data here, where the C6 (99 aa) and C7 (179 aa) hinges in the R11 CAR construct were insufficient to eliminate $ROR1^+$ MCL cells with R11 CAR T cells *in vitro*. In another study, Hudecek et al.[10] reported that ROR1 CARs based on the mAbs 2A2 and R12 work best with a short hinge of 12 aa, as the epitopes of these two scFvs are located in the immunoglobulin-like domain located near the NH_2 terminus of ROR1, distal from the membrane.[37] In contrast, our R12 CAR T cells showed efficient lysis of $ROR1^+$ cells, irrespective of the incorporated hinge (99, 179 and 229 aa). One reason for these divergent results might be that, due to the MACS enrichment of genetically modified T cells providing us cells with high CAR expression levels, our CAR T cells are more efficient killers, especially considering that Hudecek et al. used effector-to-target cells ratios from 30:1 to 1:1, while ratios from 1:1 to 0.01:1 were sufficient in our cytotoxic assays. Two additional target antigen structures, where the hinge length appears to be critical for existing CARs using scFvs against membrane-proximal epitopes, are NCAM and the oncofetal antigen 5T4, both targetable with an IgG1-derived CH_2CH_3 long hinge.[8] The glycosylation of the targeted antigen can be another factor where epitopes embedded within heavily glycosylated regions of a protein can only be targeted with long and flexible hinges. CARs against MUC1, whose glycosylation is frequently dysregulated in malignancies, also rely on the incorporation of a longer hinge derived from IgD (103 aa) to be functional.[12]

Casucci et al.[19] included surface sequences of the human low-affinity p75 NGFR into CAR constructs as hinge and demonstrated that NWL, the longest version (222 aa) with the complete extracellular sequence of NGFR, was best suited for both staining of transduced CAR T cells for flow-cytometric analysis and selection on MACS columns, albeit with a rather low yield of $\leq$40%. However, the authors did not compare the NGFR hinge with a standard hinge such as CD8, thus the feasibility of their hinge for clinical purposes is still unclear.

In contrast, we have shown here that our intermediate CD34 C6 hinge (99 aa) is an excellent candidate for a wide range of scFvs, as C6-hinged CAR constructs on CAR T cells show excellent efficacy against

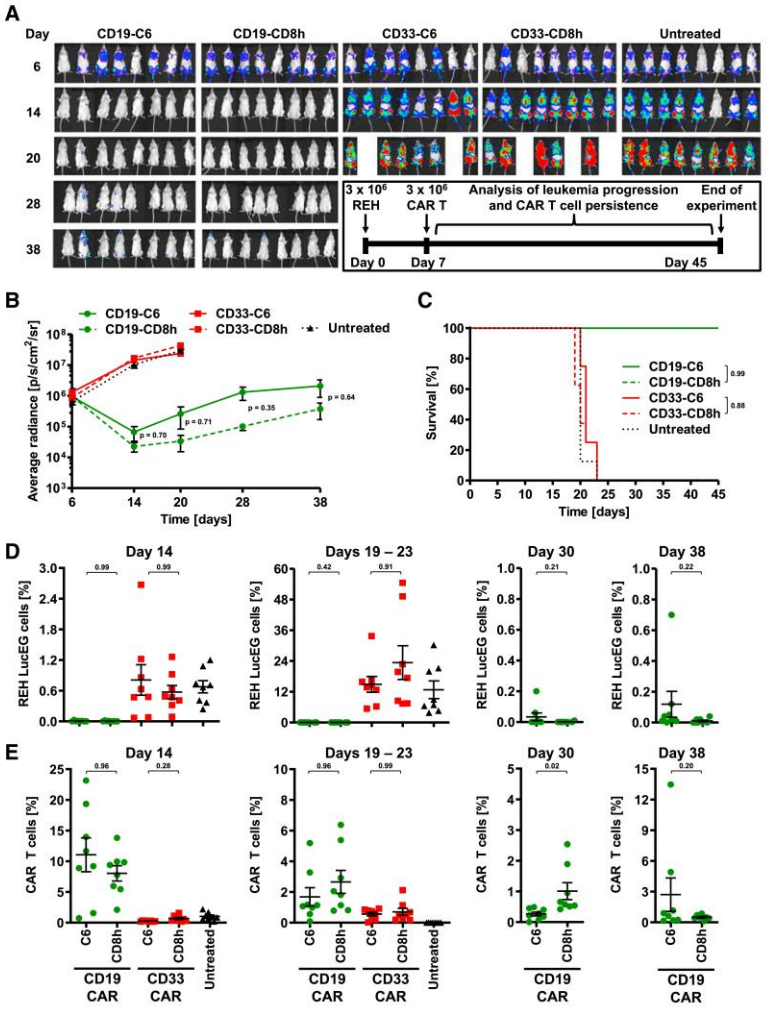


Figure 7. C6-hinged CD19 CARs efficaciously control ALL disease progression *in vivo*

Primary human T cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP in *cis* with C6-hinged or ΔCD34 in *cis* with CD8-hinged CD19 or CD33 CARs. NSG mice (8 mice/group) were xenografted with 3×10^6 cells of the human ALL cell line REH and received a single injection of 3×10^6 MACS-enriched CAR T cells 7 days later. (A and B) At days 6, 14, 20, 28, and 38, mice were monitored for REH persistence via luminescence imaging. (C) Kaplan-Meier survival curves of the four treatment groups as well as the untreated mice. Blood samples were analyzed by flow cytometry on days 14, 19–23, 28 and 38 for (D) REH and (E) CAR T cell persistence. p values were calculated by mixed-model ANOVA followed by Tukey's multiple comparison testing (B), log-rank test (C), or one-way ANOVA followed by Dunnett's multiple comparison testing for days 14 and 19–23 or unpaired t test for days 30 and 38 (D and E). Data are presented as means ± SEM.

CD5, CD19, CD33, CD123 and ROR1 (R12) target antigens on malignant cells *in vitro* and also *in vivo* with CD19 CARs.

Safety and clinical implications

The idea of including a marker that is not naturally expressed on the target cells in a CAR construct for selection of genetically modified cells for clinical settings is not new.[46] High purities and high yields are especially important if allogeneic donor T cells need to be controlled *in vivo* in patients. e.g. by co-expressing a suicide gene. Zhan et al.[47,48] used the surface and transmembrane sequences of human CD34 fused to the thymidine kinase enzyme of the herpes simplex virus for clinical safety testing of mismatched donor T cell infusions in patients after stem cell transplantation. Philip et al.[34] went one step further and created a highly compact sort/suicide gene by combining the binding epitope for the QBend-10 and two epitopes for the rituximab mAbs in a single sequence placed on a CD8α-derived stalk. Although the authors reported that transduced T cells can be enriched by using the CD34 MACS technology, the data presented on this topic in the paper is still sparse[34] and we can imagine, based on our own experiences in using the hinges C2–C4, that the CD34 microbeads-mediated selection of transduced T cells which harbor only short CD34 sequences is rather inefficient.

Here, we have established a novel hinge for CARs that allows rapid detection of CAR T cells, e.g. from peripheral blood and in functional analyses, and also efficient enrichment of the CAR T cells under GMP conditions prior to infusion into patients. Direct comparison of our C6 hinge and the widely used CD8α hinge demonstrated *in vitro,* using numerous CARs and multiple leukemic cell lines, and *in vivo* in NSG mice that both hinges mediated similar cytotoxicities, that no non-specific off-target effects occur and that the cytokine profile of both hinges is identical. Interestingly, extending the C6 hinge by 80 additional amino acids (C7) was not possible, as we observed non-specific binding of C7-hinged CD19 and R12 CARs to AML-M4/M5 cell lines, similarly to what has been described for CARs containing CH_2CH_3 hinges from human immunoglobulins.[14] Moreover, we recently demonstrated that our C6 hinge worked in CAR constructs recognizing solid tumor-associated antigens[49,50] and also here did not result in off-target activation of the genetically modified T cells, thus suggesting again that the C6 hinge seems to be well suited for CAR constructs for clinical products.

In summary, we believe that our CD34-derived C6 hinge is an ideal candidate as an essential element for the next generations of CAR constructs, which need to contain additional functional elements to address the heterogeneity of antigen expression on the malignant cells and the tumor-associated immune suppression. These adoptive cellular therapies could also be combined with supportive or complementary treatment components in more complex protocols to influence the homeostasis in the tumor microenvironment and thus drive CAR T cell therapy forward.

MATERIALS AND METHODS

Construct generation

The self-inactivating (SIN) lentiviral vector for stable high-level co-expression of two transgenes in primary human T cells contains a *Thosea asigna* virus T2A site and the viral U3 regions from the myeloproliferative sarcoma virus (MPSV) as previously published.[35] The aa sequence for the different CD34-derived hinges C2–C7 were derived from a human codon usage-optimized version of truncated human CD34 (#P28906-2; GeneArt, Thermo Fisher Scientific, Schwerte, Germany).[51] The CD34-derived inserts were generated by PCR amplification and inserted into codon-usage optimized CD19 (clone FMC63) CAR vector.[35] For some constructs, we inserted the EGFP (#C5MKY7) as a fluorescent marker in front of the T2A site and different ΔCD34-hinged CD19 CAR genes in second position after the T2A site. Moreover, the C6 and C7 hinges were inserted into codon-optimized CAR constructs against CD5 (clone H65[52,53]), ROR1 (clones R11 and R12[10,11,37]), CD33 (clone DRB2[54]) and CD123 (clone 43[36]) with the tagBFP marker being in 5′ of the T2A site. When IgG4-derived CH_2CH_3[35] or CD8-derived hinges were used in the CAR constructs, the truncated CD34 isoform (ΔCD34) was inserted in front of T2A to enable CD34 microbead-mediated MACS enrichment of the genetically modified CAR T cells.

Cell culture

All cell lines were obtained from DSMZ (Braunschweig, Germany) and grown in medium as recommended (DMEM GlutaMAX, RPMI1640 GlutaMAX, penicillin/streptomycin and fetal calf serum [FCS] were acquired from Thermo Fisher Scientific). Primary human T cells were collected from peripheral blood of healthy adult volunteers who gave informed consent according to the protocols (#4687 and #2019-623) approved by the local ethics committee (Universitätsklinikum Düsseldorf, Germany) and separated by density-gradient centrifugation using Ficoll-Paque (Cytiva Europe, Freiburg, Germany) according to the manufacturer's protocol. The PBMCs were cultivated on CD3- (Thermo Fisher Scientific) and CD28- (BD Biosciences, Heidelberg, Germany) coated nontissue culture-treated 6-well plates in Iscove's modified Dulbecco's medium (Sigma-Aldrich, Darmstadt, Germany) with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (all from Thermo Fisher Scientific) and 100 U/mL IL-2 (Proleukin, Novartis, Basel, Switzerland) to activate and specifically expand the T cells.

Lentiviral vector production and transduction of eukaryotic cells

Vesicular stomatitis virus-G (VSV-G)-pseudotyped replication-deficient lentiviral particles were produced after transfection into HEK293T cells and used for transduction of cell lines and CD3/CD28-prestimulated primary human T cells in the presence of 10 μg/mL protamine phosphate (Sigma-Aldrich) as described previously.[55]

Cell enrichment

Three to four days after transduction, CAR T cells and Jurkat cells were enriched using CD34 microbeads based on the CD34 antibody QBend-10 (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. In brief, cells were stained with CD34 microbeads, loaded onto MS MACS columns, which were washed three times, and then eluted. The fractions (preMACS, flowthrough and postMACS) were analyzed by flow cytometry for EGFP, BFP and ΔCD34 expression after staining with the QBend-10 CD34 antibody directly conjugated to phycoerythrin (PE) (Thermo Fisher Scientific). After enrichment on MACS columns, T cells were cultured for 1–2 days in 100 U/mL IL-2 until further usage or analysis.

Functional *in vitro* cytotoxicity assays

Enriched and non-enriched CAR T cells were cultured with EGFP-expressing Mino, MOLM-14, REH, Granta519, JeKo-1, HL60, THP-1, LAMA84, MEG01, MONOMAC6, K562, and NB4 cells at various effector-to-target cell ratios in round-bottomed 96-well plates. After 16 h, the cultures were harvested, the cells incubated with propidium iodide for live/dead cell discrimination, and the samples analyzed on a MACSQuant-X (Miltenyi Biotec). EGFP expression was used to discriminate between effector and target cells. Samples with HL60, THP-1, LAMA84, MEG01, MONOMAC6, K562 and NB4 cells were stained with CD33-APC (Miltenyi Biotec) for discrimination between target and effector cells. The specific lysis was calculated as 1 − (number of viable GFP-positive cells/number of control GFP-positive cells) × 100%. Negative lysis rates were set to 0%.

Functional *in vitro* cytokine secretion assays

Cytokine secretion by CAR T cells was analyzed using the MACSPlex Cytotoxic T and NK Cell Kit (Miltenyi Biotec) according to the manufacturer's instructions. Supernatants for these analyses were collected after 16 h of co-cultivation of CAR T cells and the malignant target cells in round-bottomed 96-well plates at effector-to-target cell ratios of 1:1. The supernatants were stored at −20°C until analysis. Per analysis, 50 μL of undiluted supernatants was used.

In vivo xenograft model

All *in vivo* studies were approved by the state animal research committee (LANUV, NRW, Germany) and animals were cared for according to guidelines of the Federation of European Laboratory Animal Science Associations. Eight- to ten-week-old female NSG mice (Charles River Laboratories, Sulzfeld, Germany) were xenografted with 3×10^6 REH cells, stably expressing a human codon-optimized firefly luciferase-EGFP fusion protein (REH/ffluc-EGFP).[43] Seven days later, 3×10^6 C6- or CD8-hinged CD19 or

CD33 CAR T cells were injected without conditioning. At days 6, 14, 20, 28 and 38, the persistence of REH cells was assessed by luminescence. Mice were injected with D-luciferin (OZ Biosciences, Marseilles, France) and the luciferase activity was measured after 5 min in a Caliper IVIS Lumina II system (PerkinElmer, Rodgau, Germany) with an exposure time of 15 s. Luminescence was analyzed using the Living Image software (PerkinElmer). At days 14, 19–23, 30 and 38, blood samples were analyzed by flow cytometry for persistence of REH and CAR T cells by assessing EGFP, CD19 and CD45 expression for REH cells and BFP, CAR (ΔCD34), CD3 and CD45 expression for CAR T cells using CD34-PE, CD3-PerCP-Vio700, CD19-PE-Vio770 and CD45-APC directly conjugated monoclonal antibodies (the last three from Miltenyi Biotec), respectively.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.2.0. P values were, depending on experimental setup, calculated by unpaired t test, one-way analysis of variance (ANOVA), two-way ANOVA, mixed-model ANOVA, or log-rank test followed by Tukey's or Dunnett's multiple comparison testing where indicated. p values of lysis curves are summarized in Table S1, and p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.11.003.

ACKNOWLEDGMENTS

We gratefully acknowledge the healthy donors who provided peripheral blood for the *in vitro* and *in vivo* studies. We would like to thank Jörg Schipper, MD, director of the ENT clinic, for his support of this research project. We are in debt to Wolfgang Schulz, PhD, and Michèle Hoffmann, PhD, both from the department of Urology, Heinrich Heine University, for the use of the MACSQuant-X. This work was supported, in part, by funding from the Medical Research School Düsseldorf, DSO, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, the Essener Elterninitiative zur Unterstüzung krebskranker Kinder e.V. and within the framework of the iCAN33 project, funded by the European Regional 470 Development Fund NRW (ERDF, German EFRE-0801320) 2014–2020.

AUTHOR CONTRIBUTIONS

A.B., T.I., K.R., M.W., K.S., N.G., C.W., and H.H. planned the experiments. A.B., T.I., C.H., D.S., and K.R. conducted the experiments. A.B., T.I., C.H., K.R. and H.H. analyzed data. A.B., C.H., K.S., C.W., and H.H. wrote the manuscript. All authors approved the final manuscript.

DECLARATION OF INTERESTS

H.H., C.W., T.I., and K.R. are inventors on a patent describing the CD34 hinge. All other authors declare no competing interests.

REFERENCES

1. June, C.H., and Sadelain, M. (2018). Chimeric antigen receptor therapy. N. Engl. J. Med. *379*, 64–73. https://doi.org/10.1056/NEJMra1706169.
2. Larson, R.C., and Maus, M.V. (2021). Recent advances and discoveries in the mechanisms and functions of CAR T cells. Nat. Rev. Cancer *21*, 145–161. https://doi.org/10.1038/s41568-020-00323-z.
3. Albinger, N., Hartmann, J., and Ullrich, E. (2021). Current status and perspective of CAR-T and CAR-NK cell therapy trials in Germany. Gene Ther. https://doi.org/10.1038/s41434-021-00246-w.
4. Jonnalagadda, M., Mardiros, A., Urak, R., Wang, X., Hoffman, L.J., Bernanke, A., Chang, W.C., Bretzlaff, W., Starr, R., Priceman, S., et al. (2015). Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy. Mol. Ther. *23*, 757–768. https://doi.org/10.1038/mt.2014.208.
5. Qin, L., Lai, Y., Zhao, R., Wei, X., Weng, J., Lai, P., Li, B., Lin, S., Wang, S., Wu, Q., et al. (2017). Incorporation of a hinge domain improves the expansion of chimeric antigen receptor T cells. J. Hematol. Oncol. *10*, 68. https://doi.org/10.1186/s13045-017-0437-8.
6. Watanabe, N., Bajgain, P., Sukumaran, S., Ansari, S., Heslop, H.E., Rooney, C.M., Brenner, M.K., Leen, A.M., and Vera, J.F. (2016). Fine-tuning the CAR spacer improves T-cell potency. Oncoimmunology *5*, e1253656. https://doi.org/10.1080/2162402X.2016.1253656.
7. Fujiwara, K., Tsunei, A., Kusabuka, H., Ogaki, E., Tachibana, M., and Okada, N. (2020). Hinge and transmembrane domains of chimeric antigen receptor regulate receptor expression and signaling threshold. Cells *9*. https://doi.org/10.3390/cells9051182.
8. Guest, R.D., Hawkins, R.E., Kirillova, N., Cheadle, E.J., Arnold, J., O'Neill, A., Irlam, J., Chester, K.A., Kemshead, J.T., Shaw, D.M., et al. (2005). The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. J. Immunother. *28*, 203–211. https://doi.org/10.1097/01.cji.0000161397.96582.59.
9. Moritz, D., and Groner, B. (1995). A spacer region between the single chain antibody- and the CD3 zeta-chain domain of chimeric T cell receptor components is required for efficient ligand binding and signaling activity. Gene Ther. *2*, 539–546.
10. Hudecek, M., Lupo-Stanghellini, M.T., Kosasih, P.L., Sommermeyer, D., Jensen, M.C., Rader, C., and Riddell, S.R. (2013). Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. Clin. Cancer Res. *19*, 3153–3164. https://doi.org/10.1158/1078-0432.CCR-13-0330.
11. Hudecek, M., Sommermeyer, D., Kosasih, P.L., Silva-Benedict, A., Liu, L., Rader, C., Jensen, M.C., and Riddell, S.R. (2015). The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. Cancer Immunol. Res. *3*, 125–135. https://doi.org/10.1158/2326-6066.CIR-14-0127.
12. Wilkie, S., Picco, G., Foster, J., Davies, D.M., Julien, S., Cooper, L., Arif, S., Mather, S.J., Taylor-Papadimitriou, J., Burchell, J.M., and Maher, J. (2008). Retargeting of human T cells to tumor-associated MUC1: The evolution of a chimeric antigen receptor. J. Immunol. *180*, 4901–4909. https://doi.org/10.4049/jimmunol.180.7.4901.
13. Almasbak, H., Walseng, E., Kristian, A., Myhre, M.R., Suso, E.M., Munthe, L.A., Andersen, J.T., Wang, M.Y., Kvalheim, G., Gaudernack, G., and Kyte, J.A. (2015). Inclusion of an IgG1-Fc spacer abrogates efficacy of CD19 CAR T cells in a xenograft mouse model. Gene Ther. *22*, 391–403. https://doi.org/10.1038/gt.2015.4.
14. Hombach, A., Hombach, A.A., and Abken, H. (2010). Adoptive immunotherapy with genetically engineered T cells: Modification of the IgG1 Fc 'spacer' domain in the extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and unintended initiation of an innate immune response. Gene Ther. *17*, 1206–1213. https://doi.org/10.1038/gt.2010.91.
15. Turtle, C.J., Hanafi, L.A., Berger, C., Gooley, T.A., Cherian, S., Hudecek, M., Sommermeyer, D., Melville, K., Pender, B., Budiarto, T.M., et al. (2016). CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J. Clin. Invest. *126*, 2123–2138. https://doi.org/10.1172/JCI85309.
16. Cerrano, M., Ruella, M., Perales, M.A., Vitale, C., Faraci, D.G., Giaccone, L., Coscia, M., Maloy, M., Sanchez-Escamilla, M., Elsabah, H., et al. (2020). The advent of CAR

T-cell therapy for lymphoproliferative neoplasms: Integrating research into clinical practice. Front. Immunol. *11*, 888. https://doi.org/10.3389/fimmu.2020.00888.

17. Valton, J., Guyot, V., Boldajipour, B., Sommer, C., Pertel, T., Juillerat, A., Duclert, A., Sasu, B.J., Duchateau, P., and Poirot, L. (2018). A versatile safeguard for chimeric antigen receptor T-cell immunotherapies. Sci. Rep. *8*, 8972. https://doi.org/10.1038/s41598-018-27264-w.

18. Koristka, S., Ziller-Walter, P., Bergmann, R., Arndt, C., Feldmann, A., Kegler, A., Cartellieri, M., Ehninger, A., Ehninger, G., Bornhauser, M., and Bachmann, M.P. (2019). Anti-CAR-engineered T cells for epitope-based elimination of autologous CAR T cells. Cancer Immunol. Immunother. *68*, 1401–1415. https://doi.org/10.1007/s00262-019-02376-y.

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

20. Liu, L., Sommermeyer, D., Cabanov, A., Kosasih, P., Hill, T., and Riddell, S.R. (2016). Inclusion of Strep-tag II in design of antigen receptors for T-cell immunotherapy. Nat. Biotechnol. *34*, 430–434. https://doi.org/10.1038/nbt.3461.

21. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517. https://doi.org/10.1056/NEJMoa1407222.

22. Cruz, C.R., Micklethwaite, K.P., Savoldo, B., Ramos, C.A., Lam, S., Ku, S., Diouf, O., Liu, E., Barrett, A.J., Ito, S., et al. (2013). Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: A phase 1 study. Blood *122*, 2965–2973. https://doi.org/10.1182/blood-2013-06-506741.

23. Badbaran, A., Berger, C., Riecken, K., Kruchen, A., Geffken, M., Muller, I., Kroger, N., Ayuk, F.A., and Fehse, B. (2020). Accurate in-vivo quantification of CD19 CAR-T cells after treatment with axicabtagene ciloleucel (Axi-Cel) and tisagenlecleucel (Tisa-Cel) using digital PCR. Cancers *12*, 1970. https://doi.org/10.3390/cancers12071970.

24. Zhang, Q., Hu, H., Chen, S.Y., Liu, C.J., Hu, F.F., Yu, J.M., Wu, Y.H., and Guo, A.Y. (2019). Transcriptome and regulatory network analyses of CD19-CAR-T immunotherapy for B-all. Genomics Proteomics Bioinformatics *17*, 190–200. https://doi.org/10.1016/j.gpb.2018.12.008.

25. Keu, K.V., Witney, T.H., Yaghoubi, S., Rosenberg, J., Kurien, A., Magnusson, R., Williams, J., Habte, F., Wagner, J.R., Forman, S., et al. (2017). Reporter gene imaging of targeted T cell immunotherapy in recurrent glioma. Sci. Transl. Med. *9*, eaag2196. https://doi.org/10.1126/scitranslmed.aag2196.

26. Jena, B., Maiti, S., Huls, H., Singh, H., Lee, D.A., Champlin, R.E., and Cooper, L.J. (2013). Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. PLoS One *8*, e57838. https://doi.org/10.1371/journal.pone.0057838.

27. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.Z.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: A phase 1 dose-escalation trial. Lancet *385*, 517–528. https://doi.org/10.1016/S0140-6736(14)61403-3.

28. Chen, P.H., Lipschitz, M., Wright, K., Armand, P., Jacobson, C.A., Roberts, Z.J., Rossi, J.M., Bot, A., Go, W.Y., and Rodig, S.J. (2018). Analysis of CAR-T and immune cells within the tumor micro-environment of diffuse large B-cell lymphoma post CAR-T treatment by multiplex Immunofluorescence. Blood *132*. https://doi.org/10.1182/blood-2018-99-113644.

29. Ayuk, F., Fehse, B., Janson, D., Berger, C., Riecken, K., and Kroger, N. (2020). Excellent proliferation and persistence of allogeneic donor-derived 41-BB based CAR-T cells despite immunosuppression with cyclosporine A. Haematologica *105*, E322–E324. https://doi.org/10.3324/haematol.2019.245969.

30. Abramson, J.S., Palomba, M.L., Gordon, L.I., Lunning, M.A., Wang, M., Arnason, J., Mehta, A., Purev, E., Maloney, D.G., Andreadis, C., et al. (2020). Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): A multicentre seamless design study. Lancet *396*, 839–852. https://doi.org/10.1016/S0140-6736(20)31366-0.

31. AbuSamra, D.B., Aleisa, F.A., Al-Amoodi, A.S., Jalal Ahmed, H.M., Chin, C.J., Abuelela, A.F., Bergam, P., Sougrat, R., and Merzaban, J.S. (2017). Not just a marker: CD34 on human hematopoietic stem/progenitor cells dominates vascular selectin binding along with CD44. Blood Adv. *1*, 2799–2816. https://doi.org/10.1182/bloodadvances.2017004317.

32. Shah, G.L., Scordo, M., Kosuri, S., Herrera, D.A., Cho, C., Devlin, S.M., Borrill, T., Carlow, D.C., Avecilla, S.T., Meagher, R.C., et al. (2018). Impact of toxicity on survival for older adult patients after CD34(+) selected allogeneic hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. *24*, 142–149. https://doi.org/10.1016/j.bbmt.2017.08.040.

33. Berger, M.D., Branger, G., Leibundgut, K., Baerlocher, G.M., Seipel, K., Mueller, B.U., Gregor, M., Ruefer, A., and Pabst, T. (2015). $CD34^+$ selected versus unselected autologous stem cell transplantation in patients with advanced-stage mantle cell and diffuse large B-cell lymphoma. Leuk. Res. *39*, 561–567. https://doi.org/10.1016/j.leukres.2015.03.004.

34. Philip, B., Kokalaki, E., Mekkaoui, L., Thomas, S., Straathof, K., Flutter, B., Marin, V., Marafioti, T., Chakraverty, R., Linch, D., et al. (2014). A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. Blood *124*, 1277–1287. https://doi.org/10.1182/blood-2014-01-545020.

35. Roellecke, K., Virts, E.L., Einholz, R., Edson, K.Z., Altvater, B., Rossig, C., von Laer, D., Scheckenbach, K., Wagenmann, M., Reinhardt, D., et al. (2016). Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies. Gene Ther. *23*, 615–626. https://doi.org/10.1038/gt.2016.38.

36. Stein, C., Kellner, C., Kugler, M., Reiff, N., Mentz, K., Schwenkert, M., Stockmeyer, B., Mackensen, A., and Fey, G.H. (2010). Novel conjugates of single-chain Fv antibody fragments specific for stem cell antigen CD123 mediate potent death of acute myeloid leukaemia cells. Br. J. Haematol. *148*, 879–889. https://doi.org/10.1111/j.1365-2141.2009.08033.x.

37. Yang, J., Baskar, S., Kwong, K.Y., Kennedy, M.G., Wiestner, A., and Rader, C. (2011). Therapeutic potential and challenges of targeting receptor tyrosine kinase ROR1 with monoclonal antibodies in B-cell malignancies. PLoS One *6*, e21018. https://doi.org/10.1371/journal.pone.0021018.

38. Mitsuoka, C., Sawada-Kasugai, M., Ando-Furui, K., Izawa, M., Nakanishi, H., Nakamura, S., Ishida, H., Kiso, M., and Kannagi, R. (1998). Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis X. J. Biol. Chem. *273*, 11225–11233. https://doi.org/10.1074/jbc.273.18.11225.

39. Hernandez Mir, G., Helin, J., Skarp, K.P., Cummings, R.D., Makitie, A., Renkonen, R., and Leppanen, A. (2009). Glycoforms of human endothelial CD34 that bind L-selectin carry sulfated sialyl Lewis x capped O- and N-glycans. Blood *114*, 733–741. https://doi.org/10.1182/blood-2009-03-210237.

40. Steentoft, C., Vakhrushev, S.Y., Joshi, H.J., Kong, Y., Vester-Christensen, M.B., Schjoldager, K.T.B.G., Lavrsen, K., Dabelsteen, S., Pedersen, N.B., Marcos-Silva, L., et al. (2013). Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. Embo J. *32*, 1478–1488. https://doi.org/10.1038/emboj.2013.79.

41. Chauhan, J.S., Rao, A., and Raghava, G.P.S. (2013). In silico platform for prediction of N-, O- and C-glycosites in eukaryotic protein sequences. PLoS One *8*, e67008. https://doi.org/10.1371/journal.pone.0067008.

42. Guedan, S., Calderon, H., Posey, A.D., Jr., and Maus, M.V. (2019). Engineering and design of chimeric antigen receptors. Mol. Ther. Methods Clin. Dev. *12*, 145–156. https://doi.org/10.1016/j.omtm.2018.12.009.

43. Gu, D., Liu, H., Su, G.H., Zhang, X., Chin-Sinex, H., Hanenberg, H., Mendonca, M.S., Shannon, H.E., Chiorean, E.G., and Xie, J. (2013). Combining hedgehog signaling inhibition with focal irradiation on reduction of pancreatic cancer metastasis. Mol. Cancer Ther. *12*, 1038–1048. https://doi.org/10.1158/1535-7163.MCT-12-1030.

44. Di Stasi, A., Tey, S.K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A.G., Grilley, B., et al. (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. N. Engl. J. Med. *365*, 1673–1683. https://doi.org/10.1056/NEJMoa1106152.

45. Alabanza, L., Pegues, M., Geldres, C., Shi, V., Wiltzius, J.J.W., Sievers, S.A., Yang, S.C., and Kochenderfer, J.N. (2017). Function of novel anti-CD19 chimeric antigen

receptors with human variable regions is affected by hinge and transmembrane domains. Mol. Ther. *25*, 2452–2465. https://doi.org/10.1016/j.ymthe.2017.07.013.

46. Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponzoni, M., Rossini, S., Mavilio, F., Traversari, C., and Bordignon, C. (1997). HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science *276*, 1719–1724. https://doi.org/10.1126/science.276.5319.1719.

47. Zhan, H., Gilmour, K., Chan, L., Farzaneh, F., McNicol, A.M., Xu, J.H., Adams, S., Fehse, B., Veys, P., Thrasher, A., et al. (2013). Production and first-in-man use of T cells engineered to express a HSVTK-CD34 sort-suicide gene. PLoS One *8*, e77106. https://doi.org/10.1371/journal.pone.0077106.

48. Eissenberg, L.G., Rettig, M.P., Ritchey, J.K., Prior, J.L., Schwarz, S.W., Frye, J., White, B.S., Fulton, R.S., Ghobadi, A., Cooper, M.L., et al. (2015). [(18)F]FHBG PET/CT imaging of CD34-TK75 transduced donor T cells in relapsed allogeneic stem cell transplant patients: Safety and feasibility. Mol. Ther. *23*, 1110–1122. https://doi.org/10.1038/mt.2015.48.

49. Haist, C., Schulte, E., Bartels, N., Bister, A., Poschinski, Z., Ibach, T.C., Geipel, K., Wiek, C., Wagenmann, M., Monzel, C., et al. (2021). CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells. Oral Oncol. *116*, 105259. https://doi.org/10.1016/j.oraloncology.2021.105259.

50. Grunewald, C.M., Haist, C., König, C., Petzsch, P., Bister, A., Nößner, E., et al. (2021). Epigenetic priming of bladder cancer cells with decitabine increases cytotoxicity of human EGFR and CD44v6 CAR engineered T-cells. Front. Immunol. https://doi.org/10.3389/fimmu.2021.782448.

51. Fehse, B., Richters, A., Putimtseva-Scharf, K., Klump, H., Li, Z., Ostertag, W., Zander, A.R., and Baum, C. (2000). CD34 splice variant: An attractive marker for selection of gene-modified cells. Mol. Ther. *1*, 448–456. https://doi.org/10.1006/mthe.2000.0068.

52. Mamonkin, M., Rouce, R.H., Tashiro, H., and Brenner, M.K. (2015). A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies. Blood *126*, 983–992. https://doi.org/10.1182/blood-2015-02-629527.

53. Studnicka, G.M., Soares, S., Better, M., Williams, R.E., Nadell, R., and Horwitz, A.H. (1994). Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementarity-modulating residues. Protein Eng. *7*, 805–814. https://doi.org/10.1093/protein/7.6.805.

54. Stamova, S., Cartellieri, M., Feldmann, A., Arndt, C., Koristka, S., Bartsch, H., Bippes, C.C., Wehner, R., Schmitz, M., von Bonin, M., et al. (2011). Unexpected recombinations in single chain bispecific anti-CD3-anti-CD33 antibodies can be avoided by a novel linker module. Mol. Immunol. *49*, 474–482. https://doi.org/10.1016/j.molimm.2011.09.019.

55. Wiek, C., Schmidt, E.M., Roellecke, K., Freund, M., Nakano, M., Kelly, E.J., Kaisers, W., Yarov-Yarovoy, V., Kramm, C.M., Rettie, A.E., and Hanenberg, H. (2015). Identification of amino acid determinants in CYP4B1 for optimal catalytic processing of 4-ipomeanol. Biochem. J. *465*, 103–114. https://doi.org/10.1042/BJ20140813.

NEUARTIGE AUF NGFR-BASIERENDE *HINGE* ZUR SCHNELLEN UND EFFIZIENTEN ANREICHERUNG UND DETEKTION VON CAR-T-ZELLEN

Titel: Optimized NGFR-derived hinges for rapid and efficient enrichment and detection of CAR T cells in vitro and in vivo

Autoren: Arthur Bister, Tabea C. Ibach, **Corinna Haist**, Gina Gerhorst, Denise Smorra, Soldierer Maren, Katharina Roellecke, Martin Wagenmann, Kathrin Scheckenbach, Norbert Gattermann, Constanze Wiek*, Helmut Hanenberg*

* Diese Autoren haben gleichermaßen zu dieser Arbeit beigetragen

Eigenleistung: 10 %

- Hilfestellung bei der Durchführung der Experimente, Schreiben und Korrektur des Manuskripts

Veröffentlicht in: Molecular Therapy - Oncolytics

Impact Factor: 7,200

DOI: 10.1016/j.omoto.2022.05.012

Molecular Therapy
Oncolytics
Original Article

Optimized NGFR-derived hinges for rapid and efficient enrichment and detection of CAR T cells *in vitro* and *in vivo*

A. Bister,[1,2] T. Ibach,[2] C. Haist,[1,2] G. Gerhorst,[2] D. Smorra,[1] M. Soldierer,[1] K. Roellecke,[2] M. Wagenmann,[2] K. Scheckenbach,[2] N. Gattermann,[3] C. Wiek,[2,4] and H. Hanenberg[1,2,4]

[1]Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Hufelandstr. 55, 45122 Essen, Germany; [2]Department of Otorhinolaryngology, Head & Neck Surgery, Heinrich Heine University, Düsseldorf, Germany; [3]Department of Hematology, Heinrich Heine University, Düsseldorf, Germany

Chimeric antigen receptor (CAR) T cell therapy has demonstrated unprecedented success with high remission rates for heavily pretreated patients with hematological malignancies. The hinge connecting the extracellular antigen recognition unit to the transmembrane domain provides the length and flexibility of the CAR constructs and ensures that the CAR can reach the target antigen and mediate recognition and killing of target cells. The hinge can also include specific amino acid sequences to improve CAR expression, influence T cell proliferation, and facilitate CAR T cell detection, enrichment, and even elimination. Here, we report the generation of two novel hinge domains derived from the low-affinity p75 chain of the human nerve growth factor receptor (NGFR), termed N3 and N4, which, when incorporated into the CAR backbone, allow detection as well as high-grade enrichment of CAR T cells with GMP-compatible immunomagnetic reagents. After optimizing the MACS protocol for excellent CAR T cell purity and yield, we demonstrated that N3- and N4-hinged CAR T cells are as efficacious as their CD8-hinged counterparts *in vitro* against hematological blasts and also *in vivo* in the control of acute monocytic leukemia in an immunodeficient mouse xenograft model. Thus, both hinges could potentially be an integral part of future CAR designs and universally applicable in clinical applications.

INTRODUCTION

More than three decades after the first concept studies,[1] the amazing clinical success of chimeric antigen receptor (CAR) T cell therapy in the last couple of years has transformed the clinical care of patients with poor-prognosis hematological malignancies.[2] For clinical CAR T cell therapy, autologous T cells from patients with leukemia and lymphoma are transduced *in vitro* with lentivirally expressed CAR constructs that typically combine the antigen recognition ability of monoclonal antibodies in *cis* with functional domains of T cell receptor signaling, including epitope recognition, activation, and expansion, in a single molecule.[2] Currently, six different CAR products have received market approval for hematological malignancies, namely Kymriah, Yescarta, Tecartus, and Breyanzi for CD19-positive leukemias or lymphomas, and Abecma and Carvykti for BCMA-positive multiple myelomas.[3,4]

The second-generation CAR constructs used in these six formulations contain single-chain variable fragments (scFvs) of monoclonal antibodies linked in *cis* via a hinge/spacer and a transmembrane region to the intracellular signaling domains of the CD3ζ-chain and the co-stimulatory receptors CD28 or 4-1BB/CD137.[2,3] Importantly, the design of this single chimeric protein ensures that the CAR molecules mediate MHC-independent T cell activation and killing of malignant as well as normal cells if the target antigen is expressed.[2,3] Although the hinge can theoretically influence the function of CAR constructs, only few candidates for this domain in CARs have been thoroughly investigated.[3,5–18] One key aspect to consider for the length and flexibility of a hinge is the location of the epitope recognized by the CAR scFvs within the three-dimensional structure of the target antigen.[5,13] In addition, incorporating a specific hinge can improve the protein expression and stability of the CAR and can modulate the expansion, proliferation, and stimulation of the CAR T cells.[9,10,14,18] Most clinically used CARs contain hinges derived from human CD8 or CD28, which appear to be safe to use as they are naturally expressed on T cells and by themselves do not confer additional features to the CAR T cells.[19–22] However, instead of using the hinge as neutral element that simply connects the scFvs to the transmembrane and signaling domains, endeavors have been made to modulate the hinge for improvement of T cell stimulation (patent EP3184548A1) for *in vivo* detection and/or elimination of CAR T cells[15,23] and for enrichment of transduced T cells *in vitro* before infusion into patients.[12,23,24]

Received 8 February 2022; accepted 27 May 2022;
https://doi.org/10.1016/j.omto.2022.05.012.

[4]These authors contributed equally

Correspondence: Helmut Hanenberg, Department of Pediatrics III, University Children's Hospital, University of Duisburg-Essen, Hufelandstr. 55, 45122 Essen, MD, Essen, Germany.
E-mail: Helmut.Hanenberg@uk-essen.de

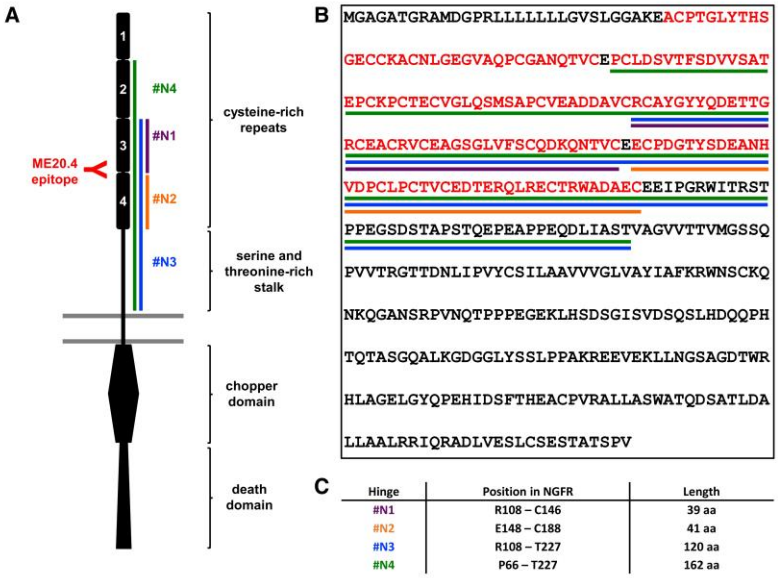


Figure 1. Structure and amino acid sequence of NGFR- and NGFR-derived hinges

(A) NGFR consists extracellularly of four cysteine-rich domains (CRDs) and a serine/threonine-rich (S/T-rich) stalk. Intracellularly, it carries a chopper and a death domain. The NGFR antibody (clone ME20.4) presumably binds an epitope in the third and/or fourth cysteine-rich repeat. The novel NGFR-derived hinges consist of the third CRD (N1, 39 aa), the fourth CRD (N2, 41 aa), the third and fourth CRD plus the stalk (N3, 120 aa), or the second, third, and fourth CRD plus the stalk (N4, 162 aa). (B) The amino acid sequences of the hinges are indicated in the NGFR amino acid sequence by lines in the corresponding color. The CRDs are shown in red. (C) Tabular overview of the NGFR-derived hinges, their length and position within NGFR.

Several methods to quantify CAR T cell persistence in patients exist: Idiotypic antibodies[25,26] and tagged antigens[27,28] bind the CAR construct directly and thus allow to precisely visualize the CAR expression on protein level; however, both detection reagents have to be adapted whenever a new antigen is targeted.[25] CAR T cell persistence can also be measured on the mRNA level by qRT-PCR,[31–33] ddPCR,[27] or RNA-sequencing;[34] however, this analysis will not address protein translation or stability issues. Cell surface markers[29,30] can be co-expressed with CAR constructs in the same vector, however this approach will increase vector size, thus influencing transduction efficiency[35], and both transgenes will not necessarily be expressed at similar ratios.[36] Ultimately, as flow cytometric methods will allow to specifically assess the CAR T cell phenotype, including activation and exhaustion markers,[7,12] the inclusion of an epitope recognizable by antibodies into the hinge of CARs combines the advantages of all these strategies.

The extracellular sequence of nerve growth factor receptor (NGFR) contains a 28 amino acid (aa) leader peptide, four cysteine-rich domains (CRDs) of approximately 40 aa each and a serine/threonine (S/T)-rich stalk followed by a single-pass transmembrane domain.[37] For more than 30 years, this structure, with and also without the 155 aa cytoplasmic tail, has been used as a marker for successful gene transfer in research as well as clinical settings.[38-40] In 2002, the description of insertional mutagenesis in a murine transplantation model with a splice-active oncoretroviral vector, where the cytoplasmatically truncated NGFR (ΔNGFR) was expressed off the strong 5′ LTR, questioned the safety of using the ΔNGFR cDNA as transgene for human clinical applications.[41] However, Bonini et al.[42] reported the successful transduction of more than 7×10^9 bone marrow cells with subsequent infusion into over 900 mice, rats and dogs without a single oncogenic transformation and Ciceri et al.[38] used the ΔNGFR as a magnetic cell sorting (MACS) selection marker for suicide gene-expressing allogeneic T cells in clinical studies without any insertional mutagenesis. In 2018, Casucci et al.[12] systematically investigated the potential of the ΔNGFR as a hinge for CAR T cells, testing different lengths of the extracellular sequences in lentiviral CAR expression constructs. Although detection of the CAR construct on transduced T cells was readily possible with an NGFR antibody, the authors could not efficiently enrich ΔNGFR-hinged CAR T cells with directly coupled LNGFR/CD271 microbeads.[12] In a follow-up study, the same group further improved the hinge regarding cytotoxicity by including additional amino acids from the stalk, but still failed to efficiently enrich their NGFR-hinged CAR T cells with GMP-compatible microbeads.[17]

Here, we describe the successful establishment of two novel NGFR-derived hinges termed N3 and N4, which mediate efficient selection of CAR T cells against hematological cancers and are functionally indistinguishable from a clinically well-established human CD8-derived hinge[12,17] *in vitro* and *in vivo* in immunodeficient mice.

RESULTS

Design of NGFR-derived hinges for CARs

The NGFR antibody clone ME20.4 used by Miltenyi Biotec in their NGFR microbeads for MACS selection binds an epitope in the third and/or fourth CRD in the extracellular part of NGFR (personal communication with Miltenyi Biotec 2015).[12] Therefore, we designed four new NGFR sequences around the third and fourth CRD to be included as hinges into our CAR constructs (Figure 1): The two short hinges N1 (39 aa) and N2 (41 aa) only consisted of the third or fourth CRD, respectively. N3 (120 aa) contained the third and fourth CRD plus the S/T-rich stalk and N4 (162 aa) additionally the second CRD. For control constructs (described below), we used the complete codon-optimized surface and transmembrane regions of NGFR, but deleted the cytoplasmic chopper and death domains (Figure 1).

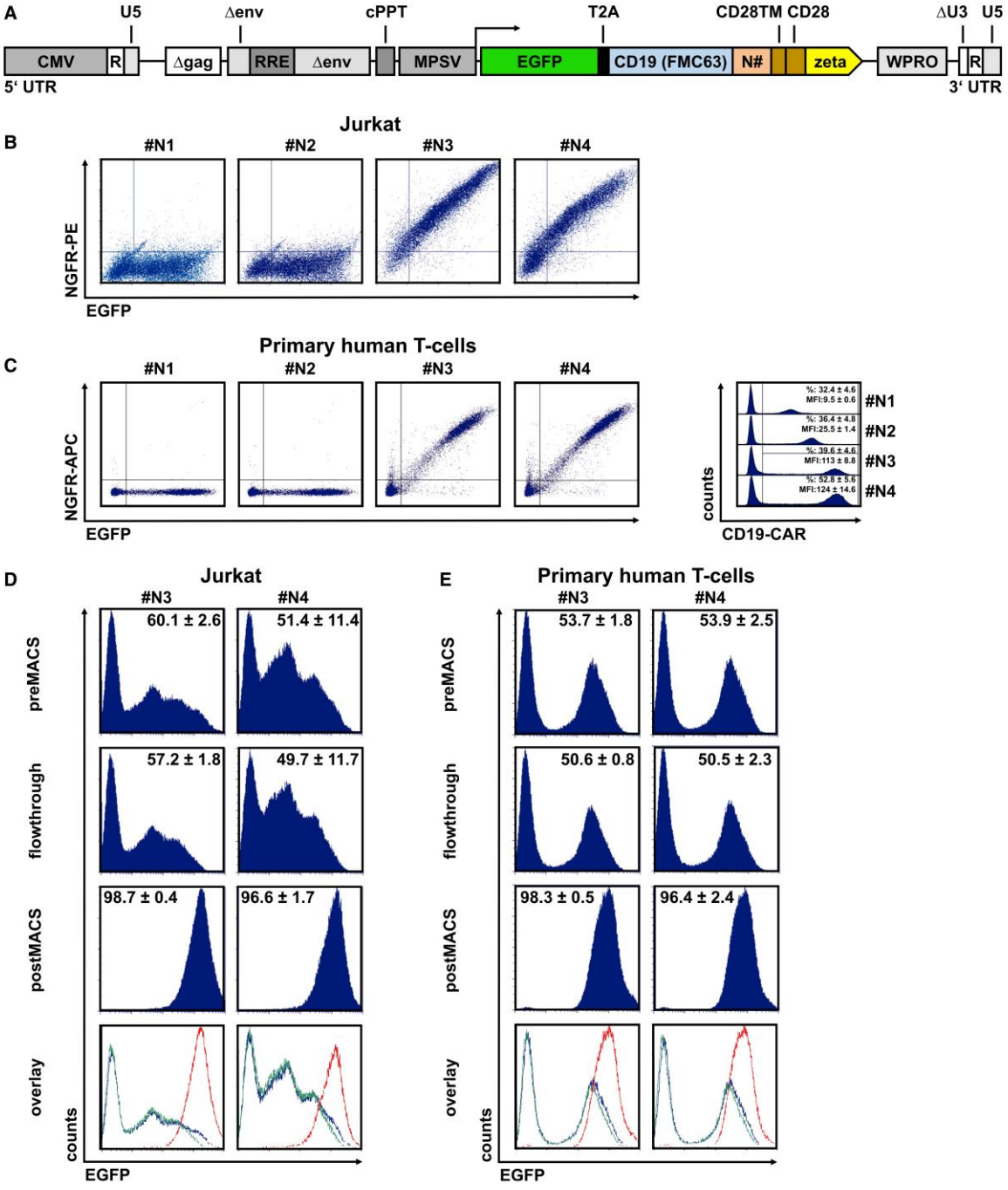


Figure 2. Expression and enrichment of NGFR-hinged CARs in Jurkat and primary human T cells

Jurkat or primary human T cells were lentivirally transduced with constructs co-expressing EGFP in *cis* with N1-, N2-, N3-, or N4-hinged CD19 CARs depicted in (A). Three days after transduction Jurkat cells (B) or primary human T cells (C) were flow cytometrically analyzed for their CAR expression levels (via staining with NGFR-PE, clone

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NGFR-hinged CAR expression and enrichment on MACS MS columns

The fragments N1-N4 were inserted into a previously published human codon-optimized CD19 CAR construct in a bicistronic lentiviral vector (Figure 2A), thereby replacing the 229 aa IgG1-derived CH_2CH_3 hinge.[43] To test whether the CARs with the four NGFR hinges are stably expressed on the surface of transduced cells and bind the ME20.4 NGFR antibody, we transduced Jurkat and primary human T cells with VSV-G-pseudotyped bicistronic lentiviral vectors co-expressing EGFP and N1-N4-hinged CD19 CARs (Figure 2A). After 5 days, the transduced cells were stained with ME20.4-PE and then analyzed for NGFR as well as EGFP expression by flow cytometry. Although the EGFP expression in Jurkat (Figure 2B) and primary human T cells (Figure 2C) was comparable for all four constructs, only N3- and N4-hinged CD19 CARs showed a clear co-expression of NGFR and EGFP (Figures 2B and 2C). We also co-stained the CAR T cells with the CD19 CAR detection reagent developed by Miltenyi Biotec. While both the N1- as well as the N2-hinged CD19 CAR bound the CD19 CAR detection reagent and thus the CARs were indeed expressed on the surface of the T cells, the expression levels were much lower compared to the N3- and N4-hinged constructs (Figure 2C), demonstrating that the NGFR monoclonal antibody cannot bind N1- and N2-hinged CARs and suggesting that the N1 and N2 constructs were less stable. Since N1 and N2 could not be used for detection or selection of CAR T cells, these hinges were not included in further experiments.

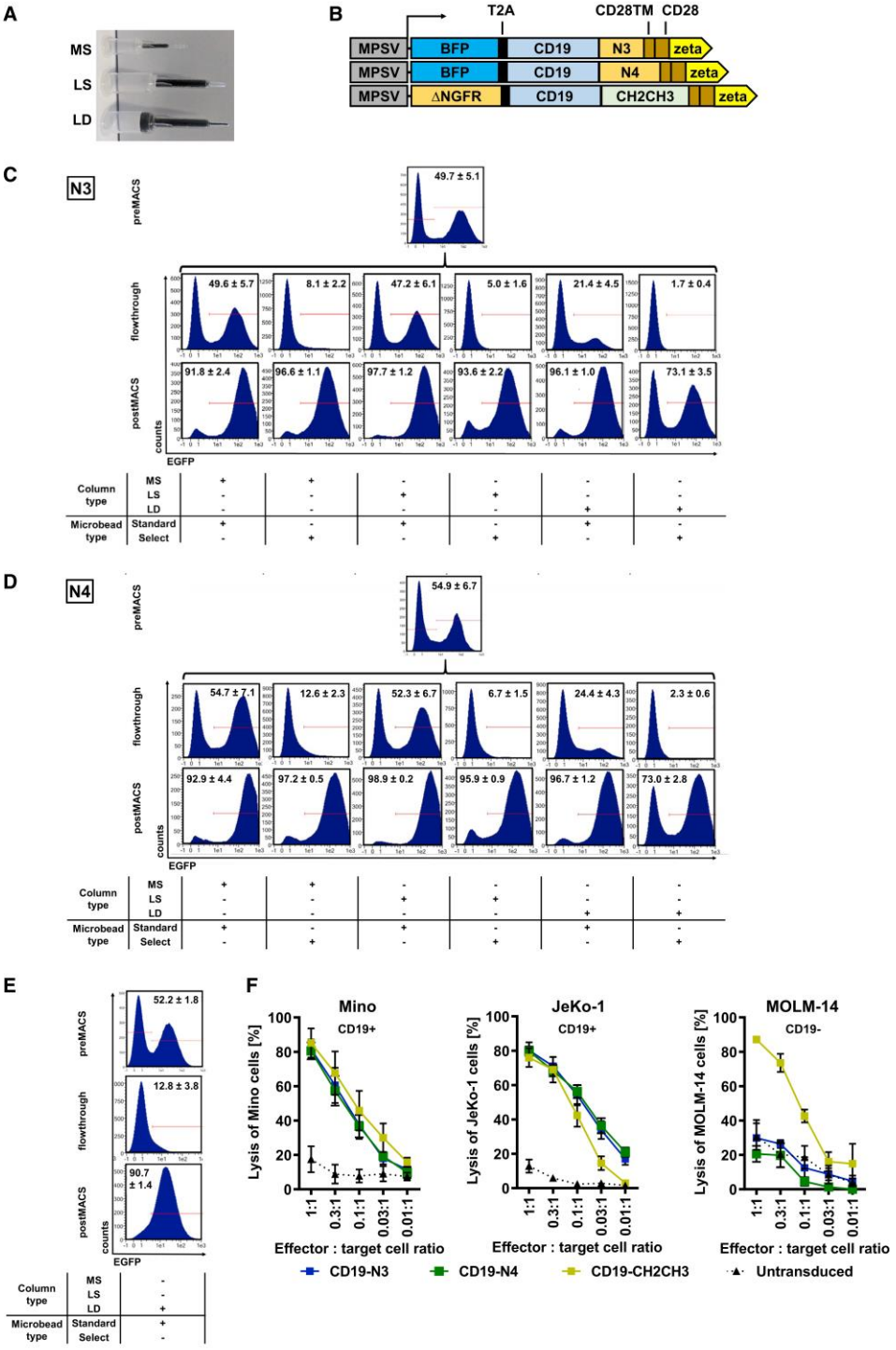
Next, we transduced Jurkat and primary human T cells with VSV-G pseudotyped lentiviral particles introducing the N3- and N4-hinged CD19 CAR bicistronic vectors (Figure 2A) and then used *Standard* CD271 microbeads on MACS MS columns once to enrich for stably transduced cells. To analyze the efficiency of the enrichment process, the three cellular fractions of the MACS separation, the cells after transduction and prior to enrichment (preMACS), the cells not retained by the MACS columns in the magnetic field (flowthrough), and the cells harvested from the columns after removing the magnet (postMACS) were analyzed by flow cytometry for their EGFP expression. Before selection, 60.1% ± 2.6% (N3) and 51.4% ± 11.4% (N4) of the Jurkat cells (Figure 2D, preMACS) and 53.7% ± 1.8% (N3) and 53.9% ± 2.5% (N4) of the T cells (Figure 2E, preMACS) were EGFP positive. The enrichment with the CD271 microbeads on the columns led to highly purified populations: 98.7% ± 0.4% (N3) and 96.6% ± 1.7% (N4) for Jurkat cells (Figure 2D, postMACS) and 98.3% ± 0.5% (N3) and 96.4% ± 2.4% (N4) of the T cells (Figure 2E, postMACS) were transduced/EGFP positive, respectively. However, the enrichment processes were highly inefficient, as the flowthroughs contained 57.2% ± 1.8% (N3) and 49.7% ± 11.7% (N4) EGFP-positive Jurkat (Figure 2D, flowthrough) and 50.6% ± 0.8% (N3) and 50.5% ± 2.3% (N4) transduced T cells (Figure 2E, flowthrough), respectively. Thus, before any functional testing, we had to strongly reduce the loss of CAR T cells in the flowthrough of the columns while maintaining pure populations.

Minimizing the loss of CAR T cells on MACS columns

Three different types of column (mini selection [MS], large selection [LS], and large depletion [LD]; Figure 3A), which vary in the length and density of the iron particles, and two different types of CD271 microbead reagents (*Standard*, *Select*), which differ in their antibody-microbead conjugation, are available from Miltenyi Biotec. Thus, we systematically tested whether specific combinations of the column and microbead types (MS + *Standard*, MS + *Select*, LS + *Standard*, LS + *Select*, LD + *Standard*, and LD + *Select*) can be utilized to avoid the high losses of transduced T cells in the flowthrough. To this end, primary human T cells were transduced with bicistronic lentiviral vectors co-expressing EGFP and N3- or N4-hinged CD19 CAR constructs (Figure 3B). Three to 4 days later, the transduced T cells were selected with combinations of three columns and the two CD271 microbead types. Subsequently, samples from preMACS, flowthrough, and postMACS were analyzed by flow cytometry for EGFP expression.

Before enrichment, 49.7% ± 5.1% and 54.9% ± 6.7% of T cells expressed EGFP and hence the N3 or N4 CAR construct, respectively (Figures 3C and 3D; preMACS). All enrichment protocols, except for enrichment with *Select* microbeads in combination with LD columns, led to purities of CAR T cells above 90% (Figures 3C and 3D; postMACS). However, the combination of *Select* microbeads and LD columns resulted in purities of just 73.1% and 73.0%, respectively (Figures 3C and 3D; postMACS). Enrichment with *Standard* microbeads on MS and LS columns also was highly inefficient, since >95% of the CAR T cells were lost in the flowthrough (Figures 3C and 3D; flowthrough). Enrichment with *Standard* microbeads on LD columns was more efficient, since the flowthroughs contained only 21.4% and 24.4% N3- and N4-hinged CD19 CAR T cells, respectively (Figures 3C and 3D; flowthrough). Importantly, the most efficient enrichments were achieved by combining *Select* microbeads with MS or LS columns, thereby reaching purities of 93.6% ± 2.2% to 97.2% ± 0.5% (Figures 3C and 3D; postMACS), while the flowthrough only contained 5.0% ± 1.6% to 12.6% ± 2.3% CAR T cells (Figures 3C and 3D; flowthrough). Since enrichment with *Select* microbeads and MS columns reached a slightly higher purity and the MS magnet enables eight simultaneous separations, in contrast to the LS/LD magnet with a capacity of just four columns, we continued with the MS/*Select* combination for enrichment of N3- and N4-hinged CAR T cells.

ME20.4, or CD19 CAR detection reagent stained with anti-biotin-APC) and EGFP expression. Subsequently, N3- and N4-hinged CAR Jurkat cells (D) or primary human T cells (E) were enriched via magnetic cell sorting (using CD271 microbeads in combination with MS columns) and the three fractions (preMACS, flowthrough, and postMACS) were flow cytometrically analyzed for EGFP expression. In the overlays, the preMACS analysis was depicted as a blue line, the flowthrough as a green line, and the postMACS measurements as a red line. Representative blots were used and data were shown as means ± SEM of at least three biological replicates.



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N3- and N4-hinged CD19 CARs lyse tumor cells as efficacious as their CH_2CH_3-hinged counterparts

Next, we compared the lytic capabilities of the new N3- and N4-hinged CD19 CARs with the killing mediated by the original CD19 CAR with an IgG1-derived CH_2CH_3 hinge.[36,43] To this end, primary human T cells were transduced with bicistronic lentiviral vectors co-expressing BFP and CD19-N3 or CD19-N4 or a construct with ΔNGFR and CD19-CH_2CH_3 (Figure 3B). Four days after transduction, N3- and N4-hinged CD19 CAR T cells were enriched with *Select* microbeads on MS columns, while ΔNGFR/CD19-CH_2CH_3 CAR T cells were enriched with *Standard* microbeads on LD columns, which allowed to enrich these cells to purities of 90.7% ± 1.4% (Figure 3E). Co-culturing the three CD19 CAR T cell populations differing in the hinge sequence overnight with EGFP-expressing Mino ($CD19^+$) or JeKo-1 ($CD19^+$) mantle cell lymphoma (MCL) and MOLM-14 acute monocytic leukemia (AML) ($CD19^-$) cells revealed that CD19-N3 and CD19-N4 CAR T cells killed the $CD19^+$ Mino and JeKo-1 cells as efficaciously as CD19-CH_2CH_3 CAR T cells (Figure 3F). Importantly, the $CD19^-$ MOLM-14 cells were not eliminated by N3- and N4-hinged CD19 CAR T cells, while the CH_2CH_3-hinged CD19 CAR T cells nonspecifically eradicated MOLM-14 cells (Figure 3F). This nonspecific activation of the CH_2CH_3-hinged CAR by MOLM-14 cells is most likely due to the presence of Fcγ receptors on these cells[44] and was described previously.[7,24]

N3- and N4-hinged CD19 CARs function comparably to their CD8-hinged counterparts

To establish our NGFR-derived hinges as potential candidates for clinical use, we compared the efficacy of N3- and N4-hinged CD19 and CD33 CAR constructs to counterparts that contained a 48 aa hinge region derived from the human CD8 α-chain.[45,46] Importantly, this CD8 sequence is used as the hinge in multiple CAR constructs[3] including the clinically approved CAR T cell products Kymriah[46] and Abecma.[47] To enable enrichment of CD8-hinged CAR T cells with CD271 microbeads, we also included the ΔNGFR cDNA in the vector with the CD8-hinged constructs. Primary human T cells were transduced with bicistronic lentiviral vectors co-expressing BFP and N3- or N4-hinged or ΔNGFR and CD8-hinged CD19 or CD33 CARs (Figure 4A), CAR T cells enriched via MACS and then co-cultured with EGFP-expressing Mino ($CD19^+$ $CD33^-$), REH ($CD19^+$ $CD33^-$), or MOLM-14 cells ($CD19^-$ $CD33^+$). When comparing the expression of the N3- and N4-hinged CARs with the CD8-hinged counterpart using the CD19 CAR detection reagent, we noticed that expression levels of the two NGFR-hinged CARs were at least comparable with the expression of the CD19-CD8h CAR construct (Figure 4B).

The N3- and N4-hinged CD19 CAR T cells lysed the $CD19^+$ cell lines Mino and REH, but not MOLM-14, as efficaciously as the CD19 CAR construct with the CD8 hinge (Figure 4C). Remarkably, the cytokine profiles of the three CD19 CAR T cell products were almost identical when analyzing IFN-γ, GM-CSF, and TNF-α in supernatants of the co-cultures with Mino and REH cells Figure 4D). It was noteworthy that co-cultures of the CD19 CAR T cells with the MCL Mino cells generally induced higher levels of cytokines compared with co-cultures with B cell precursor ALL REH cells. The CD33 CAR T cells remained nonresponsive against Mino cells, as the lysis remained at background levels (Figure 4C) and no inflammatory cytokines were induced, comparable with the incubation of the cell lines with untransduced T cells (Figure 4D). Co-culture of $CD19^+$ $CD33^-$ REH cells with N3/N4-hinged CD33 CAR T cells showed no specific lysis and no secretion of cytokines, while the CD8-hinged CD33 CAR T cells minimally lysed REH cells, albeit without induction of cytokines (Figures 4C and 4D). The $CD33^+$ $CD19^-$ MOLM-14 cells were efficaciously eliminated by all three CD33 CARs and the N3/N4-hinged CARs again proved to be as efficacious as the CD8-hinged CARs (Figure 4C). In the co-cultures with MOLM-14 cells, the CD33 CARs but not the CD19 CARs induced secretion of IFN-γ, GM-CSF, and TNF-α by T cells, comparable with the secretion profiles observed for CD19 CAR T cells upon co-culture with REH cells (Figure 4D).

N3 and N4 hinges can be used as the hinge in different CAR constructs

After establishing that N3- and N4-hinged CD19 and CD33 CAR constructs are as efficacious and specific as their CD8-hinged counterparts, we constructed three additional CARs with scFvs against ROR1, CD5, and CD123 with N3 or N4 hinges and also included the N3- or N4-hinged CD33 CAR constructs as controls in these experiments (Figure 5A). T cells were transduced with bicistronic lentiviral vectors co-expressing BFP and N3- or N4-hinged ROR1, CD5, CD33, or CD123 CARs; CAR T cells were enriched by MACS and co-cultured with EGFP-expressing Mino, JeKo-1, or MOLM-14 cells.

While Mino cells ($ROR1^+$, $CD5^+$, $CD33^-$, $CD123^-$) were only partially eliminated by N3-hinged ROR1 and CD5 CAR T cells (Figure 5B), the specific lysis increased when the cells were incubated with the N4-hinged counterparts (Figure 5C). Untransduced as well as CD33 and CD123 CAR T cells did not exhibit any cytotoxicity against

Figure 3. Enrichment of N3- and N4-hinged CD19 CAR T cells can be optimized by applying different microbeads or columns

(A) Photograph of the three columns used. Primary human T cells were lentivirally transduced with constructs co-expressing EGFP in *cis* with CD19-N3, CD19-N4, or CD19-CH_2CH_3 depicted in (B). Three days after transduction CD19-N3 cells (C) or CD19-N4 CAR T cells (D) were enriched with combinations of *Standard* or *Select* microbeads and MS, LS, or LD columns. (E) CD19-CH_2CH_3 CAR T cells were stained with *Standard* microbeads and separated on LD columns. Afterwards, the three fractions (preMACS, flowthrough, and postMACS) were flow cytometrically analyzed for EGFP expression. Representative blots are shown. Values are indicated as percentages and data are depicted as means ± SEM of at least three biological replicates. (F) MACS-enriched CD19-N3, CD19-N4, or CD19-CH_2CH_3 CAR T cells were co-cultured with EGFP-expressing Mino, JeKo-1, or MOLM-14 cells at various effector:target cell ratios. After 16 h co-culture, propidium iodide was added for viable/dead discrimination and samples were analyzed by flow cytometry for lysis of target cells by effector cells. Data were depicted as means ± SEM of at least three biological replicates.

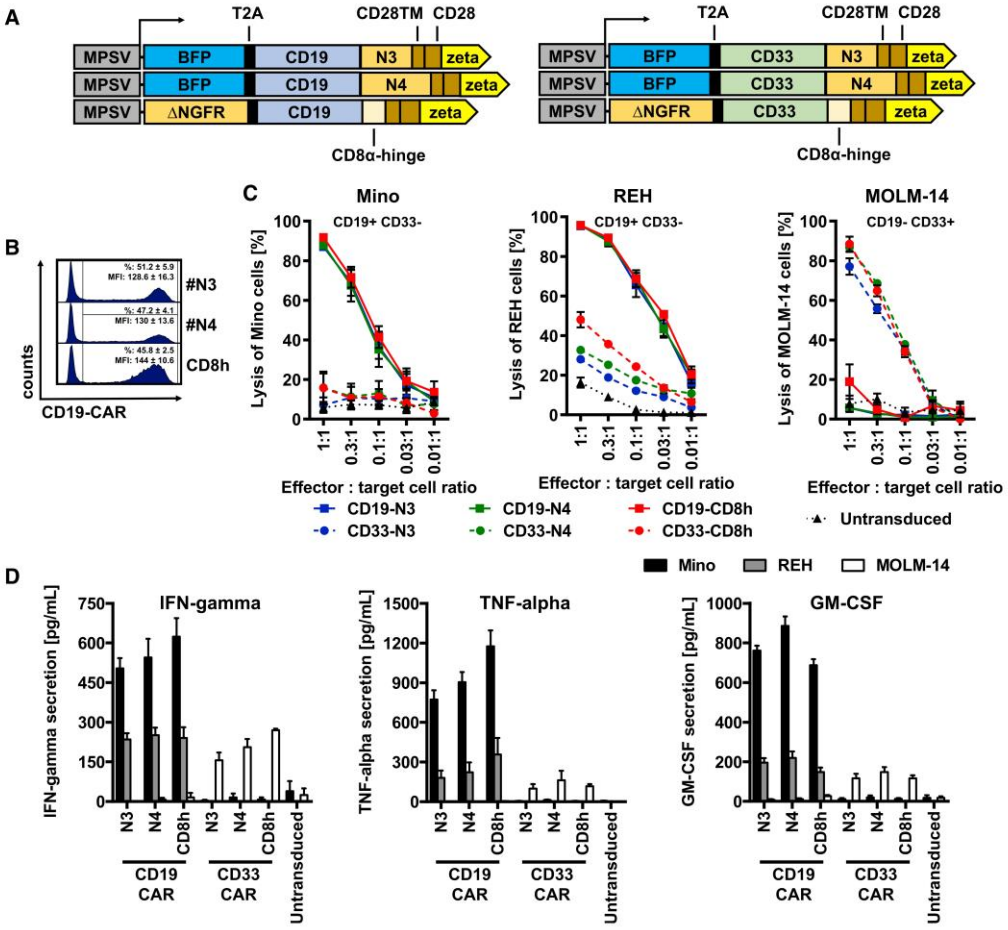

Figure 4. N3- and N4-hinged CD19 and CD33 CARs are as efficacious as CD8-hinged counterparts *in vitro*

Primary human T cells were lentivirally transduced with constructs co-expressing BFP in *cis* with N3- or N4-hinged CD19 or CD33 CARs or ΔNGFR in *cis* with CD8-hinged CD19 or CD33 CARs depicted in (A). (B) Three days after transduction, the T cells were flow cytometrically analyzed for CAR expression (via staining with CD19 CAR detection reagent) and enriched via magnetic cell sorting (CD271 MACSelect microbeads plus MS columns). Subsequently, CAR T cells were co-cultured with EGFP-expressing Mino, REH, or MOLM-14 cells at various effector:target cell ratios. (C) After 16 h co-culture, propidium iodide was added for viable/dead discrimination and samples were analyzed by flow cytometry for lysis of target cells by effector cells. (D) Co-culture supernatants were analyzed for the presence of IFN-γ, GM-CSF, and TNF-α by MACSPlex. Data were depicted as means ± SEM of at least three biological replicates.

Mino cells, regardless of the hinge used in the construct. In contrast, elimination of JeKo-1 cells (ROR1^{+}, CD5^{low+}, CD33^{-}, CD123^{-}) by ROR1-N3 and ROR1-N4 as well as CD5-N3 and CD5-N4 CAR T cells was comparable and not influenced by the hinge length. Noteworthy, the CD5 CAR constructs mediated only limited cytotoxicity, as CD5 is only expressed on a subpopulation of JeKo-1 cells.[24] Once again, the CD33 and CD123 CAR T cells with either N3 or N4 hinges remained inert, as both target antigens are not expressed on JeKo-1 cells (Figures 5B and 5C). MOLM-14 cells (ROR1^{-}, CD5^{-}, CD33^{+}, CD123^{+}) were efficaciously and specifically eliminated by N3- and N4-hinged CD33 and CD123 CAR T cells, respectively, but not by the CD5 and ROR1 CAR T cells (Figures 5B and 5C). In summary, using the N3 or N4 sequences as hinge in ROR1, CD5, CD33, and CD123 CAR T cells specifically eliminated antigen-positive cells without causing nonspecific lysis.

N3-, N4-, and CD8-hinged CD33 CAR T cells exert equal control of AML blasts *in vivo*

An important preclinical test for the efficacy and safety of the N3 and N4 hinges in CARs in a more complex model is the *in vivo* leukemia control of N3/N4-hinged CAR T cells compared to the control of CAR T cells with a CD8 hinge, thus allowing to see minimal differences and *off-target* cell toxicity over time. First, we engrafted NSG mice with 3.5 × 10^{6} MOLM-14 cells that had been equipped by a

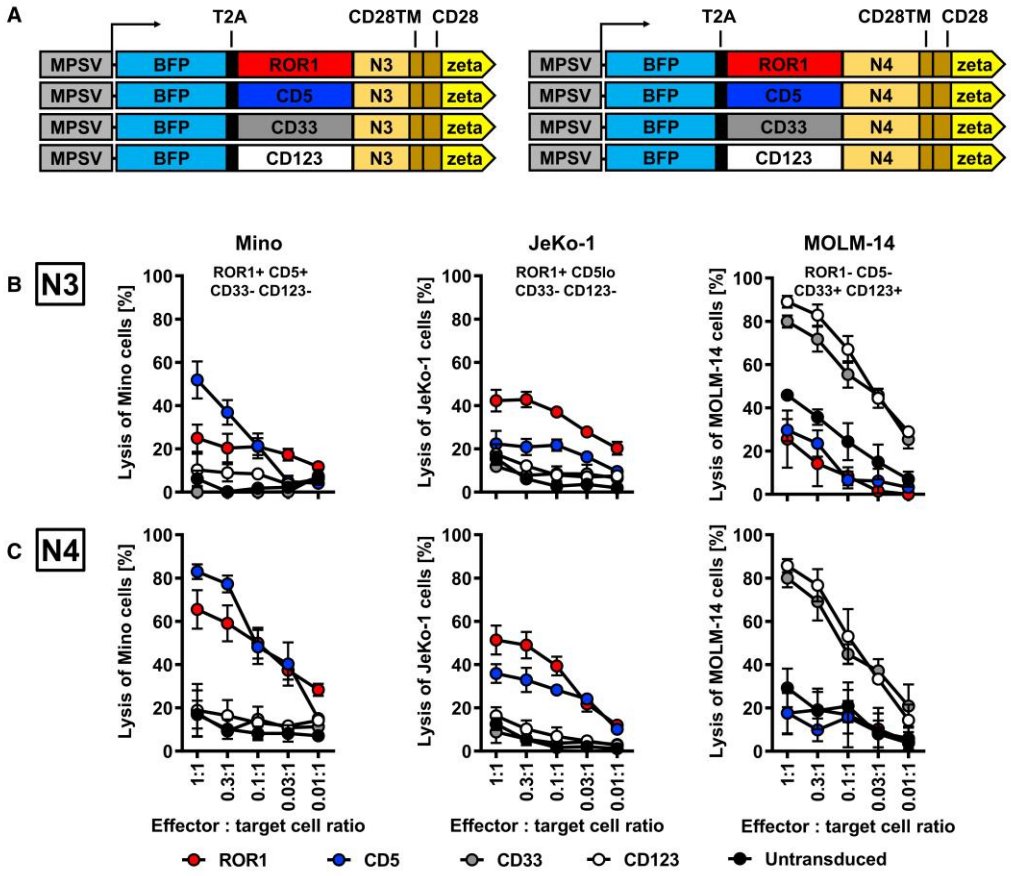

Figure 5. N3- and N4-hinged CARs eliminate malignant cells depending on antigen expression *in vitro*

Primary human T cells were lentivirally transduced with constructs co-expressing BFP in *cis* with N3- or N4-hinged ROR1, CD5, CD33, or CD123 CARs depicted in (A). Three days after transduction, CAR T cells were enriched via magnetic cell sorting (CD271 MACSelect microbeads plus MS columns for all constructs). Subsequently, N3-hinged (B) and N4-hinged (C) CAR T cells were co-cultured with EGFP-transduced Mino, JeKo-1, or MOLM-14 cells at various effector:target cell ratios. After 16 h co-culture, propidium iodide was added for viable/dead discrimination and samples were analyzed flow cytometrically for lysis of target cells by effector cells. Data were depicted as means ± SEM of at least three biological replicates.

lentiviral vector with a luciferase-EGFP fusion gene (LucEG).[24] The engraftment of the blasts was assessed 6 days later by luminescence imaging, revealing robust leukemia growth in almost all mice (Figure 6A). We then intravenously administered 3.5×10^6 CD19 or CD33 CAR T cells that contained N3, N4, or CD8 as hinges (Figure 4A) and which had been enriched by MACS to >90% purity. Mice in the control group did not receive a human T cell graft. The persistence of MOLM-14/LucEG cells and CAR T cells was analyzed on days 13, 20, 27, and 34 via luminescence imaging and/or flow cytometry after staining of peripheral blood with directly conjugated antibodies for murine cells (mCD18), human cells (CD45), T cells (CD3), and CAR expression (CD271). The content of AML blasts in the bone marrows was determined by flow cytometry upon sacrifice of the animals.

Untreated NSG mice experienced rapid progression of the AML, as shown by luminescence imaging (Figures 6A and 6B) and blood analysis (Figure 6D), and had to be sacrificed between days 21 and 25 (Figure 6C). The analyses of the animals at sacrifice demonstrated that the bone marrow was strongly infiltrated by MOLM-14/LucEG cells (Figure 6E). Treatment of the animals with CD19 CAR T cells, irrespective of the hinge, did not improve survival, as the disease rapidly progressed also in these animals (Figures 6A–6E). Although CD19 CAR T cells do not recognize MOLM-14 cells (Figure 4B), these CAR T cells still persisted for about three weeks, until sacrifice of the animals, in the bloodstream of the mice (Figure 6F) and could be detected at low levels in the bone marrow at sacrifice (Figure 6G). Importantly, the persistence of CD19 CAR T cells occurred irrespective of the hinge used, and did not influence the leukemia

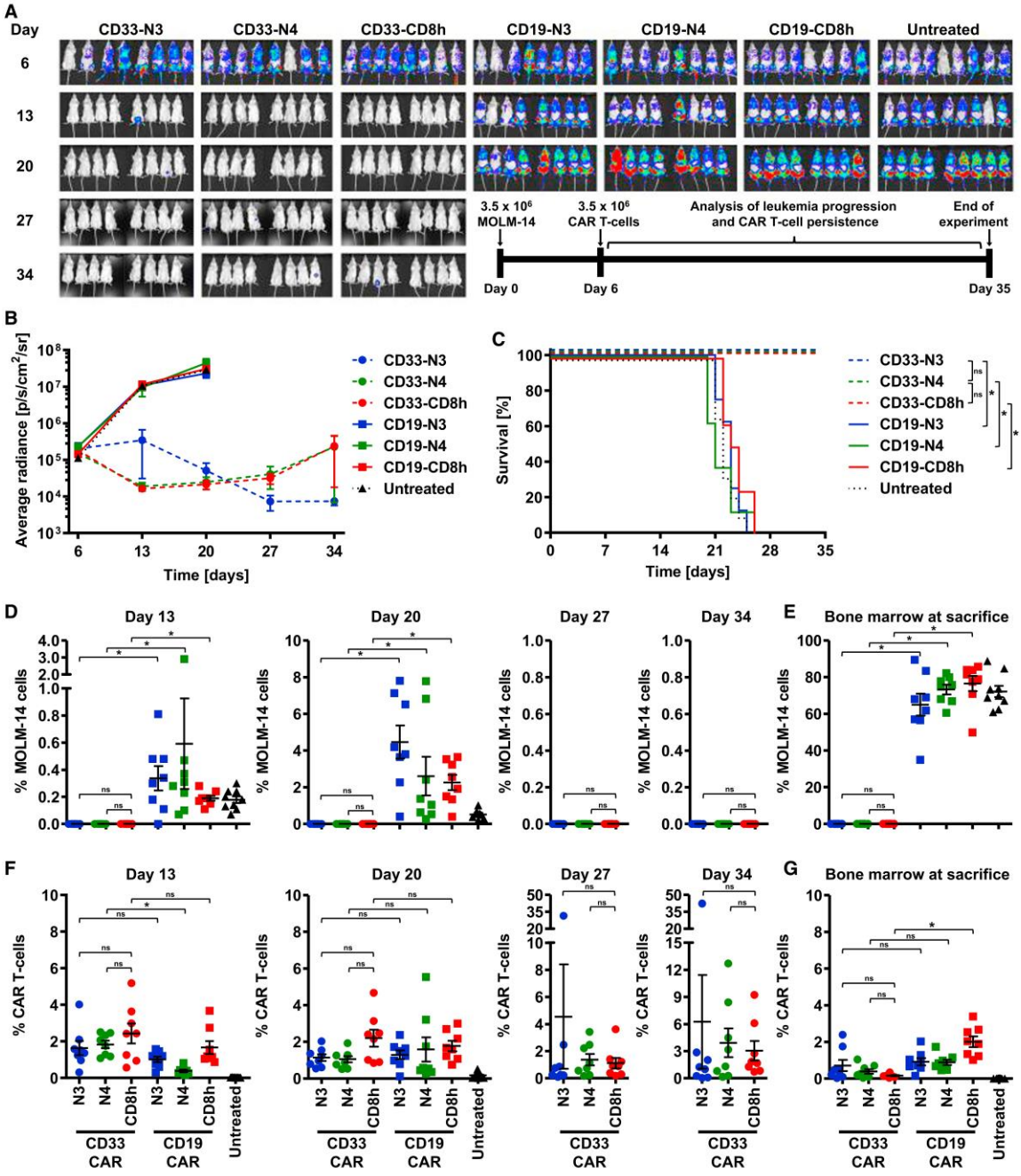

Figure 6. N3- and N4-hinged CD19 and CD33 CARs control AML progression as efficacious as CD8-hinged counterparts

NOD-SCID-gamma mice were xenografted with 3.5 × 10^6 MOLM-14/LucEG cells. After 6 days, mice were xenografted with a single dose of 3.5 × 10^6 N3-, N4-, or CD8-hinged CD19 or CD33 CARs or left untreated. To monitor disease progression, luminescence was measured on days 6, 13, 20, 27, and 34 (A and B), blood was flow

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control/survival of the animals. Treatment of the animals by administration of CD33-CAR T cells strongly reduced the leukemia burden and persistence and thus improved the overall survival (Figure 6). Here, it was obvious that N3- and N4-hinged CAR T cells proved to be as efficacious as their CD8-hinged counterparts (Figures 6A–6C). Blood analysis demonstrated that CD33 CAR T cells had engrafted well and persisted in the blood stream for up to 34 days, comparably to the animals that had received CD19 CAR T cells (on days 13 and 20; Figure 6F). Upon sacrifice of the animals, the BM samples of mice in all three CD33 CAR groups were devoid of AML blasts (Figure 6E), despite the previous findings that one to two animals in each group temporarily showed signs of extramedullary disease at single time points (Figure 6A). Finally, we did not observe any toxicity in mice treated with CD8-, N3-, or N4-hinged CAR T cells, suggesting that our NGFR-derived hinges are as efficacious and safe to use as the CD8 hinge.

DISCUSSION

In this work, we developed and validated two human NGFR/CD271-based hinges, N3 and N4, for inclusion in CAR constructs that facilitate both efficient enrichment of the genetically modified CAR T cells using the MACS technology (Miltenyi Biotec) and detection of CAR-positive cells *in vitro* and *in vivo* using staining with directly coupled antibodies and flow cytometry. Although N3 (120 aa) was shorter than N4 (162 aa), both hinges were highly comparable in their MACS enrichment efficiency with *Select* microbeads, their cytotoxicity, and their cytokine induction against leukemia and lymphoma cell lines *in vitro* as well as in their *in vivo* control of AML blasts in NSG mice. However, in CARs against ROR1 and CD5, the N4-hinged counterpart executed slightly higher killing of Mino cells compared with the N3 hinge. Whether this is specific for this cell line or depends on the CAR affinity needs to be evaluated in future studies.

Other groups already used amino acid sequences of the NGFR/CD271 surface domain as selection and detection marker after retroviral gene transfer into hematopoietic stem/progenitor cells as well as T cells.[38,42] Recently, Casucci et al.[12] included four different amino acid stretches binding the CD271 monoclonal antibody ME20.4 as hinges into a CD44v6 CAR construct: The longest hinge of 222 aa (NWL) contained the complete wild-type surface unit of NGFR, and a second hinge contained all four repeats but without the S/R-rich stalk (NWS). To prevent NGF binding,[48] they further constructed two hinges (NML and NMS) by introducing mutations in the fourth CRD of NWL and NWS. Importantly, only the NWL hinge with the complete surface unit of NGFR allowed selection of transduced T cells with directly conjugated microbeads, remarkably however this procedure was quite inefficient (yield of ~40%). For the other three hinges, the author even had to use a two-step procedure with a PE-labeled CD271 antibody followed by anti-PE microbeads,[12] which certainly will be a problem for applications in humans. Although none of the hinges appears satisfactory for the purification process (loss of >50% transduced cells in the flowthrough),[12] a lentiviral vector co-expressing a high-affinity CD44v6 CAR with the NWL hinge and herpes simplex virus 1 thymidine kinase suicide gene[39] is currently being employed in a phase I/IIa trial for AML and MM (NCT04097301).

In a more recent publication, the same group reported three new variants of the non-mutated full-length NWL hinge by shortening the S/R-rich stalk.[17] The shortest of these constructs, NWN2, with a length of 173 aa, was functionally almost undistinguishable from the NWL isoform in all assays. Strikingly, however, the CD44v6.NWN2 CAR T cells could also not be efficiently selected with ME20.4-based MACS microbeads; instead, the authors relied once again on a two-step procedure with ME20.4-PE staining followed by sheep-anti-mouse IgG1-coated microbeads, leading to purities of 90%, but yields for NWL and NWN2 CAR T cells of only 40% and 33%, respectively.[17]

Our novel NGFR-derived hinges are shorter and more efficient

Our two hinges allowed detecting the expression of CAR constructs on genetically modified T cells from *in vitro* and *in vivo* samples. They also facilitated very efficient and pure enrichment of CAR T cells, which is paramount if the intended use of the hinge is to produce pure CAR T cell products that can be controlled in patients due to co-expression of a suicide gene in the vector. For most CARs recognizing noncritical membrane-distant epitopes, we believe that the N3 hinge with 120 aa will suffice; however, more membrane-proximal located epitope as described for a ROR1 scFv[6] or CD44v6 isoforms[17] might require a longer hinge, such as N4. Importantly, the specific requirements for the hinge have to be determined for every CAR and targeted epitope on the malignant cells.

Our work here clearly demonstrated that enrichment of transduced cells via MACS needs to be optimized by determining the optimal combination of microbeads and columns. Based on our findings, we suspect that Casucci et al. as well as Stornaiuolo et al.[12,17] used a nonoptimal MACS protocol, leading to a loss of more than 50% of their CAR T cells on the columns. While the *Select* microbeads appear to be more highly concentrated, which improves retention of the cells within the magnetic field and thus MACS efficiency, the loss of genetically modified CAR T cells in the flowthrough of columns can partly also be avoided by using columns with a higher capacity e.g. the LD columns. However, we currently do not know whether this approach will translate well into the selection protocol used on the CliniMACS or Prodigy devices. In addition, the fact that our NGFR-derived

cytometrically analyzed on days 13, 20, 27, and 34 (D), and bone marrow was flow cytometrically analyzed upon sacrifice of the animals (E). To monitor CAR T cell persistence, blood was flow cytometrically analyzed on days 6, 13, 20, 27, and 34 (F) and bone marrow was flow cytometrically analyzed upon sacrifice of the animals (G). (C) Kaplan-Meyer-Survival curves for the seven treatment groups with eight animals per group. p values were calculated by (C) log rank test or (D–G) one-way ANOVA with Dunn's correction for multiple comparisons. The asterisks indicated statistical significance; ns, not significant. Data were depicted as means ± SEM with eight to nine mice per treatment group.

hinges N3 and N4 contain the entire S/R-rich stalk also seems to be important for high-level CAR expression, as Stornaiuolo et al.[17] reported the shortening of the stalk to be associated with larger amounts of CAR constructs remaining in the intracellular compartment. Finally, in contrast to the two previous publications from Casucci et al. and Stornaiuolo et al.,[12,17] we rigorously demonstrated here that our NGFR-derived hinges are functionally indistinguishably from an already clinically used CD8 hinge in *in vitro* assays and *in vivo* in a xenotransplantation model for AML, but still facilitate efficient recognition and also selection of CAR T cells.

Safety

A hinge cannot only affect CAR function but can also cause unwanted side effects due nonspecific CAR signaling and T cell activation.[6,7] A well-characterized example of this phenomenon are hinges based on the second and third constant heavy chain domain of human IgG1 or IgG4 (CH_2CH_3). These hinges provide the necessary flexibility, stability, and length for multiple scFvs in CAR constructs to reach their epitopes.[13,49] However, CH_2CH_3 hinges can also cause *off-target off-tumor* toxicities by binding to Fc receptors on myeloid cells, thereby mediating activation-induced cell death of the T cells and nonspecific lysis of Fc receptor-positive cells, such as macrophages, monocytes, or NK cells.[6,7,50] Indeed, as a positive control, we were able to reproduce scFv-independent activation of CH_2CH_3-hinged CAR T cells after co-culture with FcγR expressing MOLM-14 cells.[44] In contrast, our N3 and N4 hinges were not activated by target antigen-negative cells in any of our experiments *in vitro* and *in vivo*, thus suggesting that no *off-target* effects are mediated by the hinges. Finally, Casucci et al.[12] observed that incubation of NGFR-hinged CAR T cells, containing the entire surface domain of NGFR, with supraphysiological concentrations of NGF, did not affect the transduced cells, probably as important transmembrane and cytoplasmic sequences required for NGF binding and receptor dimerization were missing in these constructs.[48,51,52]

Two hinges for Split-CAR approaches

We previously reported the development of a CD34-derived hinge with a 99 aa sequence of human CD34,[24] which contained the 16 aa epitope of CD34 recognized by the antibody clone QBend-10.[53] Thus, we now potentially have two different hinges available for efficient CAR T cell enrichment under GMP-compliant conditions. Individual staining and/or selection procedures enabled by two hinges are important for *Split-CAR* approaches where two CAR constructs need to be present/monitored on the same cell. Here, the classical intracellular signaling domains CD3ζ and CD28 of a second-generation CAR are split upon two distinct CAR constructs, where each scFv targets a different antigen. Consequently, the T cell is only fully activated by co-current CD28 and CD3ζ signaling, when both *Split-CAR* constructs bind their specific antigens on the target cells.[54] Ideally, this system allows to reduce *on-target off-tumor* toxicities associated with the adoptive CAR T cell treatment, as only a pathologic co-expression of two target antigens leads to the full activation of the CAR T cells and therefore killing of the target cells. However, for this approach to work, the expression of the two CARs needs to be carefully balanced and the expression of the two target antigens on the malignant cells relatively constant.[54] We currently are focusing our *Split-CAR* work on MCL as a difficult to treat hematological malignant entity in adults and used the two hinges, N3 and CD34, to co-target combinations of CD19, CD5, and ROR1 (Bister et al., unpublished data).

CONCLUSION

In summary, we developed two novel human NGFR-derived hinges of different lengths that allow manufacturing of pure CAR T cell products, using MACS technology to monitor the CAR expression levels on T cells and flow cytometry to detect the presence of transduced T cells in preclinical models and eventually in patients. Despite being slightly larger, the two hinges proved to be as effective as a human CD8-based hinge *in vitro* as well as *in vivo* without any signs of *off-target* toxicities in the tested systems.

MATERIALS AND METHODS

Construct generation

Human NGFR/CD271 (NP_002498.1) truncated after the transmembrane domain at aa position 276 was codon-optimized for human codon usage and synthesized by GeneArt (Thermo Fisher Scientific, Schwerte, Germany). Hinge fragments of different lengths were generated by PCR amplification and inserted into our self-inactivating lentiviral vector expressing a FMC63-based CD19 CAR with a CH_2CH_3 hinge off a viral MPSV U3 promoter.[24,43,55] We also used a T2A site for expression of two transgenes.[36] ΔNGFR-hinged CD19 CARs were inserted behind and EGFP or tagBFP (referred to as BFP) in front of the T2A site.[56] Moreover, the N3 and N4 hinges were cloned into codon-optimized CARs recognizing the following human target antigen structures: ROR1 (clone R12[5,57]), CD5 (clone H65[58,59]), CD33 (clone DRB2[60]), and CD123 (clone 43[61]). CAR constructs with a human CD8-derived hinge/without a ΔNGFR-derived hinge were co-expressed with the codon-optimized cytoplasmically truncated NGFR[36] to enable enrichment with directly labeled NGFR microbeads.

Cell culture

The acute T cell leukemia cell line Jurkat, the MCL cell lines Mino and JeKo-1, the acute lymphoblastic leukemia (ALL) cell line REH, and the AML cell line MOLM-14 (all purchased from DSMZ, Braunschweig, Germany) were maintained in RPMI-1640 GlutaMAX medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Thermo Fisher Scientific). HEK293T cells (DSMZ) were cultured in DMEM GlutaMAX (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Antigen expression profiles of the used cell lines were published previously.[24]

Primary human T cells were isolated from peripheral blood of healthy donors who gave written and informed consent according to the protocols (no. 4687 and no. 2019-623) approved by the ethics committee of the University Hospital Düsseldorf. Peripheral blood (PB) mononuclear cells were separated via density gradient centrifugation using

Ficoll-Paque Plus (Cytiva Europe, Freiburg, Germany) according to the manufacturer's instructions. To activate and expand T cells, PBMCs were cultured in IMDM (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (Thermo Fisher Scientific), and 100 U/mL interleukin-2 (IL-2, Proleukin, Novartis, Basel, Switzerland) on anti-human CD3- (Thermo Fisher Scientific) and anti-human CD28-coated (BD Biosciences, Heidelberg, Germany) six-well plates.

All cells were maintained at 5% CO_2, 95% humidity, and 37°C.

Production of lentiviral vectors and transduction of eukaryotic cells

Vesicular stomatitis virus G glycoprotein-pseudotyped replication-deficient lentiviral vectors were generated by polyethyleneiminie transfection (Sigma-Aldrich, Darmstadt, Germany) of 6 µg pczVSV-G, 6 µg pCD-NL/BH, and 6 µg vector plasmid into HEK 293T cells as described previously.[24,62,63] Two days after transfection, virus-containing supernatant was harvested, filtered (0.45 µm), and used for transduction of eukaryotic cells. For the transduction of Jurkat or primary human T cells, 5×10^5 cells were incubated with 2 mL virus-containing supernatant and 10 µg/mL protamine phosphate (Sigma-Aldrich) for 24 h, replenished with fresh medium, and used for experiments after 48 h.

Mino, JeKo-1, REH, or MOLM-14 cells were transduced with limited dilutions of lentiviral vectors to express EGFP and G418 resistance or a firefly luciferase-EGFP fusion protein with subsequent antibiotic selection and/or flow-assisted cell sorting as described previously.[24,62,64]

Cell enrichment via MACS and flow cytometry

Three to four days after transduction, CAR T cells were enriched with magnetic microbeads and separation columns from Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, cells were labeled with either CD271 microbeads (from now on referred to as *Standard* microbeads) or LNGFR MACSelect microbeads (from now on referred to as *Select* microbeads) and separated on MS (maximal capacity: 1×10^7 cells), LS (maximal capacity: 1×10^8 cells), and LD (maximal capacity: 5×10^8 cells) columns. The three fractions (preMACS, flowthrough, and postMACS) were flow cytometrically analyzed on a MACSQuant Analyzer X for EGFP, CAR expression, and ΔNGFR expression via staining with CD271-PE (clone ME20.4, Miltenyi Biotec).

The expression levels of CD19 CAR constructs were determined by flow cytometry using the biotin-coupled CD19 CAR detection reagent followed by staining with anti-biotin-PE or anti-bioti-APC monoclonal antibodies (all reagents from Miltenyi Biotec).

Functional *in vitro* assays

CAR-mediated cytotoxicity of CAR T cells against malignant cell lines was measured via flow cytometry. CAR T cells were co-cultured at various ratios with 2×10^4 EGFP-transduced Mino, JeKo-1, REH, or MOLM-14 cells for 16 h in U-bottom 96-well plates. Subsequently, supernatants were harvested and frozen at −20°C for cytokine analysis, cells were washed and stained with 1 µg/mL propidium iodide (Sigma-Aldrich) for dead/viable distinction, analyzed on the MACSQuant Analyzer X, and data were evaluated with the MACSQuantify Software 2.11. Tumor cell lysis was determined as 100% – (number of viable tumor cells after co-culture with CAR T cells/number of viable tumor cells without CAR T cells) × 100%. Negative lysis rates were set to be 0%.

Cytokine secretion by CAR T cells was analyzed using the MACSPlex Cytotoxic T/NK cell kit (Miltenyi Biotec) according to the manufacturer's instructions. Per analysis, 50 µL undiluted supernatant was used.

In vivo xenograft model

Animal studies were approved by the state animal research committee (LANUV, NRW, Germany) and all animals were cared for according to the guidelines set by the Federation of European Laboratory Animal Science Associations. Six- to 8-week-old female NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (NOD-SCID gamma; NSG) mice (Charles River Laboratories, Sulzfeld, Germany) were intravenously engrafted with 3.5×10^6 MOLM-14 cells stably expressing a firefly luciferase-EGFP fusion protein (LucEG). Six days later, mice were intravenously injected with 3.5×10^6 N3-, N4-, or CD8-hinged CD19 or CD33 CAR T cells. At days 6, 13, 20, 27, and 34, the persistence of MOLM-14 cells was assessed via luminescence imaging and PB analysis. For luminescence imaging, mice were intraperitoneally injected with D-luciferin (OZ Biosciences SAS, Marseilles, France) and after 5 min luminescence was measured in a Caliper IVIS Lumina II system (PerkinElmer LAS, Rodgau, Germany) with an exposure time of 15 s. PB was drawn from the tail vein, the erythrocytes lysed with BD Pharm Lyse (BD Biosciences), and the samples analyzed on a MACSQuant Analyzer X flow cytometer for EGFP, CD33, and CD45 expression for MOLM-14 cells and BFP, CAR (ΔNGFR), CD3, and CD45 expression for CAR T cells after staining with CD271-PE, CD3-PerCP-Vio700, CD45-APC, and CD33-APC-Vio770 (all from Miltenyi Biotec)

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9. p values were calculated using one-way ANOVA with Dunn's correction for multiple comparisons of log rank test. p values below 0.05 were considered statistically significant and are indicated by an asterisk.

ACKNOWLEDGMENTS

We gratefully acknowledge the healthy donors who provided peripheral blood for the *in vitro* and *in vivo* studies. We would like to thank Jörg Schipper, MD, director of the ENT clinic, for his support of this research project. We are also in debt to Wolfgang Schulz, PhD, and Michèle Hoffmann, PhD, both in the Department of Urology, Heinrich Heine University, for the use of the MACSQuant Analyzer X. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen. This work was supported, in part, by

funding from the Medical Research School Düsseldorf, DSO, Heinrich-Heine-Universität Düsseldorf, and the Forschungskommission of the Medical Faculty (KR/27/2016), Heinrich Heine University Dü, the Deutsche Forschungsgemeinschaft (CW/2021-06), the Essener Elterninitiative zur Unterstüzung krebskranker Kinder e.V. and within the framework of the iCAN33 project, funded by the European Regional 470 Development Fund NRW (ERDF, German EFRE) 2014–2020.

AUTHOR CONTRIBUTIONS

A.B., T.I., K.R., M.W., K.S., N.G., C.W., and H.H. planned the experiments. A.B., T.I., C.H., G.G., D.S., M.S., and K.R. conducted the experiments. A.B., T.I., M.S., K.R., and G.G. analyzed the data. A.B., C.H., G.G., K.S., C.W., and H.H. wrote the manuscript. All authors approved the final manuscript.

DECLARATION OF INTERESTS

H.H., C.W., T.I., and K.R. are inventors on a patent describing the NGFR hinges. All other authors declare no competing interests.

REFERENCES

1. Gross, G., Waks, T., and Eshhar, Z. (1989). Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc. Natl. Acad. Sci. USA *86*, 10024–10028. https://doi.org/10.1073/pnas.86.24.10024.

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

3. Larson, R.C., and Maus, M.V. (2021). Recent advances and discoveries in the mechanisms and functions of CAR T cells. Nat. Rev. Cancer *21*, 145–161. https://doi.org/10.1038/s41568-020-00323-z.

4. Albinger, N., Hartmann, J., and Ullrich, E. (2021). Current status and perspective of CAR-T and CAR-NK cell therapy trials in Germany. Gene Ther. *28*, 513–527. https://doi.org/10.1038/s41434-021-00246-w.

5. Hudecek, M., Lupo-Stanghellini, M.T., Kosasih, P.L., Sommermeyer, D., Jensen, M.C., Rader, C., and Riddell, S.R. (2013). Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. Clin. Cancer Res. *19*, 3153–3164. https://doi.org/10.1158/1078-0432.CCR-13-0330.

6. Hudecek, M., Sommermeyer, D., Kosasih, P.L., Silva-Benedict, A., Liu, L., Rader, C., Jensen, M.C., and Riddell, S.R. (2015). The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. Cancer Immunol. Res. *3*, 125–135. https://doi.org/10.1158/2326-6066.CIR-14-0127.

7. Hombach, A., Hombach, A.A., Abken, H., and HombAch, A. (2010). Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc 'spacer' domain in the extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and unintended initiation of an innate immune response. Gene Ther. *17*, 1206–1213. https://doi.org/10.1038/gt.2010.91.

8. Alabanza, L., Pegues, M., Geldres, C., Shi, V., Wiltzius, J.J.W., Sievers, S.A., Yang, S.C., and Kochenderfer, J.N. (2017). Function of novel anti-CD19 chimeric antigen receptors with human variable regions is affected by hinge and transmembrane domains. Mol. Ther. *25*, 2452–2465. https://doi.org/10.1016/j.ymthe.2017.07.013.

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

10. Qin, L., Lai, Y., Zhao, R., Wei, X., Weng, J., Lai, P., Li, B., Lin, S., Wang, S., Wu, Q., et al. (2017). Incorporation of a hinge domain improves the expansion of chimeric antigen receptor T cells. J. Hematol. Oncol. *10*, 68. https://doi.org/10.1186/s13045-017-0437-8.

11. Almåsbak, H., Walseng, E., Kristian, A., Myhre, M.R., Suso, E.M., Munthe, L.A., Andersen, J.T., Wang, M.Y., Kvalheim, G., Gaudernack, G., et al. (2015). Inclusion of an IgG1-Fc spacer abrogates efficacy of CD19 CAR T cells in a xenograft mouse model. Gene Ther. *22*, 391–403. https://doi.org/10.1038/gt.2015.4.

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

13. Guest, R.D., Hawkins, R.E., Kirillova, N., Cheadle, E.J., Arnold, J., O'Neill, A., Irlam, J., Chester, K.A., Kemshead, J.T., Shaw, D.M., et al. (2005). The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. J. Immunother. *28*, 203–211. https://doi.org/10.1097/01.cji.0000161397.96582.59.

14. Jonnalagadda, M., Mardiros, A., Urak, R., Wang, X., Hoffman, L.J., Bernanke, A., Chang, W.C., Bretzlaff, W., Starr, R., Priceman, S., et al. (2015). Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy. Mol. Ther. *23*, 757–768. https://doi.org/10.1038/mt.2014.208.

15. Koristka, S., Ziller-Walter, P., Bergmann, R., Arndt, C., Feldmann, A., Kegler, A., Cartellieri, M., Ehninger, A., Ehninger, G., Bornhäuser, M., and Bachmann, M.P. (2019). Anti-CAR-engineered T cells for epitope-based elimination of autologous CAR T cells. Cancer Immunol. Immunother. *68*, 1401–1415. https://doi.org/10.1007/s00262-019-02376-y.

16. Liu, L., Sommermeyer, D., Cabanov, A., Kosasih, P., Hill, T., and Riddell, S.R. (2016). Inclusion of Strep-tag II in design of antigen receptors for T-cell immunotherapy. Nat. Biotechnol. *34*, 430–434. https://doi.org/10.1038/nbt.3461.

17. Stornaiuolo, A., Valentinis, B., Sirini, C., Scavullo, C., Asperti, C., Zhou, D., Martinez De La Torre, Y., Corna, S., Casucci, M., Porcellini, S., and Traversari, C. (2021). Characterization and functional analysis of CD44v6.CAR T cells endowed with a new low-affinity nerve growth factor receptor-based spacer. Hum. Gene Ther. *32*, 744–760. https://doi.org/10.1089/hum.2020.216.

18. Watanabe, N., Bajgain, P., Sukumaran, S., Ansari, S., Heslop, H.E., Rooney, C.M., Brenner, M.K., Leen, A.M., and Vera, J.F. (2016). Fine-tuning the CAR spacer improves T-cell potency. Oncoimmunology *5*, e1253656. https://doi.org/10.1080/2162402X.2016.1253656.

19. Locke, F.L., Ghobadi, A., Jacobson, C.A., Miklos, D.B., Lekakis, L.J., Oluwole, O.O., Lin, Y., Braunschweig, I., Hill, B.T., Timmerman, J.M., et al. (2019). Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. Lancet Oncol. *20*, 31–42. https://doi.org/10.1016/S1470-2045(18)30864-7.

20. Cheng, Z., Wei, R., Ma, Q., Shi, L., He, F., Shi, Z., Jin, T., Xie, R., Wei, B., Chen, J., et al. (2018). In vivo expansion and antitumor activity of coinfused CD28- and 4-1BB-engineered CAR-T cells in patients with B cell leukemia. Mol. Ther. *26*, 976–985. https://doi.org/10.1016/j.ymthe.2018.01.022.

21. Vairy, S., Lopes Garcia, J., Teira, P., and Bittencourt, H. (2018). CTL019 (tisagenlecleucel): CAR-T therapy for relapsed and refractory B-cell acute lymphoblastic leukemia. Drug Des. Devel Ther. *12*, 3885–3898. https://doi.org/10.2147/DDDT.S138765.

22. Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T cell activation. Annu. Rev. Immunol. *27*, 591–619. https://doi.org/10.1146/annurev.immunol.021908.132706.

23. Valton, J., Guyot, V., Boldajipour, B., Sommer, C., Pertel, T., Juillerat, A., Duclert, A., Sasu, B.J., Duchateau, P., and Poirot, L. (2018). A versatile safeguard for chimeric antigen receptor T-cell immunotherapies. Sci. Rep. *8*, 8972. https://doi.org/10.1038/s41598-018-27264-w.

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

25. Jena, B., Maiti, S., Huls, H., Singh, H., Lee, D.A., Champlin, R.E., and Cooper, L.J.N. (2013). Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. PLoS One *8*, e57838. https://doi.org/10.1371/journal.pone.0057838.

26. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet *385*, 517–528. https://doi.org/10.1016/S0140-6736(14)61403-3.

27. Badbaran, A., Berger, C., Riecken, K., Kruchen, A., Geffken, M., Müller, I., Kröger, N., Ayuk, F.A., and Fehse, B. (2020). Accurate in-vivo quantification of CD19 CAR-T cells after treatment with axicabtagene ciloleucel (Axi-Cel) and tisagenlecleucel (Tisa-Cel) using digital PCR. Cancers *12*, 1970. https://doi.org/10.3390/cancers12071970.

28. Ayuk, F., Fehse, B., Janson, D., Berger, C., Riecken, K., and Kröger, N. (2020). Excellent proliferation and persistence of allogeneic donor-derived 41-BB based CAR-T cells despite immunosuppression with cyclosporine A. Haematologica *105*, E322–E324. https://doi.org/10.3324/haematol.2019.245969.

29. Abramson, J.S., Palomba, M.L., Gordon, L.I., Lunning, M.A., Wang, M., Arnason, J., Mehta, A., Purev, E., Maloney, D.G., Andreadis, C., et al. (2020). Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. Lancet *396*, 839–852. https://doi.org/10.1016/S0140-6736(20)31366-0.

30. Turtle, C.J., Hanafi, L.A., Berger, C., Gooley, T.A., Cherian, S., Hudecek, M., Sommermeyer, D., Melville, K., Pender, B., Budiarto, T.M., et al. (2016). CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J. Clin. Invest. *126*, 2123–2138. https://doi.org/10.1172/JCI85309.

31. Porter, D.L., Hwang, W.T., Frey, N.V., Lacey, S.F., Shaw, P.A., Loren, A.W., Bagg, A., Marcucci, K.T., Shen, A., Gonzalez, V., et al. (2015). Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci. Transl Med. *7*, 303ra139. https://doi.org/10.1126/scitranslmed.aac5415.

32. Maude, S.L., Frey, N., Shaw, P.A., Aplenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. *371*, 1507–1517. https://doi.org/10.1056/NEJMoa1407222.

33. Ritchie, D.S., Neeson, P.J., Khot, A., Peinert, S., Tai, T., Tainton, K., Chen, K., Shin, M., Wall, D.M., Hönemann, D., et al. (2013). Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. Mol. Ther. *21*, 2122–2129. https://doi.org/10.1038/mt.2013.154.

34. Zhang, Q., Hu, H., Chen, S.Y., Liu, C.J., Hu, F.F., Yu, J.M., Wu, Y.H., and Guo, A.Y. (2019). Transcriptome and regulatory network analyses of CD19-CAR-T immunotherapy for B-all. Genom Proteom Bioinf *17*, 190–200. https://doi.org/10.1016/j.gpb.2018.12.008.

35. Canté-Barrett, K., Mendes, R.D., Smits, W.K., van Helsdingen-van Wijk, Y.M., Pieters, R., and Meijerink, J.P.P. (2016). Lentiviral gene transfer into human and murine hematopoietic stem cells: size matters. BMC Res. Notes *9*, 312. https://doi.org/10.1186/s13104-016-2118-z.

36. Roellecke, K., Virts, E.L., Einholz, R., Edson, K.Z., Altvater, B., Rossig, C., von Laer, D., Scheckenbach, K., Wagenmann, M., Reinhardt, D., et al. (2016). Optimized human CYP4B1 in combination with the alkylator prodrug 4-ipomeanol serves as a novel suicide gene system for adoptive T-cell therapies. Gene Ther. *23*, 615–626. https://doi.org/10.1038/gt.2016.38.

37. Johnson, D., Lanahan, A., Buck, C.R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986). Expression and structure of the human NGF receptor. Cell *47*, 545–554. https://doi.org/10.1016/0092-8674(86)90619-7.

38. Ciceri, F., Bonini, C., Stanghellini, M.T.L., Bondanza, A., Traversari, C., Salomoni, M., Turchetto, L., Colombi, S., Bernardi, M., Peccatori, J., et al. (2009). Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol. *10*, 489–500. https://doi.org/10.1016/S1470-2045(09)70074-9.

39. Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponzoni, M., Rossini, S., Mavilio, F., Traversari, C., and Bordignon, C. (1997). HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science *276*, 1719–1724. https://doi.org/10.1126/science.276.5319.1719.

40. Amaria, R.N., Haymaker, C.L., Bernatchez, C., Forget, M.-A., Patel, V., Hwu, W.-J., Davies, M.A., Patel, S.P., Diab, A., Glitza, I.C., et al. (2016). A phase I/II study of lymphodepletion plus adoptive cell transfer (ACT) with T cells transduced with CXCR2 and NGFR followed by high dose interleukin-2 (IL-2) in patients with metastatic melanoma (MM). J. Clin. Oncol. *34*, TPS9594. https://doi.org/10.1200/JCO.2016.34.15_suppl.TPS9594.

41. Li, Z., Dullmann, J., Schiedlmeier, B., Schmidt, M., von Kalle, C., Meyer, J., Forster, M., Stocking, C., Wahlers, A., Frank, O., et al. (2002). Murine leukemia induced by retroviral gene marking. Science *296*, 497. https://doi.org/10.1126/science.1068893.

42. Bonini, C., Grez, M., Traversari, C., Ciceri, F., Marktel, S., Ferrari, G., Dinauer, M., Sadat, M., Aiuti, A., Deola, S., et al. (2003). Safety of retroviral gene marking with a truncated NGF receptor. Nat. Med. *9*, 367–369. https://doi.org/10.1038/nm0403-367.

43. Landmeier, S., Altvater, B., Pscherer, S., Meltzer, J., Sebire, N., Pule, M., Vera, J., Hotfilder, M., Juergens, H., Vormoor, J., and Rossig, C. (2010). Cytotoxic T cells transduced with chimeric anti-CD19 receptors prevent engraftment of primary lymphoblastic leukemia in vivo. Leukemia *24*, 1080–1084. https://doi.org/10.1038/leu.2010.38.

44. Matsuo, Y., MacLeod, R.A., Uphoff, C.C., Drexler, H.G., Nishizaki, C., Katayama, Y., Kimura, G., Fujii, N., Omoto, E., Harada, M., and Orita, K. (1997). Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). Leukemia *11*, 1469–1477. https://doi.org/10.1038/sj.leu.2400768.

45. Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D., Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G., et al. (2009). Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol. Ther. *17*, 1453–1464. https://doi.org/10.1038/mt.2009.83.

46. Schuster, S.J., Bishop, M.R., Tam, C.S., Waller, E.K., Borchmann, P., McGuirk, J.P., Jäger, U., Jaglowski, S., Andreadis, C., Westin, J.R., et al. (2019). Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. N. Engl. J. Med. *380*, 45–56. https://doi.org/10.1056/NEJMoa1804980.

47. Munshi, N.C., Anderson, L.D., Jr., Shah, N., Madduri, D., Berdeja, J., Lonial, S., Raje, N., Lin, Y., Siegel, D., Oriol, A., et al. (2021). Idecabtagene vicleucel in relapsed and refractory multiple myeloma. N. Engl. J. Med. *384*, 705–716. https://doi.org/10.1056/NEJMoa2024850.

48. Yan, H., and Chao, M.V. (1991). Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. J. Biol. Chem. *266*, 12099–12104. https://doi.org/10.1016/s0021-9258(18)99070-8.

49. Moritz, D., and Groner, B. (1995). A spacer region between the single chain antibody- and the CD3 zeta-chain domain of chimeric T cell receptor components is required for efficient ligand binding and signaling activity. Gene Ther. *2*, 539–546.

50. Ravetch, J.V., and Bolland, S. (2001). IgG fc receptors. Annu. Rev. Immunol. *19*, 275–290. https://doi.org/10.1146/annurev.immunol.19.1.275.

51. Shamovsky, I.L., Ross, G.M., Riopelle, R.J., and Weaver, D.F. (2008). The interaction of neurotrophins with the p75NTR common neurotrophin receptor: a comprehensive molecular modeling study. Protein Sci. *8*, 2223–2233. https://doi.org/10.1110/ps.8.11.2223.

52. Vilar, M., Charalampopoulos, I., Kenchappa, R.S., Simi, A., Karaca, E., Reversi, A., Choi, S., Bothwell, M., Mingarro, I., Friedman, W.J., et al. (2009). Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron *62*, 72–83. https://doi.org/10.1016/j.neuron.2009.02.020.

53. Philip, B., Kokalaki, E., Mekkaoui, L., Thomas, S., Straathof, K., Flutter, B., Marin, V., Marafioti, T., Chakraverty, R., Linch, D., et al. (2014). A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. Blood *124*, 1277–1287. https://doi.org/10.1182/blood-2014-01-545020.

54. Kloss, C.C., Condomines, M., Cartellieri, M., Bachmann, M., and Sadelain, M. (2013). Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat. Biotechnol. *31*, 71–75. https://doi.org/10.1038/nbt.2459.

55. Rössig, C., Pscherer, S., Landmeier, S., Altvater, B., Jürgens, H., and Vormoor, J. (2005). Adoptive cellular immunotherapy with CD19-specific T cells. Klin Padiatr. *217*, 351–356. https://doi.org/10.1055/s-2005-872521.

56. Haist, C., Schulte, E., Bartels, N., Bister, A., Poschinski, Z., Ibach, T.C., Geipel, K., Wiek, C., Wagenmann, M., Monzel, C., et al. (2021). CD44v6-targeted CAR T-cells specifically eliminate CD44 isoform 6 expressing head/neck squamous cell carcinoma cells. Oral Oncol. *116*, 105259. https://doi.org/10.1016/j.oraloncology.2021.105259.

57. Yang, J., Baskar, S., Kwong, K.Y., Kennedy, M.G., Wiestner, A., and Rader, C. (2011). Therapeutic potential and challenges of targeting receptor tyrosine kinase ROR1 with monoclonal antibodies in B-cell malignancies. PLoS One *6*, e21018. https://doi.org/10.1371/journal.pone.0021018.

58. Studnicka, G.M., Soares, S., Better, M., Williams, R.E., Nadell, R., and Horwitz, A.H. (1994). Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementarity-modulating residues. Protein Eng. *7*, 805–814. https://doi.org/10.1093/protein/7.6.805.

59. Mamonkin, M., Rouce, R.H., Tashiro, H., and Brenner, M.K. (2015). A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies. Blood *126*, 983–992. https://doi.org/10.1182/blood-2015-02-629527.

60. Stamova, S., Cartellieri, M., Feldmann, A., Arndt, C., Koristka, S., Bartsch, H., Bippes, C.C., Wehner, R., Schmitz, M., von Bonin, M., et al. (2011). Unexpected recombinations in single chain bispecific anti-CD3-anti-CD33 antibodies can be avoided by a novel linker module. Mol. Immunol. *49*, 474–482. https://doi.org/10.1016/j.molimm.2011.09.019.

61. Stein, C., Kellner, C., Kügler, M., Reiff, N., Mentz, K., Schwenkert, M., Stockmeyer, B., Mackensen, A., and Fey, G.H. (2010). Novel conjugates of single-chain Fv antibody fragments specific for stem cell antigen CD123 mediate potent death of acute myeloid leukaemia cells. Br. J. Haematol. *148*, 879–889. https://doi.org/10.1111/j.1365-2141.2009.08033.x.

62. Wiek, C., Schmidt, E.M., Roellecke, K., Freund, M., Nakano, M., Kelly, E.J., Kaisers, W., Yarov-Yarovoy, V., Kramm, C.M., Rettie, A.E., and Hanenberg, H. (2015). Identification of amino acid determinants in CYP4B1 for optimal catalytic processing of 4-ipomeanol. Biochem. J. *465*, 103–114. https://doi.org/10.1042/BJ20140813.

63. Grunewald, C.M., Haist, C., König, C., Petzsch, P., Bister, A., Nößner, E., Wiek, C., Scheckenbach, K., Köhrer, K., Niegisch, G., et al. (2021). Epigenetic priming of bladder cancer cells with decitabine increases cytotoxicity of human EGFR and CD44v6 CAR engineered T-cells. Front. Immunol. *12*, 782448. https://doi.org/10.3389/fimmu.2021.782448.

64. Gu, D., Liu, H., Su, G.H., Zhang, X., Chin-Sinex, H., Hanenberg, H., Mendonca, M.S., Shannon, H.E., Chiorean, E.G., and Xie, J. (2013). Combining hedgehog signaling inhibition with focal irradiation on reduction of pancreatic cancer metastasis. Mol. Cancer Ther. *12*, 1038–1048. https://doi.org/10.1158/1535-7163.MCT-12-1030.

3. DISKUSSION

Die erste Zulassung der CAR-T-Zelltherapie im Jahre 2017 kann als der Beginn einer vielversprechenden neuartigen Therapieoption für Patienten mit bösartigen Erkrankungen bezeichnet werden [100]. Bislang ist der Therapieerfolg auf B-Zellneoplasien beschränkt, da die Ansprechraten der CAR-T-Zelltherapie gegen solide Tumore vergleichsweise gering sind und häufig zu schwerwiegenden Nebenwirkungen im Patienten führen [39]. Die unterschiedlichen physikalischen und pathophysiologischen Eigenschaften hämatologischer und solider Tumore implizieren, dass die CAR-T-Zelltherapie an die Besonderheiten solider Tumore angepasst werden muss, denn im Gegensatz zu hämatologischen Tumoren sind die malignen Zellen Teil eines TMEs, in der eine Vielzahl von Faktoren, darunter hemmende Rezeptoren/Zellen/Zytokine, physikalische Barrieren, Hypoxie und der Tumorstoffwechsel, Immunreaktionen unterdrückt und das Tumorwachstum fördert. Um diese Hindernisse zu überwinden, muss nicht nur der CAR weiterentwickelt, sondern auch Kombinationstherapien untersucht werden, die die Zytotoxizität der CARs steigert, die Tumorzellen und das TME beeinflusst und somit das volle Potential der zellulären Immuntherapie entfalten.

Aus diesem Grund habe ich während meiner Arbeit Hindernisse und Probleme, auf welche die CAR-T-Zelltherapie für solide Tumore stößt, aufgegriffen und in folgenden Schwerpunkten bearbeitet:

- Auswahl von hoch-affinen scFvs und Validierung von EGFR und CD44v6 als Zielantigene für die CAR-T-Zelltherapie gegen HNSCC
- Optimierung des CAR-Designs für eine vereinfachtere Nutzung und verbesserte Produktion der CAR-T-Zellen
- Epigenetische Stimulation der Tumorzellen für sensibleres Ansprechen auf die CAR-T-Zelltherapie und Entgegenwirken des TME

EGFR UND CD44V6 ALS CAR-T-ZELLANTIGENE FÜR HNSCC

Die Nutzung eines geeigneten Zielantigens zur Eliminierung von Tumorzellen stellt einen wichtigen Faktor in der CAR-T-Zelltherapie dar und bestimmt beträchtlich das Potential des Behandlungserfolgs. Aus diesem Grund wird die Identifikation neuer Zielantigene, einzelnen oder in pathologischen Kombinationen auf soliden Tumoren exprimiert, immer häufiger in klinischen und präklinischen Studien thematisiert [101–103].

Prinzipiell können die aktuell genutzten therapeutischen Zielantigene in zwei Gruppen eingeteilt werden, die TSAs und die TAAs. Die TSAs weisen, wie ihr Name bereits andeutet, eine hohe Spezifität für Zellen des Tumors auf, da diese ausschließlich von Tumorzellen

und nicht von gesunden Geweben exprimiert werden. Meist wird diese Spezifität mithilfe von tumorspezifischen somatischen Mutationen, wie z.B. bei β-Catenin, oder durch onkovirale Antigene, wie HPV 16 und 18, hervorgerufen [102]. Ein interessanter Kandidat für HNSCC stellt auch das tumorspezifische EGFRvIII dar. Diese EGFR Variante, gekennzeichnet durch eine genomische Deletion der Aminosäuren 6-273 [104], wurde durch ihre erfolgreichen präklinischen und humanen Studien zur Behandlung von Glioblastomen bekannt [105–107] und wird ebenfalls auf ungefähr 40 % aller HNSCCs exprimiert [108].

Im Gegensatz dazu sind TAAs sowohl auf Tumor- als auch auf normalem Gewebe vorhanden. Insgesamt lassen sich drei Gruppen von TAAs unterscheiden: **Differenzierungsantigene**, wie gp100 oder MART-1, sind Zelloberflächenproteine, welche in Abhängigkeit der Gewebedifferenzierung oder Zellaktivierung herauf bzw. herunter reguliert werden [101]. Dadurch kann eine genaue Abgrenzung zwischen Zielantigen exprimierendem Tumor und dem umliegenden Gewebe erfolgen. Oft werden auch **Tumor-Hoden-Antigene (*cancer testis* antigens)** als Zielantigene genutzt [109], die ebenfalls in HNSCCs exprimiert werden (wie z.B.: Mitglieder der MAGE Familie oder NY-ESO-1) [102,110]. Zu der Gruppe mit den am häufigsten für die CAR-T-Zelltherapie genutzten Zielkandidaten gehören die **überexprimierten Antigene**, welche sich in ihrer Expressionsstärke auf den gesunden und malignen Geweben deutlich voneinander unterscheiden. Während die Überexpression des Zielantigens auf malignen Zellen zu einer Immunantwort führen kann, wird diese durch die niedrige Expression auf Normalgewebe meist nicht ausgelöst.

In dieser Arbeit wurden **zwei Vertreter der überexprimierten Antigene** für die Eliminierung von HNSCCs mittels CAR-T-Zellen untersucht. Das erste Antigen stellt das in Kapitel 2.3 bereits beschriebene EGFR dar, welches ausgewählt wurde, weil eine sehr starke Expression der ErbB-Tyrosin-Kinase-Mitglieder (wie z.B. EGFR, Her2neu) in Verbindung mit einer schlechten Krankheitsprognose bei HNSCC steht und der Einsatz von blockierenden Antikörpern zur Hemmung der Wachstumsfaktoren bereits klinische Erfolge erbrachte [102]. Der FDA und EMA zugelassene chimäre Antikörper Cetuximab (Erbitux®) gegen EGFR lässt sich nicht nur zur Behandlung von HNSCCs alleine nutzen [111], sondern wurde auch in dieser Arbeit - in modifizierter Form als antigenbindende Domäne in einem CAR - *in vitro* genutzt, um maligne Zellen unterschiedlicher Entitäten abzutöten.

Das zweite Antigen, eine onkogene Spleißvariante von CD44, wird in Kapitel 2.1 genauer beschrieben. CD44v6 ist für ein homogenes Expressionsmuster in HNSCCs bekannt und wird bei Überexpression auf den Tumorzellen zusätzlich mit Radio- und Chemoresistenz in Verbindung gebracht [112]. Deshalb könnte die CAR-T-Zelltherapie gegen CD44v6 bei fehlender Wirksamkeit der standardmäßigen Behandlungen genutzt werden.

Die scFvs beider CARs basieren auf hoch-affinen Antikörpern (Cetuximab und BIWA8), welche nach der klinischen Verabreichung aufgrund von ihrer Expression auf Normalgewebe (CD44v6: epidermale Keratinozyten, Zervix, Cornea, Tonsillen [113]; EGFR: epidermale Keratinozyten, Nephros, Gastrointestinaltrakt [114]), ungewünschte Toxizität im Patienten zeigten. Während Cetuximab hauptsächlich für reversible kutane Nebenwirkungen bekannt ist [115], führte die Nutzung von radioimmuntherapeutischen CD44v6-Antikörpern zu oralen Mukositis und Myelotoxizität, bei einem Patienten mit letalem Ausgang [116].

Spezifität

Ein *on-target off-tumor* Effekt wird in vielen Studien der Gabe hoch-affiner Antikörper bzw. CAR-T-Zellen zugeschrieben und kann durch die Verringerung der Affinität umgangen werden [117]. Gering-affine EGFR- oder ErbB2-CARs konnten im Vergleich zu ihrem hoch-affinen Gegenpart besser zwischen schwach exprimierten TAAs auf Normalgewebe und überexprimierten TAAs auf Tumorgewebe differenzieren, was letztlich die Spezifität für den Tumor deutlich erhöhte [43,114]. Allerdings zeigten niedrig-affine CARs im Umkehrschluss oft eine schlechtere Anti-Tumor-Wirkung [42,118]. Ebenfalls konnten gering-affine GD2-CARs sich schnell teilende Neuroblastoma-Zellen nicht vollständig lysieren [119]. Da die CAR-T-Zelltherapie für HNSCCs zukünftig vermutlich nach oder in Kombination mit den standardmäßigen Behandlungsmethoden durchgeführt wird, sollen mit ihrer Hilfe alle verbleibenden malignen Zellen eliminiert werden. Die damit verbundene vollständige Lyse des Tumors setzt somit eine starke Anti-Tumor-Wirkung voraus, weshalb in dieser Arbeit gezielt hoch-affine CARs etabliert wurden.

Um sicher zu gehen, dass das potentielle Auftreten von *on-target off-tumor* Effekten durch die hoch-affinen scFv vermieden werden kann, wurde in unsere CAR-Vektoren ein zweites Transgen (blau-fluoreszierendes Protein (BFP)) als Platzhalter für die Integration von Sicherheitsmechanismen, wie z.B. eines Suizidgens oder eine zweite scFv zur Expression eines Split-CARs eingebaut.

Bei Split-CARs werden die beiden kostimulatorischen Domänen eines zweite Generation CARs voneinander getrennt und mit unterschiedlichen scFvs gegen zwei Zielantigene ausgestattet, so dass nur die zeitgleiche und gleichmäßige Bindung beider CARs zu einer vollständigen T-Zellaktivierung führt [120]. Die vielversprechenden Ergebnisse erster präklinischen Studien, wie z.B. ein ErbB2-MUC1-Split-CAR gegen ErbB2 und MUC1 positive Brustkrebs Zelllinien BT20, MCF7 oder ZR-75, zeigten eine effiziente Lyse der Zielantigen positiven Tumorzellen und die ErbB2- und MUC1-abhängige Proliferation der CAR-T-Zellen [121].

Eine Kombination, der in dieser Arbeit etablierten hoch-affinen CARs gegen EGFR und CD44v6 in Form eines Split-CARs würde die *on-target off-tumor* Toxizität nicht reduzieren, da beide Antigene auf epidermalen Keratinozyten exprimiert werden. Jedoch wären beide Zielantigene zusammen mit Antigenen, die nicht auf normalen Hautzellen exprimiert vorliegen, denkbar.

Falls dennoch Normalgewebe von den CAR-T-Zellen angegriffen wird, kann die Integration eines Suizidgens das Management von unerwarteten Begleiterscheinungen erleichtern und gleichzeitig die *in vivo* Depletion der modifizierten Zellen nach Abschluss der Therapie ermöglichen [122]. Prinzipiell kodiert ein Suizidgen für ein Protein, das ein Prodrug in einen zytotoxischen Metaboliten umwandeln kann. Wichtig ist es auch, dass die Co-Expression des Suizidgens mit dem CAR für seine Effektorzelle keine negative Auswirkung auf die Proliferation oder Zellviabilität hat, bis das passende Prodrug verabreicht wird. Denn erst die Aktivierung des Prodrugs löst den intrinsischen Apoptose-Signalweg der Effektorzelle aus, wodurch diese schließlich eliminiert wird [123]. Das erste und am besten charakterisierte System, das *in vivo* genutzt wurde, ist die Herpes-Simplex-Virus-Thymidin-Kinase (HSV-tk) in Kombination mit ihrem Prodrug Ganciclovir [124]. Obwohl die HSV-tk in vielen klinischen Studien effektiv und sicher als Suizidgen eingesetzt wurde, wirkt sie stark immunogen, so dass die transduzierten T-Zellen vom Immunsystem des Empfängers eliminiert wurden [125,126]. Aus diesem Grund wird in den letzten Jahren zunehmend das induzierbare Caspase 9 (iCasp9) System genutzt [125,126]. Der iCasp9-vermittelte Suizid basiert auf der Dimerisierung pro-apoptotisch wirkender Signaleinheiten aus dem humanen Caspase 9 Protein, weshalb dieses System weniger immunogen als die virale HSV-tk ist. In ersten klinischen Studien konnte das iCASP9 System auch schon erfolgreich eingesetzt werden [125,126]. So erhielten Patienten mit rezidivierender Leukämie allodepletierte Spender-T-Zellen, die nach Transduktion iCasp9 exprimierten. Nach Auftreten der Spender-gegen-Wirt-Krankheit (GVHD) auf der Haut oder Leber, wurde die Zahl der zirkulierenden transgenen T-Zellen innerhalb von 30 Minuten nach der Gabe des Prodrugs um 90 % reduziert, ohne dass es zu schweren systemischen Nebenwirkungen kam [127]. Weitere klinischen Studien, in denen iCasp9 kombiniert mit CAR-T-Zellen gegen CD19, GD2 und Mesothelin eingesetzt werden sollen, rekrutieren zur Zeit Patienten [125,126].

Eine weitere Möglichkeit zur Erhöhung der Sicherheit der Applikation von CAR-T-Zellen stellt die kontrollierte CAR-Expression auf der Effektorzelle dar. Dazu wird die Genexpression unter Kontrolle eines induzierbaren Promoters gestellt, meist das Tet-ON-System, um die Tumorlyse mittels reversibler Expression des CARs durch die Gabe von Doxycyclin zu regulieren [128,129]. Obwohl die Expression z.B. eines CD19-CARs unter Tet-induzierbarem Promoter sehr effizient sein kann, konnte auch ohne die Zugabe von

Doxycyclin eine sehr schwache Expression des CARs an der Oberfläche der T-Zellen beobachtet werden, die jedoch nicht zur Ausschüttung von Zytokinen nach Co-Kultur mit CD19-positiven Zelllinien (Raji, CD19-K562, SU-DHL6) führte [128]. Im Mausmodell konnten diese Resultate bestätigt werden: bei einer Behandlung mit Tet-CD19CAR-T-Zellen und Doxycyclin kam es zu einer statistisch signifikanten Rückbildung des Tumors, wohingegen ohne die Zugabe von Doxycyclin das Tumorwachstum nicht unterdrückt werden konnte [128]. Weitere präklinische Studien mit induzierbaren CAR-Konstrukten zeigten, dass mithilfe dieser Regulation ebenfalls das gefürchtete Zytokin-Freisetzungssyndrom (CRS, *Engl.: cytokine release syndrom*), bei welchem durch die maximale Aktivierung des T-Zell-Systems und den schnellen Zerfall von Krebszellen massiv pro-inflammatorische Zytokine freigesetzt werden und ein Herz- und Kreislaufversagen auftreten kann [130], besser kontrolliert werden kann.

Der *on-target off-tumor* Effekt und die damit verbundenen Nebenwirkungen wurde bei der Behandlung von soliden Tumoren bislang hauptsächlich nach der intravenösen Injektion der CAR-T-Zellen beobachtet. Da die Effektorzellen bis zum Erreichen des Tumorgewebes einen langen Weg zurücklegen müssen, erhöht sich die Wahrscheinlichkeit für einen *on-taget off-tumor*-Effekt. Erste Ansätze verfolgen daher die Idee, die CAR-T-Zellen direkt in den Tumor zu injizieren und somit die Effektorzellen dort zu lokalisieren. Obwohl die Ergebnisse einer Phase-I-Studie bislang noch nicht endgültig veröffentlicht wurden, verblieben die Effektorzellen nach intratumoraler Injektion von T4 PanErbB-CAR-T-Zellen zur Behandlung von HNSCCs größtenteils im Tumorgewebe, so dass ein *on-target off-tumor*-Effekt ausblieb [131]. Auch die direkte Injektion von mRNA c-MET-CAR-T-Zellen in Mammakarzinome wurde von den Patienten gut toleriert - ganz ohne das Auftreten eines CRS [132]. In beiden Studien wurden vereinzelt transduzierte Immuneffektorzellen im Blutkreislauf gefunden, welche aber keine Nebenwirkungen oder *on-target off-tumor*-Effekte auslösten.

Bei HNSCCs als gut zugängliche lokalisierte Tumoren führte die Kombination einer CAR-T-Zelltherapie mit der direkten Injektion von IL-12 in den Tumor zu einer lokalen Stimulation und erhöhten Resistenz der T-Zellen gegenüber des TMEs, was sich wiederrum positiv auf das *homing* der anderen Effektorzellen auswirkt [133]. Mithilfe der direkten Verabreichung von ILs in den Tumor kann die verabreichte Dosis leicht kontrolliert werden, so dass eine systemische Toxizität mit potentiellem letalen Ausgang, weitgehend ausgeschlossen werden kann [134]. Vierte Generation CARs, die zusätzlich zur CAR-Expression auch noch eine Aktivierungs-abhängige Expression von z.B. ILs oder Chemokinen ermöglichen, um die geringe Proliferation und kurzfristige Persistenz der Effektorzellen vor Ort zu verbessern, werden mittlerweile in klinischen Studien eingesetzt [135]. Diese Konstrukte bieten eine gute Möglichkeit zur lokalen Zytokin- und

Chemokin-Freisetzung, insbesondere wenn der Tumor für eine direkte Injektion nicht optimal lokalisiert ist. Ein Nachteil der Nutzung vierter Generation CAR-T-Zellen ist, dass nur ein Zyto-/Chemokine ausgeschüttet werden kann. Es ist bekannt, dass verschiedene Zytokine unterschiedliche Funktionen im Zusammenhang mit der Proliferation, Differenzierung und Persistenz von T-Zellen aufweisen, so dass bei der direkten Injektion in den Tumor ein Zytokin-Cocktail zur optimalen Stimulation der Effektorzellen verabreicht werden könnte.

Ein weiterer, möglicherweise bei HNSCCs gut durchzuführender Ansatz, um das *homing* der T-Zellen zu verbessern, wurde von Sanz-Ortega *et al.* beschrieben [136]. Hier wurden T-Zellen mit magnetischen Nanopartikeln gefüllt und mithilfe eines magnetischen Feldes vermehrt im Tumor lokalisiert.

Antigendichte

Ein weiterer entscheidender Faktor für die Auswahl des geeigneten Zielantigens stellt die Antigendichte auf der einzelnen Tumorzelle dar und wie gut die Tumorzellen insgesamt aufgrund der Expression dieses Zielantigens von den CARs erkannt werden. Im besten Falle resultiert die hohe Expression eins TAA/TSA auf der Tumorzelle in einer effizienten Erkennung und somit auch Bindung des CARs an die Tumorzelle, so dass diese effektiv lysiert wird.

Um die Antigendichte von EGFR und CD44v6 in dieser Arbeit zu untersuchen, wurden die diese auf 32 primären HNSCC-Ziellinien mithilfe von direkt Fluorochrom-konjugierten mABs angefärbt und die EGFR- bzw. CD44v6-Expression mittels Durchflusszytometrie semiquantitativ gemessen. Dabei konnten wir feststellen, dass beide Zielantigene zwar unterschiedlichen stark innerhalb der untersuchten Zelllinien, jedoch innerhalb einer Linie relativ homogen exprimiert werden (2.1, 2.3).

EGFR wurde von 78 % der 32 Zelllinien exprimiert [137]: dieser Wert ist vergleichbar mit den 80-90 % EGFR-exprimierenden HNSCCs, die in der Literatur angegeben werden [138,139]. Im Vergleich zum Normalgewebe zeigen jedoch nur 10-30 % der Fälle eine tumorspezifische Überexpression von EGFR [138,140,141]. Damit nicht nur der geringe Anteil der EGFR-überexprimierenden Tumore, sondern ebenfalls die EGFR schwach positiven malignen Zellen effizient eliminiert werden können, ist die Nutzung eines hoch-affinen CARs sehr wichtig. In dieser Arbeit konnte in Kapitel 2.3 gezeigt werden, dass die EGFR-CAR-T-Zellen, deren Antigenbindungsdomäne von dem hoch-affinen Cetuximab abstammt, sogar Zielzellen lysieren, deren Zielantigendichte unter der mit Durchflusszytometrie ermittelbaren Dichte liegt.

Die Expression von CD44v6 in HNSCCs wird in der Literatur vergleichsweise kontrovers diskutiert. Während einige Studien eine abnehmende CD44v6-Expression im Laufe der Tumorentwicklung beobachteten [142,143], konnten wiederrum Andere eine Überexpression des Antigens mit der Entstehung von HNSCCs in Verbindung bringen [112,144]. In unserem Labor exprimierte der Großteil der 32 untersuchten primären HNSCC-Zelllinien CD44v6 und konnte somit für die Etablierung des CD44v6-CARs genutzt werden. Der CD44v6-CAR ist im Gegensatz zum EGFR-CAR, welcher Zelllinien auch mit sehr schwachen Antigenexpression sehr effektiv und hoch, ohne Verlust seiner Spezifität, lysiert (2.3), eher durch eine Antigendichte-abhängige Lyse der Tumorzellen gekennzeichnet (2.1).

Abdeckung und Stabilität des Zielantigens auf dem Tumor

Auch wenn - im optimalen Fall - Tumorzellen das Zielantigen stark überexprimieren, ist die Antigenexpression innerhalb eines Tumors oft sehr heterogen, so dass die Möglichkeit besteht, dass die CAR-T-Zellen die malignen Zellen mit niedriger Expression nicht lysieren [145,146]. Aus diesem Grund sollten Zielantigene ausgewählt werden, die auf möglichst allen Zellen des Tumors in hoher Dichte exprimiert werden. Gerade bei der Untersuchung von HNSCCs konnte die stark ausgeprägte Heterogenität innerhalb eines Tumors - insbesondere für die EGFR- , aber auch für die CD44v6-Expression [147] - immer wieder bestätigt werden [148]. Aus diesem Grund kann der Einsatz von hoch-affinen CAR-T-Zellen, welche aufgrund ihrer höheren Affinität gleichzeitig stärker stimuliert werden, für das Lysieren aller Tumorzellen, auch jener mit schwach exprimierten Antigenen, von Vorteil sein. Allerdings stellt sich hier die Frage, ob zukünftig der Einsatz von hoch-affinen CAR-T-Zellen, der wahrscheinlich für eine effektivere Kontrolle des gesamten heterogenen Tumors (absolut) notwendig ist, unter strenger Kontrolle der *on-target off-tumor*-Effekte, durchgeführt werden muss.

Immer mehr Studien versuchen, der Heterogenität eines Tumors durch die Optimierung der CARs zu begegnen (Abbildung 3). Der SUPRA-CAR (***s**plit, **u**niversal and **pr**ogra**a**mmable*) stellt eine Designvariante dar und wurde so angepasst, dass universale CARs an Adapter binden, die als Liganden für die Erkennung von unterschiedlichen Antigenen genutzt werden können [149] und durch diese Bindung dann eine Aktivierung der T-Zelle hervorgerufen wird [150]. Dadurch können von demselben CAR mehrere scFvs erkannt und das Problem der Tumorheterogenität umgangen werden [151]. Ein weiterer Vorteil stellt die geringe biologische Halbwertszeit der Adapter im Serum von wenigen Stunden bis Tagen dar, so dass die Adapter zwar in regelmäßigen Abständen verabreicht werden müssen, um die Lyse der Tumorzellen zu gewährleisten, gleichzeitig aber die

Applikation jederzeit schnell unterbrochen werden kann, was einen zusätzlichen Sicherheitsaspekt bietet [152].

In letzter Zeit verfolgen präklinische und sogar klinische Studien für eine CAR-T-Zelltherapie von soliden Tumoren jedoch vermehrt die Strategie, mehrere CARs gegen unterschiedliche Antigene oder auch mehrere scFvs kombiniert in einem CAR-Konstrukt als therapeutischen Ansatz zu nutzen [153]. Aufgrund der Beliebtheit von EGFR als Zielantigen wurden beide Strategien bereits mit EGFR-CARs durchgeführt. Die zeitgleiche palliative Gabe von autologen EGFR- und CD133-CAR-T-Zellen für eine 52-jährigen Patientin mit fortgeschrittenem, inoperablem und metastasiertem Gallengangkarzinom zeigte eine verbesserte Anti-Tumor-Wirksamkeit, jedoch auch einer Vielzahl von zum Teil ausgeprägten Nebenwirkungen [154]. Bei derartigen Kombinationen von CAR-T-Zellen gegen nicht-tumorspezifische Antigene muss deshalb über eine Integration von Sicherheitsmechanismen in die CAR-T-Zellen nachgedacht werden. In einer präklinischen Studie mit direkter Injektion von CAR-NK92-Zellen in die orthotopen Tumorareale bei Glioblastom in Mäusen konnten doppel-spezifische CAR-NK-Zellen gegen EGFR und EGFRvIII ebenfalls bessere Erfolge verzeichnen, als die jeweiligen CAR-NK92-Zellen mit nur einer scFv*/targeting unit* [155].

Meines Wissens wurden solche Studien noch nicht mit CD44v6 als zusätzliches Zielantigen durchgeführt. Prinzipiell wäre jedoch die Anwendung in Kombination mit einem EGFR-CAR denkbar, um die Heterogenität der HNSCCs zu überwinden. Allerdings ist hier zu beachten, dass beide Zielantigene relativ stark auf Keratinozyten in der Haut exprimierten werden und hier eine besondere Toxizität bei Akkumulation von T-Zellen in der Haut z.B. nach intravenöser Infusion zu befürchten ist.

Die Nutzung von Tandem-CAR-T-Zellen (Abbildung 3) mit zwei hoch-affinen scFvs mit zusätzlicher Integration von Sicherheitsmechanismen würden dem *Tumor-Antigen-Escape*, der in vielen klinischen Studien nach Behandlung mit CAR-T-Zellen vorgekommen ist [156], wesentlich entgegen wirken. Beim *Tumor-Antigen-Escape* wachsen nach effektiver Behandlung des Tumors einige Zeit später Tumorzellen mit modulierter Zielantigenexpression (Verlust oder Verringerung des Antigens, so dass CAR-T-Zellen dieses nicht mehr erkennen) aus und führen klinisch zum Relapse/Persistenz des Tumors. Mechanistische Untersuchungen in präklinischen und klinischen Studien zeigten, dass Tumorzellen ihr Antigenprofil an die CAR-Therapie adaptieren, indem Zielantigene mutiert oder herunterreguliert werden, so dass die Erkennung der malignen Zellen durch die CAR-T-Zellen nicht mehr möglich ist [157]. Dieses Phänomen konnte auch in HNSCC-Patienten nach der Gabe von Cetuximab beobachtet werden und würde *in vivo* vermutlich auch bei alleiniger Gabe von T-Zellen mit dem EGFR-CAR aus dieser Arbeit

erfolgen, weswegen generell die Nutzung von hoch-affinen CARs und sinnvoller Weise eine Kombination mit dem hier etablierten CD44v6-CAR eine effektivere Lyse aller Zellen des heterogenen Tumors erzielen würde. Vor Allem aus Gründen des *Tumor-Antigen-Escape* spielt die Stabilität des Zielantigens auf der Tumorzelle eine entscheidende Rolle für die langfristige Heilung des Patienten [157]. Stabil exprimierte Antigene, die nicht leicht durch externe Einflüsse reguliert werden können, sollten deshalb als Zielstrukturen für eine CAR-Therapie favorisiert werden.

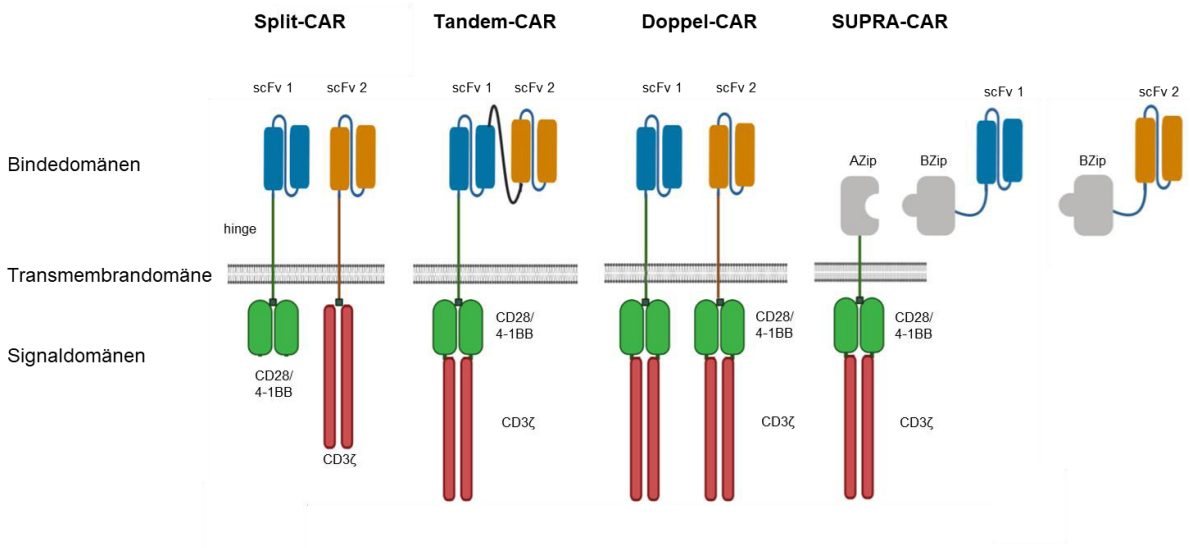

Abbildung 3: Optimierung/Anpassungen des CAR-Designs zur Überwindung der Tumorheterogenität und der *on-target off-tumor* Toxizität. Die Erkennung von mehr als einem Antigen kann die Spezifität der CAR-T-Zellen für die Tumorzellen erhöhen und so die Therapie an die oftmals heterogene Antigenexpression des Tumors anpassen und eine vermehrte Lyse des Tumorgewebes ermöglichen. Split-CAR-T-Zellen, bei denen die Aktivierung der Immuneffektorzellen durch CD3ζ und die Co-Stimulation (z.B. CD28 oder 4-1BB) über zwei CARs der ersten Generation erreicht wird, können gegen unterschiedliche Tumorantigene gerichtet werden. Die T-Zellen mit den beiden Split-CAR-Konstrukten werden erst dann zytotoxisch, sobald eine Bindung beider CARs in der gleichen T-Zelle erfolgt. Bei Tandem-CARs sind scFvs gegen zwei Antigene in einem CAR-Konstrukt über Peptidlinker gekoppelt, während bei Doppel-CARs die aktivierenden und stimulierenden Funktionen in zwei Konstrukten getrennt sind, die jeweils auf ein unabhängiges Antigen abzielen. Das SUPRA-CAR-System kann direkt mehrere Tumorantigene unter Verwendung verschiedener scFvs binden. Die Abbildung wurde modifiziert nach [158] und mit Biorender.com erstellt.

Universale Expression des Zielantigens

Da die kostspielige und langwierige Zulassung eines CAR-Konstrukts in einem Gentransfervektor ein wesentlicher limitierender Faktor für die klinische Anwendung darstellt, sollten die CARs möglichst universal einsetzbar sein, indem das Zielantigen auf Tumoren verschiedener Entitäten exprimiert wird. Wie bereits erwähnt, stellt EGFR ein beliebtes TAA dar, weshalb es als Zielantigen für CAR-T-Zellen bislang in mehreren Phase I oder Phase I/II-Studien gegen EGFR-positive solide Tumore wie Glioblastome, Lungen-, Pankreas- oder Darmkrebs-Karzinome (NCT01869166, NCT02331693, NCT03182816,

NCT03152435, NCT03182816, NCT01869166) genutzt wurde [159–161]. Aktuell werden Patienten für weitere Studien mit unter Anderem EGFR-positiven Lungenkarzinomen oder ZNS-Tumoren rekrutiert (clinicaltrails.gov).

CD44v6-CAR-T-Zellen wurden zwar bislang in noch nicht beendeten klinischen Studien eingesetzt, von denen bisher keine Daten publiziert wurden, das Zielantigen wird jedoch in hämatologischen (AML und MM) und auch soliden Tumoren überexprimiert. Besonders häufig wird die starke Expression auf Brust-, Dickdarm-, Magen-Darm-, Blasen-, Eierstock- und Lungentumoren beschrieben [162]. Die Ergebnisse der laufenden Studien mit Patienten, die an AML, MM und Brustkrebs erkrankt sind, werden die Sicherheit, Wirksamkeit und Durchführbarkeit der CD44v6-CAR-T-Zellimmuntherapie zeigen (NCT04097301, NCT04427449, NCT04430595).

Diese Arbeit bestätigte bereits die universale Einsetzbarkeit der EGFR-CAR-T-Zellen bei Plattenepithelkarzinomen der oberen Luftwege (HNSCCs) sowie der Speiseröhre und auch bei Tumoren des Ovars (2.3) und bei Urothelkarzinomen (2.2).

Insgesamt stellen also beide TAAs, EGFR und CD44v6, geeignete Kandidaten für die CAR-T-Zelltherapie dar und sind in kombinierter Form als hoch-affine CAR-T-Zellen mit Integration entsprechender Sicherheitsmechanismen für die Eliminierung verschiedener Tumorentitäten potentiell geeignet. Dennoch erfordert die CAR-T-Zelltherapie einen personalisierten Ansatz, der die Charakteristika des Tumors insbesondere bei der Wahl des passenden Zielantigens und bei dem genutzten CAR-Design berücksichtigt.

Bei der Wahl eines Zielantigens für die CAR-T-Zelltherapie sollten folgende Faktoren berücksichtigt werden:

- Hohe Tumorspezifität
- Hohe Antigendichte
- Homogene Abdeckung des Zielantigens auf den Tumorzellen
- Stabile Expression des Antigens
- Expression des Zielantigens auf verschiedenen Tumorentitäten

Folgende Ansätze können die *on-target off-tumor* Effekte reduzieren und die Sicherheit bei der Nutzung hoch-affiner CARs erhöhen:

- Verwendung von TSAs
- Injektion der CAR-T-Zellen direkt in den Tumor
- Integration eines Suizidgens
- Nutzung eines induzierbaren Promoters
- Split-CARs

Optimierung der Therapie durch Modifikationen der extrazellulären CAR-Domänen

Parallel zur Auswahl eines passenden Zielantigens kann die CAR-T-Zelltherapie durch Optimierung einzelner Faktoren des Vektors, unter anderem für den CAR-T-Zell-Herstellungsprozess unter GMP oder zur Überwachung der CAR-T-Zellen im Patienten, verbessert werden.

Typischerweise werden zu Therapiebeginn primäre T-Zellen des Patienten isoliert und mittels Vektoren und Zytokinen *ex vivo* modifiziert, um schließlich einsatzfähige autologe CAR-T-Zellen zu erhalten [163]. Die CAR-T-Zellen werden anschließend in die mit Chemotherapie (Konditionierung) vorbehandelten Patienten reinfundiert, worauf eine engmaschige stationäre Überwachung erfolgt [164]. Diese Abläufe bedürfen einer strikten zeitlichen sowie logistischen Planung, um die finanziellen Aufwendungen bzw. Verluste möglichst gering zu halten. Aktuell kostet die Verabreichung einer Dosis CD19-CAR-T-Zellen von Novartis 475.000 US$ exklusive zusätzlichen 150.000 – 200.000 US$ für Patientenbetreuung und Behandlung möglicher Komplikationen [165]. Zur Verbesserung der Effizienz und Kosteneffektivität versuchen Wissenschaftler in den großen Firmen und großen Kliniken und Instituten, die Großserienproduktion der CAR-T-Zellen an verschiedenen kritischen Produktionszwischenschritten zu optimieren. So wird unter anderem die genetische Modifikation der T-Zellen mit unterschiedlichen Vektoren (meist retrovirale und lentivirale Vektoren, aber auch nichtvirale Transposons), das T-Zellwachstum durch Zugabe unterschiedlicher Zytokine ins Expansionsmedium oder die Qualitätskontroll-Freigabetests weiterentwickelt und die Merkmale/Besonderheiten der Patienten, die auf die Therapie besonders gut bzw. gar nicht ansprechen, besser erfasst [166]. Auch in dieser Arbeit konnten Ansätze zur effizienteren Transduktion durch Modifikation der CAR-scFv und der Anreicherung von CAR-T-Zellen über neue *hinge*-Regionen etabliert werden.

Die in Kapitel 2.3 beschriebene rationale Optimierung der scFv-Antikörperdomäne von Cetuximab für die Anwendung als EGFR-CAR führte zu deutlich höheren Virustitern - im Vergleich zu dem CAR-Vektor mit der originalen Cetuximab-scFv - und resultierte in einer verbesserten Transduktionseffizienz der T-Zellen. Obwohl bekannt ist, dass die Größe des Expressionsvektors bei lentiviralem Gentransfer aufgrund von lentiviralen Transkriptions- und Verpackungsbeschränkungen mit den Titern korreliert [167], wird die Reduktion der Sequenzlänge von 200 bp der Cetuximab scFv vermutlich nicht der alleinige Faktor sein, der zu solch einer erheblichen Verbesserung in der Virusproduktion geführt hat.

Canté-Barrett *et al.* stellten fest, dass die Transduktionseffizienz von der Anordnung der Basenpaare abhängig ist [167] und Fujiwara *et al*. beschrieben, dass eine niedrige CAR-Expression auf den Effektorzellen häufig durch Aggregation des CARs mit der Membran durch posttranslationale Modifikationen verursacht werden kann [168]. Demnach scheint die Sequenzoptimierung bzw. Sequenzverkürzung der scFv ein Vorteil für die Großproduktion mit erheblichen finanziellen Vorteilen darzustellen, die zukünftig thematisiert werden sollte. Denn bislang werden die Virustiter meistens alleinig durch Änderung der Plasmidverhältnisse, unterschiedliche Zeitpunkte der Virusernte und optimierte Transfektionsreagenzien erhöht [169].

Obwohl die Nutzung viraler Vektoren mit klaren Nachteilen verbunden ist, wird für die CAR-T-Zellproduktion aktuell in den meisten zugelassenen Produkten und auch klinischen Studien γ-retroviraler oder lentiviraler Gentransfer verwendet (γ-retroviral: Yescarta/Tecartus, lentiviral: Kymriah) [170]. Die Sicherheitsbedenken z.B. aufgrund einer möglichen Insertionsmutagenese, die Kapazitätsengpässe und die bereits erwähnten sehr hohen Herstellungskosten einer GMP-konformen Vektorproduktion und die Transduktion und Expansion der autologen CAR-T-Zellen unter S2-Bedingungen steigern ganz wesentlich die Attraktivität von nicht-viralen Gentransfermethoden für die genetische Veränderung der T-Zellen [170]. Dabei werden entweder Nanopartikel mittels Endozytose oder Membranfusion von den Effektorzellen aufgenommen oder verschiedene Vektoren, wie mRNA oder DNA-basierte Transposons durch Membran-permeabilisierende Methoden (Elektroporation) in die Immuneffektorzellen eingeschleust [170]. Diese nicht-viralen Gentransfermethoden für die Produktion von CAR-T-Zellen und ihre Anwendungsmöglichen werden zurzeit vermehrt noch in präklinisichen Studien getestet. 2021 wurde jedoch schon die erste klinische Studie von SLAMF7-CAR-T-Zellen, hergestellt mit dem Transposonsystem *Sleeping Beauty*, für Patienten mit multiplen Myelomen beschrieben [171].

Neben der Erhöhung des Virustiters bringt die Integration der neuen *hinge*-Region in CAR-Konstrukte mehrere, für die Translation in die Klinik relevante Vorteile mit sich (2.4, 2.5) . Die initialen Studien nutzten für ihre CARs *hinge*-Regionen, die von humanen IgG1- oder der IgG4-CH_2CH_3-Domänen abgeleitet wurden und trotz ihrer klaren Vorteile (Flexibilität, fehlende Immunogenität und leicht anpassbare Änderung der Länge, einfache Detektion) letztendlich zu einer unspezifischen Aktivierung der Effektorzellen durch Bindung an Fcγ-Rezeptoren von Monozyten und Makrophagen führten [45,172]. Diese *off-tumor off-target*-Aktivierung resultierte in einer ungewollten Immunantwort gegen Fcγ-Rezeptor exprimierenden Immunzellen im Gewebe und dem Absterben der Effektorzellen

durch aktivierungsinduzierten Zelltod, ohne dass aber Tumorzellen vernichtet wurden [172]. Obwohl durch Mutagenese oder Deletion der CH_2-Bindungsstellen *in vivo* die Funktionalität mehrerer CARs ohne das Auftreten von ungewollten Nebeneffekten bestätigt werden konnte [46], fokussierten andere Ansätze die Verwendung von Domänen ohne natürliche FcγR-Bindungsaktivität, wie CD28 oder CD8, die auch in den klinisch zugelassenen Produkten, wie den Yescarta- und Kymriah-CARs, genutzt werden [173].

Der Einsatz der neuen c6-*hinge*-Region in dieser Arbeit zeigte weder *in vitro* gegen solide Tumore, noch gegen hämatologische Erkrankungen im Mausmodell unspezifische Aktivierung der Immuneffektorzellen (2.4). Bei Nutzung dieser neuen *hinge* können die CAR-positiven T-Zellen zusätzlich mithilfe des MACS-Systems von Miltenyi angereichert und selektioniert werden. Dadurch kann in einem Schritt, der auch unter GMP-Bedingungen durchgeführt werden kann, eine reine Population CAR-positiver T-Zellen mit einer hoher Antigenexpressionsdichte erst gewaschen und schließlich im gewünschten Puffer eluiert werden. Somit könnten in klinischen Studien Unterschiede in der Reinheit sowie der Dosis der verabreichten Zellen vermieden und somit die Therapie besser standardisiert und die Vergleichbarkeit der Behandlungen erhöht werden.

Nicht nur in dieser Arbeit, sondern auch in weiteren Studien wurden *hinge*-Domänen ebenfalls dazu verwendet CAR-positive Untergruppen der T-Zellen zu quantifizieren und zu reinigen. Viele aktuelle Anreicherungsprotokolle in präklinischen Studien greifen auf die Expression eines sekundären Zelloberflächenmarkers (z.B. trunkiertes CD34, NGFR, EGFR, trunkiertes CD19) zurück, um eine Selektion und Detektion der CAR-T-Zellen zu ermöglichen [174]. Liu *et al.* inkludierten z.B. einen Strep-Tag II in die *hinge*-Region, um mit ihrer Hilfe die CAR-T-Zellen zu reinigen und detektieren [175].

Die Vorteile der neuen *hinge* erleichtern also direkt mehrere Produktionsschritte, ohne dass eine zusätzliche Integration eines Selektionsmarkers im Vektor erforderlich wird. Da kein zweites Transgen für die Selektion der CAR-T-Zellen notwendig ist, kann so relativ einfach der lentivirale Vektor verkleinert, die Vektorproduktion verbessert und die Transduktionseffizienz erhöht werden [167]. Zusätzlich wird - ohne zweites Transgen im Vektor - die Expression des CARs auf der Zelloberfläche erhöht, ohne dass ein besonders starker Promotor verwendet werden muss.

Im Laufe der CAR-Entwicklung wurde der *hinge*-Region immer mehr Aufmerksamkeit geschenkt und ihr Einfluss auf die Stabilität und das CAR-Expressionslevel beschrieben [176]. Auch die Expansion und Persistenz der CAR-T-Zellen kann durch die Optimierung der Hinge beeinflusst werden [177]. McComb *et al*. konnten durch die Wahl der passenden *hinge* die Anzahl der T-Zellen während der Kultivierung steigern und die Invasivität erhöhen [178]. Somit stellt die Integration unserer neuen *hinge* in das CAR-Konstrukt nicht nur das

Verbindungsstück zwischen scFv und Transmembrandomäne dar, sondern könnte ein funktionell wichtiger Teil des CARs werden, der die Anwendbarkeit der CAR-T-Zelltherapie in mehreren Bereichen vereinfacht.

Trotz all dieser Optimierungsschritte ist die CAR-Therapie unter Verwendung von Effektor-T-Lymphozyten aufgrund der hoher Produktionskosten und der lang dauernder Herstellungsprozesse von ungefähr drei Wochen für viele Patienten problematisch [179]. Ebenso leiden viele Tumorpatienten aufgrund der intensiven Vorbehandlung mit Chemotherapien an einer T-Zelldysfunktion, was bedeutet, dass die T-Zellen sich *in vitro* nicht mehr ausreichend manipulieren lassen und folglich die Therapie nicht durchführbar ist [179].

Die Produktion allogener Immuneffektorzellen würde hier nicht nur die hohen Kosten der autologen T-Zellherstellung verringern, da die vorproduzierten Zellen eines normalen Donors auch sofort für andere Patienten genutzt werden könnten. Allerdings sind die Komplikationen, die durch Gabe allogener CAR-T-Zellen entstehen würden, nämlich eine Transplantat-gegen-Wirt-Reaktion oder die perakute Eliminierung der Effektorzellen durch das Immunsystem des Spenders, noch nicht beherrschbar. Trotzdem werden zurzeit mehrere Strategien für allogene CAR-T-Zelltherapien in präklinischen Studien getestet. Nachdem die TCR-abhängige Erkennung von Antigenen der CAR-T-Zellen durch unterschiedliche Methoden (CRISPR/Cas9 [180], TALEN [181], Zink-Finger-Nukleasen [182]) mittels Knockout der TCR α- und/oder β-Ketten ausgeschaltet wurde, können allogene CAR-T-Zellen jetzt theoretisch bei verschiedenen Erkrankungen des Menschen systematisch in klinischen Phase I/II-Studien getestet werden [179].

Im Vergleich zur CAR-T-Zelltherapie bietet die Nutzung von allogenen NK-Zellen als genetisch modifizierte Immuneffektorzellen viele Vorteile. So weisen allogene CAR-NK-Zellen, deren Nutzung aufgrund der fehlender HLA-Restriktion bei der Erkennung ihrer Zielzellen kein Risiko einer Transplantat-gegen-Wirt-Krankheit mit sich bringen, jedoch funktionelle Ähnlichkeit zu $CD8^+$ T-Zellen und deren zytotoxischen Mechanismen auf [183], können *off-the-shelf* produziert [184] und nicht nur aus peripherem Blut durch Apherese, sondern ebenfalls aus Nabelschnurblut gewonnen werden [185], wodurch weitere Produktionsschwierigkeiten gelöst werden würden. Zusätzlich treten aufgrund der relativ kurzen Lebensdauer von NK-Zellen *in vivo* und aufgrund der fehlenden Selbststimulation durch autologe *feedback-loops* nur selten schwerwiegende *on-target off-tumor* Effekte auf; auch schwere toxische Nebenwirkungen, wie das sogenannte *Cytokine-release syndrome*, wurden im Vergleich zur Gabe von CAR-T-Zellen, die größere Mengen

proinflammatorischer Zytokine wie TNF-α, IL-1 und IL-6 freisetzen und über Wochen massiv im Patienten expandieren können, nicht verzeichnet [185]. Zumindest für die Therapie von hämatologischen Malignome z.B. gegen CD19, CD20 oder CD22 erscheinen deshalb allogene CAR-NK-Zellen zurzeit sehr attraktiv zu sein.

Modifikationen der scFv-Sequenz und Integration einer neuen *hinge*-Region können zu produktionstechnischen und klinischen Vorteilen führen:

- Effizientere Transduktion durch erhöhte Virustiter mittels Verkürzung der scFv
- Höhere Virustiter durch Verkleinerung des Vektors, da aufgrund der neuen *hinge* kein zweites Transgen als Selektionsmarker fungieren muss
- Anreicherung, Selektion und einfache Detektion CAR-positiver T-Zellen mithilfe der neuen *hinge*

KOMBINIERTE CAR-T-ZELLTHERAPIE

Um Patienten eine möglichst kosteneffiziente und wirksame CAR-T-Zell-Monotherapie zu ermöglichen, werden zurzeit viele Ansätze zur effizienten Modifikation von autologen oder auch allogenen Effektorzellen verfolgt. Trotzdem waren die Ansprechraten in klinischen Studien zur Behandlung von soliden Tumoren mit der alleinigen Gabe von CAR-T-Zellen nur sehr gering [186–189], so dass eine zusätzliche Manipulation der Tumorzellen im Patienten möglicherweise erst zur vollen Potentialentfaltung der CAR-T-Zelltherapie führen könnte [190].

Die Patienten, die mit CAR-T-Zellen behandelt werden, haben meist primär nicht ansprechende (*refractory*) bzw. rezidivierte Malignome. Dies bedeutet, dass sie bereits mit den Standardtherapien (wie z.B. Operation, Bestrahlung, Chemotherapie) behandelt wurden, aber die Tumoren nicht ausreichend angesprochen haben. Vor der Gabe von CAR-T-Zellen erhalten die Patienten immunsuppressive Chemotherapeutika, die als Vorbehandlung primär das Immunsystem des Patienten hemmen sollen, damit die infundierten CAR-T-Zellen besser anwachsen und so die Anti-Tumor-Immuntherapie verstärkt wird; außerdem wird durch die Chemotherapie die Proliferation und Zytokinproduktion der CAR-T-Zellen, die ja nicht diesen genotoxischen Substanzen exponiert waren, angeregt werden [191]. Zusätzlich werden Tumorzellen für den Angriff von Immunzellen sensibilisiert, indem das TME modifiziert, immunsuppressive Zellen eliminiert oder die T-Zellmigration gefördert wird [192]. Die genauen Mechanismen sind noch nicht in

voller Gänze bekannt, jedoch werden z.B. durch verschiedene Chemotherapeutika Mannose-6-phosphat-Rezeptoren auf Tumorzellen überexprimiert, wodurch von T-Zellen ausgeschüttetes Granzym-B einfacher in den Tumor eindringen kann [193]. Die Vorbehandlung mit Chemo- oder Radiotherapie kann ebenfalls - durch unterschiedliche Mechanismen - eine erhöhte TAA-Expression auf den Tumorzellen induzieren und regulatorische T-Zellen sowie myeloid-abgeleitete Suppressorzellen inhibieren [190]. Lokale Bestrahlung steigert ebenfalls die Infiltration von T-Zellen in den Tumor, indem es durch eine erhöhte Chemokinexpression und/oder IFN-γ Ausschüttung ein förderlicheres TME für die Immuneffektorzellen schafft [194,195].

Alternativ können andere immuntherapeutische Ansätze, die immuninhibitorische Rezeptoren auf Effektorzellen oder Immuncheckpoint-Liganden auf Tumorzellen inhibieren, mit der CAR-T-Zelltherapie verknüpft werden [29]. Immer mehr Studien kombinieren FDA-zugelassene Checkpoint-Inhibitoren gegen PD-1 (Nivolumab, Pembrolizumab, Pidilizumab), PD-L1 (MDX-1105, MPDL3280A) oder CTLA-4 (Ipilimumab, Tremelimumab) [196] mit der CAR-T-Zelltherapie oder lassen neuerdings die Checkpoint-Inhibitoren zusätzlich von den CAR-T-Zellen vor Ort sezernieren, um die Wirksamkeit und Persistenz der transduzierten und auch der nicht genetisch veränderten Immuneffektorzellen im Tumor zu erhöhen [190,197].

Auch monoklonale Antikörper und *small molecules* können zur Beeinflussung von Signalwegen, die wichtig für Tumorwachstum, Überleben, Angiogenese oder Metastasierung sind, eingesetzt werden [198]. Die Kombination von z.B. CAR-T-Zellen mit dem antiangiogenen Antikörper Bevacizumab (gegen VEGFR) zeigte in präklinischen Studien eine verbesserte Tumorinfiltration der Effektorzellen bei soliden Tumoren [199].

Ein weiterer, bisher noch wenig genutzter Ansatz zur Manipulation von Tumorzellen bei einer gleichzeitig durchgeführten Immuntherapie stellt die Beeinflussung von epigenetischen Regulationsmechanismen von HDACs oder DNMTs dar. Die Gabe von HDACi führt zu einer Re-Expression von stillgelegten Genen durch Reduktion der DNA-Verpackung, während Inhibitoren von DNMTs hingegen meist die Expression von Genen durch Demethylierung verringern. Beide Substanzen bewirken die Reaktivierung epigenetisch stillgelegter Gene [85], wie Zellzyklusregulatoren oder Suppressorgene, wodurch die Immunogenität der Tumorzellen beeinflusst wird.

Auch in dieser Arbeit wurde die Tumorzellbehandlung mit Inhibitoren der HDACs (Romidepsin) und DNMTs (Decitabin) im Hinblick auf Sensitivierung gegen CAR-T-Zellen

untersucht (2.2). Nach Vorbehandlung von Urothel-Karzinomzelllinien mit Romidepsin kam es verstärkt unter anderen zur Regulation von Genen, die den Zelltod verhindern, was zu einer deutlich schlechteren Toxizität der CAR-T-Zellen gegenüber den Tumorzellen führte. Umgekehrt sind die malignen Urothelzellen nach Gabe von Decitabin deutlich sensitiver gegenüber den zytotoxischen Effekten der CAR-T-Zellen und wurden vermehrt abgetötet; dies konnten wir auf die Induktion von BID und die Repression von BCL2L1 zurückführen.

Die BCL2-Familie ist Teil des mitochondrial vermittelten (intrinsischen) Apoptoseweges, bei dem durch den Einfluss von Stressstimuli zunächst die Caspase-8 das Protein BID aktiviert, welches auf der mitochondrialen Außenmembran Komplexe mit BCL-2 und BCL2L1 formt und dadurch eine Öffnung der mitochondrialen Außenmembran bewirkt [200]. Die darauf folgende Freisetzung von Cytochrom C und weiteren mitochondrialen Proteinen führt zur Bildung des Apoptosoms, welches durch Rekrutierung von ATP, dATP, Apaf-1 und Caspase-9 dann Caspase-3 aktiviert und schließlich die Fragmentierung der DNA induziert und damit den Zelltod einleitet [201].

Nicht nur in dieser Arbeit konnte die zentrale Rolle von BID als Regulator der Zytotoxizität von CAR-T-Zellen identifiziert werden. Auch Singh *et al*. demonstrierten, dass Nalm-6-Zellen mit einem durch CRISPR/Cas9 verursachten BID-Knockout eine erhöhte Resistenz gegenüber CD19-CAR-T-Zellen aufweisen und zusätzlich die CAR-Effektorzellen während der Langzeit-Koinkubation eine verminderte Persistenz und Zytokinausschüttung sowie eine niedrigere Perforin- und Granzym B-Freisetzung zeigen [202]. Andere Studien beschäftigten sich mit der Auswirkung einer verminderten BID-Expression auf T-Zellen, die, ähnlich wie bei Tumorzellen, ebenfalls zu einer Resistenz gegen Apoptose und somit zu einem erhöhten Überleben der Immuneffektorzellen führt [203,204]. Inwieweit die epigenetischen Modifikationen mittels Decitabin eine Repression von BID in CAR-T-Zellen auslösen könnte, ist bislang nicht untersucht.

Es ist jedoch erwiesen, dass anti-apoptotische BCL-2-Familienmitglieder wie BCL2L1 durch die Zugabe von Decitabin reguliert werden [205] und erhöhte Konzentrationen von BCL2L1 in der Zelle die Zellmigration und Invasion von Tumorzellen steigern. Im Vergleich zur Kontrollgruppe wurden ebenfalls mehr vaskuläre Strukturen in *in vivo* Mausmodellen für zwei soliden Tumoren (Melanom, Glioblastom) gebildet und *in vitro* die Formation von Tumorsphären unterstützt [206]. Interessantereise führt die Überexpression von BCL2L1 in CAR-T-Zellen zu einer verlängerten Persistenz sowie einer erhöhten Resistenz gegenüber des TME [207,208]. Eaton *et al.* konnten auch zeigen, dass die Überexpression von BCL2L1 das Überleben von T-Lymphozyten fördern, wenn diese in Abwesenheit von IL-2 kultiviert werden [209], was vermutlich ebenfalls positive Auswirkung auf das Überleben der Immuneffektorzellen im TME haben würde.

Obwohl die Kombination von epigenetischen Wirkstoffen und immuntherapeutischen Ansätzen bislang in klinischen Studien eher auf die Gabe von PD-1 Inhibitoren (Nivolumab, Pembrolizumab) und DNMTis (Azacytidine, Varinostat) beschränkt war [210], konnte in einer Studie gezeigte werden, dass das CAR-T-Zell-Zielantigen CD19 auf Lymphomen durch die Gabe von Decitabin soweit hochreguliert worden war, dass zwei Patienten eine komplette Remission erlebten [211]. Andere präklinische Arbeiten zeigten ebenfalls, dass gewisse Zielantigene wie z.B. Tumor-Hoden-Antigene oder TAAs durch die Behandlung von Decitabin überexprimiert werden [212]. Durch die Hochregulation dieser Zielstrukturen konnte nicht nur das Ansprechen der Tumoren auf die CAR-T-Zelltherapie gesteigert, sondern auch die Tumorheterogenität umgangen werden. In dieser Arbeit wurde die Expression der Zielantigene EGFR und CD44v6 vor und nach isolierter Behandlung der Tumorzellen mit Decitabin untersucht (2.2). Wir konnten zwar eine Tendenz einer erhöhten Expression in einigen Urothel-Zelllinien beobachten, die jedoch nur bei einigen Zelllinien signifikant war.

Neben vielversprechenden Modifikationen auf Seiten der Zielzellen können die Immuneffektorzellen ebenfalls von der epigenetischen Therapie profitieren. Zum Beispiel regulierte die Behandlung von CD123-CAR-T-Zellen mit Decitabin die Expression von Genen in diesen Zellen hoch, die die Differenzierung von naiven und Gedächtnis-T-Zellen begünstigte und die Modulation der Immunsynapse positiv beeinflussen, so dass die CAR-T-Zellen insgesamt eine steigerte Toxizität aufwiesen [213]. Indirekt wird die Situation von (CAR-)T-Zellen im TME auch verbessert, indem z.B. bei der Behandlung von Ovarialkarzinomen mit Inhibitoren von HDACs und DNMTs die Produktion der Chemokine CXCL9 und CXCL10 erhöht wurde, die beide eine vermehrte Aktivierung von T-Zellen bewirkten, so dass diese effektiver in den Tumor infiltrieren konnten [214]. Auch das Chemokingen CCL5, welches in Tumoren häufig herunter reguliert wird, kann durch die Gabe von Decitabin wieder re-exprimiert werden, wodurch ebenfalls die Tumorinfiltration der T-Zellen begünstigt wird [215]. Insgesamt erscheint deshalb die Behandlung sowohl von den Tumor- als auch den Immuneffektorzellen mit Decitabin besonders attraktiv, um generell eine effizientere Lyse von Tumorzellen durch CAR-T-Zellen zu induzieren.

Eine andere Möglichkeit, Mitglieder der BCL2-Familie zu regulieren, ist der Einsatz von BCL2-hemmenden *small molecules*/Wirkstoffen, die z.T. bereits mit verschiedensten Indikationen klinisch zugelassen sind. Die Gabe von Navitoclax konnten B-Zell-Malignome nach Koinkubation mit CD19-CAR-T-Zellen *in vitro* schneller lysiert werden [216]. Auch zeigten CD19-CAR-T-Zellen der dritten Generation einen verbesserten zytotoxischen Effekt auf mit Venetoclax vorbehandelten Leukämie und Lymphom-Zelllinien [217].

Ergänzende Maßnahmen zur CAR-T-Zelltherapie können z.B. eine Vorbehandlung des Patienten mit Chemo- und/oder Radiotherapie oder auch eine parallele Therapie mit immuntherapeutischen Ansätzen wie z.B. Checkpoint-Inhibitoren oder epigenetischen Substanzen sein. Dadurch werden oftmals folgende Punkte verbessert:

- Modifikation des TME durch Beeinflussung des Tumorwachstums, der Angiogenese und der Metastasierung
- Förderung der T-Zellmigration und erhöhte Persistenz der Effektorzellen im Tumor
- Hochregulation/Überexpression von TAA
- Erhöhte Zytotoxizität der Immuneffektorzellen

DIE ZUKUNFT DER CAR-T-ZELLTHERAPIE

Obwohl es auf dem Gebiet der adoptiven T-Zelltherapie für hämatologische Neoplasien in den letzten Jahren revolutionäre Fortschritte gab, blieben die Möglichkeiten, solide Tumore mit CAR-T-Zellen zu behandeln, begrenzt [39]. Die Identifikation sicherer und homogen exprimierter Zielmoleküle auf den malignen Zellen stellt neben den Herausforderungen durch das feindliche TME hohe Hürden für die Therapie von soliden Tumoren mit CAR-T-Zellen dar, wie bereits ausführlich diskutiert wurde. Das Vorhandensein einer immunsuppressiven, tumortoleranten und metabolisch herausfordernden Umgebung im Tumor schwächt nicht nur die Infiltration, die Aktivität und das Überleben der CAR-T-Zellen [218], sondern vermindert auch zusätzlich die Anti-Tumor-Aktivität der T-Zellen durch Exposition gegenüber von immunsuppressiven Zytokinen und Chemokinen, die von tumorassoziierten Stromazellen, Fibroblasten oder regulatorischen Immunzellen sezerniert werden; ebenfalls werden die Immuneffektorzellen durch Hypoxie und eine verminderte Nährstoffverfügbarkeit im Tumor geschwächt [219].

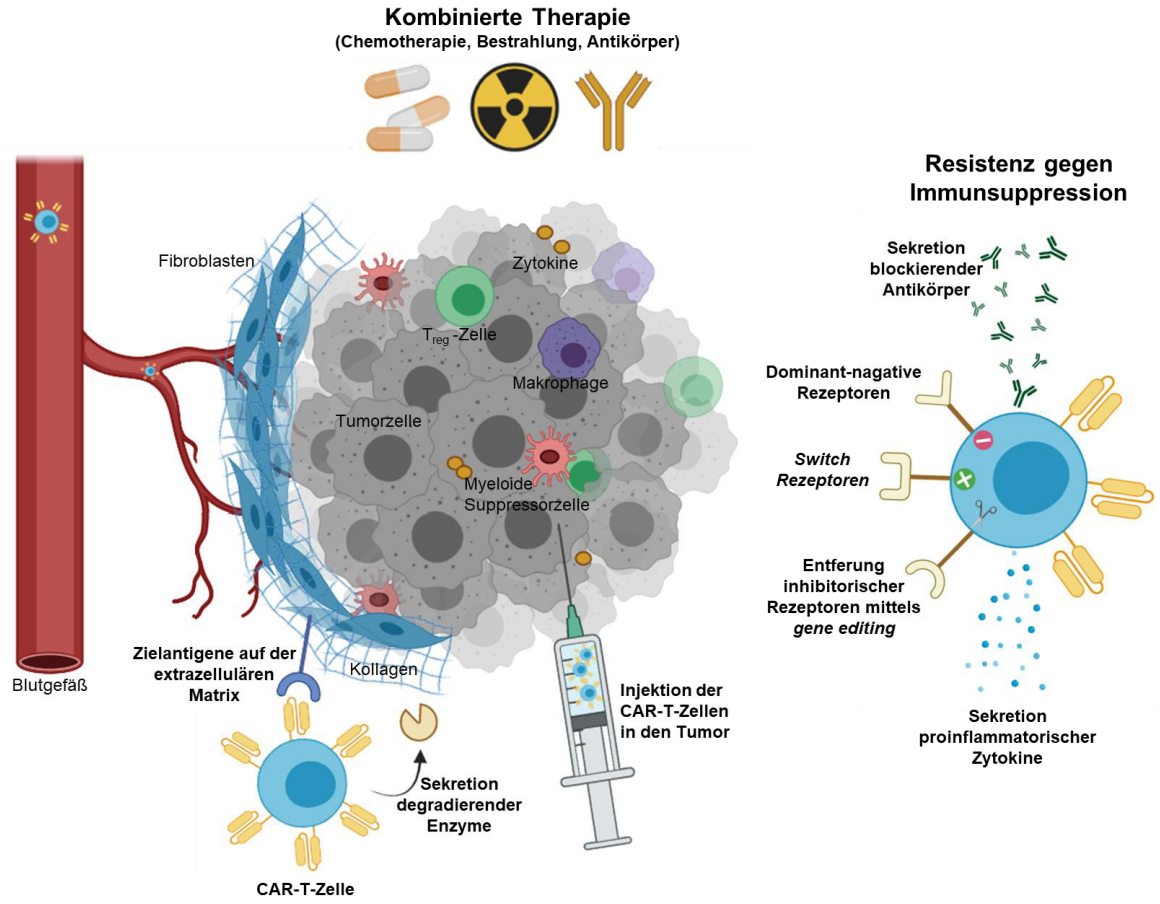

Abbildung 4: Herausforderungen des TME eines soliden Tumors und Lösungsansätze für die CAR-T-Zelltherapie. Eine schematische Darstellung der immunsuppressiven Umgebung, die CAR-T-Zellen für eine wirksame Anti-Tumor-Antwort überwinden müssen. Neben der Penetration der extrazellulären Matrix und dem Stroma, müssen CAR-T-Zellen mithilfe von kombinierten Therapieansätzen oder durch Ausstattung mit Resistenzmechanismen gegen das TME resistenter gemacht werden. Die Abbildung wurde nach [146,220] adaptiert und mit Biorender.com erstellt.

Tumorinfiltration

Der große Erfolg der CAR-T-Zelltherapie bei der Behandlung von hämatologischen Erkrankungen kann unter anderem dadurch erklärt werden, dass die zirkulierenden CAR-T-Zellen im Blutkreislauf ihre Zielzellen in den hämatologischen Organen direkt erreichen können. Im Gegensatz dazu müssen die Immuneffektorzellen bei der CAR-T-Zelltherapie von soliden Tumoren mehrere Barrieren, bestehend aus Neovaskularisierung und Stroma mit Extrazellulärer Matrix überwinden, um überhaupt zu den malignen Zellen im Tumorgewebe zu gelangen (Abbildung 4).

Die Bildung neuer Blutgefäße resultiert aus dem gestiegenen Bedarf an Nährstoffen und Sauerstoff im soliden Tumor und korreliert direkt mit seinem Wachstum. Diese Tumorvaskularisierung ist ebenfalls mit der Bildung von Stroma verbunden und erschwert

nicht nur die Infiltration der T-Zellen in den Tumor, sondern fördert auch die Infiltration bzw. Differenzierung von immunsuppressiven nicht-malignen Zellen wie tumorassoziierte Fibroblasten und Makrophagen [145]. Die Proliferation der Endothelzellen wird unter anderem durch den vaskulären endothelialen Faktor (VEGF) beschleunigt, welcher in HNSCCs häufig überexprimiert wird und in Verbindung mit einem aggressiven Krankheitsverlauf steht [221]. Monoklonale Antikörper gegen VEGF (Bevacizumab, Sunitinib, Ramucirumab, Lenvatinib) erhöhen die Permeabilität für Medikamente in den Tumor und führen in Kombination mit den Checkpoint-Inhibitoren Pembrolizumab (PD-1) oder Atezolizumab (PD-L1) zu einem Rückgang der Gefäßdichte und zur Verkleinerung des Tumors (NCT03818061, NCT03650764, NCT04199104). Obwohl VEGFR nicht auf den Tumorzellen selbst, sondern von den Endothelzellen des Tumorgefäßsystems exprimiert wird, stellt es durch die gute Zugänglichkeit für zirkulierenden T-Zellen sowie die genomische Stabilität der nicht-malignen Endothelzellen ein gutes Zielantigen für CAR-T-Zellen gegen HNSCCs dar, wenn man die Stromabildung oder die Angiogenese behindern möchte [222]. Als immuntherapeutischer Ansatz wäre daher die vorbereitende Gabe von VEGFR-CAR-T-Zellen denkbar, so dass die nachfolgend verabreichten dualen EGFR/CD44v6-CAR-T-Zellen einfacher den Tumor infiltrieren können. Allerdings müssen hier die *on-target off-Tumorendothel* Effekte kritisch überwacht werden, um nicht Blutungen in anderen nicht betroffenen Geweben im Körper zu induzieren.

Ein weiterer Faktor, der die Infiltration der CAR-T-Zellen in den soliden Tumor erheblich einschränkt, ist das Tumor-umliegende Stroma, welches aus einer extrazellulären Matrix (Kollagen, Fibronektin, Glykosaminoglykane, Proteoglykane usw.) besteht, die von den Tumorzellen oder den tumorassoziierten Fibroblasten gebildet wird [146]. Diese Barriere schützt den Tumor vor dem Immunsystem und ganz wesentlich auch vor der Therapie mit verschiedenen Wirkstoffen wie Chemotherapeutika, monoklonale Antikörper oder Checkpoint-Inhibitoren; deren Aufnahme könnte jedoch durch die lokale Vorbehandlung mit z.B. Kollagenase oder Hyaluronidase verstärkt werden [146]. Modifizierte CAR-T-Zellen, die zusätzlich zur CAR-Expression Stroma-degradierende Enzyme wie Heparanase sezernieren, zeigten im Mausmodell eine bessere Infiltration des Tumors und eine erhöhte Anti-Tumor-Aktivität [223].

Da aufgrund der guten Lokalisation von HNSCCs eine direkte Injektion der CAR-T-Zellen in den Tumor erfolgen kann, rückt die Problematik der Tumorinfiltration bei dieser Entität in den Hintergrund. Vielmehr sollten CAR-T-Zellen für HNSCCs dahingehend verbessert werden, dass ihre Proliferation und Aktivierung unterstützt wird und sie im feindlichen TME besser überleben.

Physikalische und metabolische Besonderheiten im Tumor

Schon vor 70 Jahren wurde geringer Sauerstoffgehalt in Tumorzellen mit einer Resistenz gegenüber Radiotherapie in Verbindung gebracht [224] und stellt auch heute einen wichtigen Faktor für zukünftige immunbasierte Therapien dar. Mithilfe des Hypoxie-Signalweges wird die Anpassung an den niedrigen Sauerstoffgehalt in den Tumorzellen gesteuert. Unter Normoxia wird der Hypoxie-induzierbarer Faktor 1-α (HIF-1α) in kürzester Zeit durch Prolylhydroxylase-Domäneproteine hydroxyliert und im Anschluss durch den Von-Hippel Lindau Komplex polyubiquitiniert, so dass HIF-1α letztendlich kontinuierlich protosomal abgebaut wird und kein *signalling* auslösen kann. Bei reduziertem Sauerstoffgehalt werden die Hydroxylasen inaktiv, so dass HIF-1α als Transkriptionsfaktor in den Zellkern eindringen und mit HIF-1β einen Komplex bilden kann, der über die Bindung an Hypoxie-Reaktions-Elemente die zellulären Anpassungen an die Hypoxie wie z.B. Angiogenese oder einen *switch* der Energiebereitstellung von aerob auf anaerob vermittelt [225].

Hypoxie induziert auch immunsuppressive Signalwege im TME, die mithilfe kombinatorischer therapeutischer Ansätze behandelt werden können (Abbildung 4). Es ist bekannt, dass HIF-1α hauptverantwortlich für die Expression von Checkpoint-Inhibitoren und das erhöhte Auftreten von immunsuppremierenden Zellen im Tumor ist, weshalb bereits Inhibitoren gegen HIF-1α (2ME2, PT2977) oder Medikamente, die dessen Degradation fördern, in klinischen Studien angewendet werden [146,225]. Auch Patienten mit HNSCCs werden in einer Phase II Studie (NCT02538510) mit der Kombination des HDACi Vorinostat mit Pembrolizumab behandelt, wodurch es zur Degradation von HIF-1α kommen soll [226].

Erste *in vitro* Studien passten das Design eines CARs an die hypoxischen Bedingungen im Tumor an, indem Hypoxie-Reaktions-Elemente mit dem Promoter des CAR-Konstruktes fusioniert wurden, so dass der CAR bei Anwesenheit von Sauerstoff degradiert und *on-target off-tumor* Toxizität reduziert wird [227]. Die Wahl der kostimulatorischen Domänen des CARs soll ebenfalls Einfluss auf die Aktivierung der CAR-T-Zellen unter Hypoxie nehmen, wobei aber genaue Mechanismen noch nicht identifiziert sind [146].

Ein ganz wesentlich zusätzlich limitierender Faktor stellt der Metabolismus der Tumorzellen dar, die gut an die geringen verfügbaren Mengen an Nährstoffen im TME adoptiert sind; bei T-Zellen führt dieser Mangel allerdings zu einer Suppression der Proliferation und Aktivierung [149]. Der Nährstoffmangel ist im Speziellen durch einen niedrigen Gehalt an essentiellen Aminosäuren und einen niedrigen Glukosespiegel im Tumorgewebe bedingt. Besonders Tryptophan und Arginin sind Aminosäuren, die für die ordnungsgemäße T-Zell-Funktion essentiell sind, aber im TME nur in geringen

Konzentrationen vorliegen [219]. Das im Tumor von immunregulatorischen Zellen vieler Krebsarten gebildete Enzym Indolamin-2,3-Dioxygenase wandelt Tryptophan zu Kynurenin um, so dass der Tryptophanspiegel im TME sinkt, mTOR in den T-Zellen gehemmt wird und diese inaktiviert werden [228]. Diesen Mechanismus bestätigen Ninomiya *et al*., indem sie zeigten, dass CD19-CAR-T-Zellen Indolamin-2,3-Dioxygenase-exprimierende Lymphome nicht kontrollieren können [229]. Die Wichtigkeit von ausreichender Menge Aminosäuren und Nährstoffen im TME konnte ebenfalls in anderen Studien bestätigt werden. So erhöhte die *in vitro* Zugabe von Arginin [230] sowie von Kalium [231] die antitumorale Aktivität von T-Zellen erheblich.

Ebenfalls wird durch den erhöhten Glukosestoffwechsel im Tumor die Laktatproduktion verstärkt, welche einen inhibitorischen Effekt auf die Proliferation, Aktivierung, Zytokinproduktion und Zytotoxizität der T-Zellen zeigt [232]. Die damit verbundene Übersäuerung des Tumors fördert die Metastasierung der Tumorzellen und die Bildung von Fibroblasten, wodurch die Infiltration der Immuneffektorzellen in den Tumor erschweren [233].

Wie bereits mehrfach in den Publikationen beschrieben und diskutiert, würde hier eine intratumorale Injektion von CAR-T-Zellen, die durch zusätzlich genetische Veränderungen widerstandsfähiger für das TME sind, helfen, einen Großteil der her beschriebenen Probleme zu umgehen.

Tumor-induzierte Immunevasionsmechanismen

Sowohl Tumorzellen als auch Tumor-infiltrierende Immunzellen exprimieren eine Reihe von Liganden, die durch ihre Bindung an hemmende Rezeptoren wie z.B. TIM-3 [234], CTLA-4 [235], TIGIT [236] oder PD-1 [237] auf den T-Zellen ihre Anti-Tumor-Aktivität unterdrücken. Wie im vorherigen Kapitel bereits beschrieben, werden Checkpoint-Inhibitoren erfolgreich gegen viele Krankheiten klinisch eingesetzt und neuerdings auch in Kombination mit CAR-T-Zelltherapie genutzt.

Darüber hinaus versuchen unterschiedliche Ansätze dieser Hemmung der T-Zell-Aktivität und -Funktion entgegenzuwirken (Abbildung 4), indem Gene für inhibitorische Rezeptoren oder Signalmoleküle mittels CRISPR/Cas9 oder TALEN entfernt werden [238–241]. Obwohl diese Methoden vielversprechende Ergebnisse zeigen, kann die Entfernung von Checkpoint-Inhibitoren zur unkontrollierten Proliferation der T-Zellen und somit zu einer *off-target* Aktivierung führen [61]. Erste klinische Studien, die CAR-T-Zellen mit PD-1 Knockout zur Behandlung solider Tumore einsetzen, befinden sich zurzeit in der Rekrutierung von Patienten (NCT03747965, NCT03525782, NCT03706326, NCT03399448). Die Ergebnisse einer Phase-I-Studie, in der Patienten T-Zellen erhielten, welchen die Gene TRAC, TRBC

und PDCD1 entfernt wurden, boten keine Anzeichen für eine erhöhte Toxizität dieser Zellen [242].

Aktuelle Ansätze greifen die Inhibition der T-Zellen durch Checkpoint-Rezeptoren auf, indem die trunkierte extrazelluläre Liganden-Bindedomäne eines Checkpoints wie z.B. PD-1 mit zytoplasmatischen Signaldomänen eines CARs fusioniert werden (auch *activating chimeric switch receptors* genannt), um die inhibitorischen Signale in eine Aktivierung der T-Zelle umzuwandeln [243]. Das gleiche Prinzip wurde genutzt, um der verringerten Proliferation und Zytotoxizität der CAR-T-Zellen, verursacht durch die Sekretion von antientzündlichen Zytokinen wie TGF-ß (*transforming growth factor-ß*) durch Tregs, entgegen zu wirken [244]. Dabei wurden die TGF-β-Signale in für T-Zellen stimulierende 4-1BB- oder IL-12-Signale umgewandelt, wodurch eine relative Resistenz der (CAR-)T-Zellen gegen immunsuppressive Signale entsteht [245].

Eine weitere Möglichkeit, das TGF-β-Signal zu umgehen, wird zurzeit in einer Phase-I-Studie getestet, in der Patienten mit metastasierendem Prostatakrebs gegen das Prostata-spezifischen Membranantigen (PSMA) gerichtete CAR-T-Zellen erhalten, die mit einem dominant-negativen TGF-β-Rezeptor (PSMA-TGFβRDN CAR) ausgestattet sind (NCT04227275). Der dominant-negativer Rezeptor enthält zwar die intakte Ligandenbinderegion (der extrazelluläre Teil des Rezeptors), die intrazelluläre enzymatische Region ist aber größtenteils deletiert, so dass die Bindung des Liganden an den Rezeptor stattfinden kann, eine Dimerisierung der Rezeptoren und die damit verbundene Aktivierung des inhibierenden Signalweges jedoch blockiert ist. Obwohl frühere Versuche mit dem PSMA-TGFβRDN CAR keine Hinweise auf mögliche Toxizität des in der Studie genutzten CAR-Konstrukts zeigten [246], wurde in einem Artikel aus *The Lancet* (Vol 22, July 2021) darauf hingewiesen, dass zwei Patienten in dieser Studie an Immuneffektorzell-assoziiertem Neurotoxizitätssyndrom verstorben sind und deshalb die Studie abgebrochen wurde. Ob die Todesfälle durch den dominant-negativen Rezeptor oder das Zielantigen des CARs verursacht wurden, muss noch geklärt werden. Um der tumorbedingten T-Zell-Inhibition durch TGF-β trotzdem etwas entgegen setzen zu können, ist auch eine Sekretion von TGF-β-neutralisierenden Antikörpern in das TME möglich, die von den dementsprechend genetisch veränderten CAR-T-Zelle gebildet werden [233].

Um die Aktivität und Aggressivität der CAR-T-Zellen zusätzlich zu steigern, können CAR-T-Zellen auch so modifiziert werden, dass entzündungsfördernde Zytokine wie IL-12, IL-15 und IL-18 von ihnen überexprimiert werden, um eine erhöhte Proliferations-, Überlebensfähigkeit und Resistenz gegen inhibitorische Signale hervorzurufen [244]. Dadurch wird nicht nur die Funktion der Immuneffektorzellen gestärkt, sondern ebenfalls ein regulatorischer Effekt auf Tregs oder myeloide Supressorzellen (mittels IL-2, IL-12) ausgeübt [233].

Mögliche Ansätze, um CAR-T-Zellen im TME zu stärken oder dieses auch direkt anzugreifen:

- Tumorvaskularisierung mit CAR-T-Zellen angreifen
- CAR-T-Zellen exprimieren Matrix-degradierende Enzyme
- CAR-Expression mittels Sauerstoffgehalt regulieren
- Inhibitorische Signale in den CAR-T-Zellen durch dominant-negative Rezeptoren umgehen
- Inhibitorische Signale in den CAR-T-Zellen durch *Switch*-Rezeptoren in aktivierende Signale umwandeln
- CAR-T-Zellen sekretieren neutralisierende Antikörper

Insgesamt konnte diese Arbeit zeigen, dass die sehr komplexe und facetten-reiche CAR-T-Zelltherapie zur Behandlung von soliden Tumoren aufgrund der ausgeprägten Tumorheterogenität und der inhibierenden Faktoren des TME trotz intensiver Forschung immer noch viele Herausforderungen bereithält und es dringend notwendig ist, die Ansprechraten der Patienten zu optimieren und die Toxizität zu begrenzen. Wie schon diskutiert, gibt es viele unterschiedliche Ansätze zur Überwindung dieser Hürden, die jedoch nicht alle einfach miteinander kombiniert werden können. Aus diesem Grund sollte idealerweise eine personalisierte Therapie mit umfassender Charakterisierung des Tumors für jeden Patienten erfolgen. Nur so kann das Wissen um die genaue Zusammensetzung des Tumors und seine Eigenschaften genutzt werden, um die Immuntherapie mit CAR-T-Zellen und auch Antikörpern als einen Teil der maßgeschneiderten Kombinationstherapie so zu gestalten, dass die Anti-Tumor-Immunantwort gestärkt und gleichzeitig die inhibierenden Zellen im TME unterdrückt werden.

Obwohl noch so viele Fragen im Hinblick auf die CAR-T-Zelltherapie zur Behandlung solider Tumore nicht beantwortet sind, bin ich froh mit den Arbeiten meiner Dissertation die Forschung einen kleinen Schritt, auf dem langen Weg zur Heilung vieler Patienten, voran gebracht zu haben.

4. LITERATUR

1. Hunter, K. D., Parkinson, E. K. & Harrison, P. R. Profiling early head and neck cancer. *Nature Reviews Cancer* **5**, 127–135 (2005).
2. Seliger, B. *et al.* Immune Escape Mechanisms and Their Clinical Relevance in Head and Neck Squamous Cell Carcinoma. *Int. J. Mol. Sci.* **21**, 7032 (2020).
3. Cohen, E. E. W. *et al.* The Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of squamous cell carcinoma of the head and neck (HNSCC). *J. Immunother. Cancer* **7**, 184 (2019).
4. Aupérin, A. Epidemiology of head and neck cancers: an update. *Curr. Opin. Oncol.* **32**, 178–186 (2020).
5. Durst, M., Gissmann, L., Ikenberg, H. & Zur Hausen, H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3812–3815 (1983).
6. Ang, K. K. *et al.* Human Papillomavirus and Survival of Patients with Oropharyngeal Cancer. *N. Engl. J. Med.* **363**, 24–35 (2010).
7. Kobayashi, K. *et al.* A Review of HPV-Related Head and Neck Cancer. *J. Clin. Med.* **7**, 241 (2018).
8. Fakhry, C. & Gillison, M. L. Clinical implications of human papillomavirus in head and neck cancers. *J. Clin. Oncol.* **24**, 2606–2611 (2006).
9. Goldson, T. M., Han, Y., Knight, K. B., Weiss, H. L. & Resto, V. A. Clinicopathological predictors of lymphatic metastasis in HNSCC: Implications for molecular mechanisms of metastatic disease. *J. Exp. Ther. Oncol.* **8**, 211–221 (2010).
10. Vermorken, J. B. Head and neck cancer: Medical treatment in head and neck cancer. *Ann Oncol.* **16 Suppl**, 258–64 (2015).
11. Pantel, K. & Brakenhoff, R. H. Dissecting the metastatic cascade. *Nature Reviews Cancer* **4**, 448–456 (2004).
12. Nauta, I. H. *et al.* Evaluation of the eighth TNM classification on p16-positive oropharyngeal squamous cell carcinomas in the Netherlands and the importance of additional HPV DNA testing. *Ann. Oncol.* **29**, 1273–1279 (2018).
13. Talavera, A. *et al.* Nimotuzumab, an Antitumor Antibody that Targets the Epidermal Growth Factor Receptor, Blocks Ligand Binding while Permitting the Active Receptor Conformation. *Cancer Res* **69**, 5851–5860 (2009).
14. Guigay, J. *et al.* TPExtreme randomized trial: TPEx versus Extreme regimen in 1st line recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). *J. Clin. Oncol.* **37**, 6002–6002 (2019).
15. Bonner, J. A. *et al.* Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol.* **11**, 21–28 (2010).
16. Baysal, H. *et al.* Cetuximab-induced natural killer cell cytotoxicity in head and neck squamous cell carcinoma cell lines: investigation of the role of cetuximab sensitivity and HPV status. *Br. J. Cancer* **123**, 752–761 (2020).
17. Lawrence, M. S. M. S. *et al.* Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* **517**, 576–582 (2015).
18. Alsahafi, E. *et al.* Clinical update on head and neck cancer: molecular biology and ongoing challenges. *Cell Death and Disease* **10**, 1–17 (2019).
19. Colevas, A. D. *et al.* Safety and clinical activity of atezolizumab in head and neck cancer: Results from a phase I trial. *Ann. Oncol.* **29**, 2247–2253 (2018).
20. Segal, N. H. *et al.* Safety and efficacy of durvalumab in patients with head and neck squamous cell carcinoma: results from a phase I/II expansion cohort. *Eur. J. Cancer* **109**, 154–161 (2019).
21. Shibata, H., Saito, S. & Uppaluri, R. Immunotherapy for Head and Neck Cancer: A Paradigm Shift From Induction Chemotherapy to Neoadjuvant Immunotherapy. *Front. Oncol.* **11**, 3533 (2021).
22. Kadapathri, A., Chaudhary, S., Thomas, L., Balan, A. & K R, S. Immunotherapy for head and neck cancer – The current scenario. *IP Int. J. Compr. Adv. Pharmacol.* **5**, 146–150 (2021).
23. Kumari, S., Curado, S., Mayya, V. & Dustin, M. L. T cell antigen receptor activation and actin cytoskeleton

remodeling. *Biochimica et Biophysica Acta - Biomembranes* **1838**, 546–556 (2014).
24. Rudolph, M. G., Stanfield, R. L. & Wilson, I. A. How TCRs bind MHCs, peptides and coreceptors. *Annu. Rev. Immunol.* **24**, 419–466 (2006).
25. Rock, K. L., Reits, E. & Neefjes, J. Present Yourself! By MHC Class I and MHC Class II Molecules. *Trends in Immunology* **37**, 724–737 (2016).
26. Mariuzza, R. A., Agnihotri, P. & Orban, J. The structural basis of T-cell receptor (TCR) activation: An enduring enigma. *Journal of Biological Chemistry* **295**, 914–925 (2020).
27. Almeida, L., Lochner, M., Berod, L. & Sparwasser, T. Metabolic pathways in T cell activation and lineage differentiation. *Seminars in Immunology* **28**, 514–524 (2016).
28. Trapani, J. A. & Smyth, M. J. Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology* **2**, 735–747 (2002).
29. Ruella, M. & Kalos, M. Adoptive immunotherapy for cancer. *Immunol. Rev.* **257**, 14–38 (2014).
30. Rosenberg, S. A. *et al.* Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma. *N. Engl. J. Med.* **319**, 1676–1680 (1988).
31. Qian, X., Wang, X. & Jin, H. Cell transfer therapy for cancer: past, present, and future. *Journal of immunology research* **2014**, 525913 (2014).
32. Rosenberg, S. A. *et al.* Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* **86**, 1159–1166 (1994).
33. Mehta, G. U. *et al.* Outcomes of Adoptive Cell Transfer with Tumor-infiltrating Lymphocytes for Metastatic Melanoma Patients with and Without Brain Metastases. *J. Immunother.* **41**, 241–247 (2018).
34. Andersen, R. *et al.* Long-Lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated il2 regimen. *Clin. Cancer Res.* **22**, 3734–3745 (2016).
35. Karpanen, T. & Olweus, J. T-cell receptor gene therapy - ready to go viral? *Molecular Oncology* **9**, 2019–2042 (2015).
36. Schumacher, T. N. M. T-cell-receptor gene therapy. *Nature Reviews Immunology* **2**, 512–519 (2002).
37. Kunert, A. *et al.* TCR-engineered T cells meet new challenges to treat solid tumors: Choice of antigen, T cell fitness, and sensitization of tumor milieu. *Frontiers in Immunology* **4**, 363 (2013).
38. Maude, S. L. *et al.* Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia. *N. Engl. J. Med.* **371**, 1507–1517 (2014).
39. Larson, R. C. & Maus, M. V. Recent advances and discoveries in the mechanisms and functions of CAR T cells. *Nature Reviews Cancer* **21**, 145–161 (2021).
40. Huston, J. S. *et al.* Medical applications of single-chain antibodies. *Int. Rev. Immunol.* **10**, 195–217 (1993).
41. Caruso, H. G. *et al.* Tuning Sensitivity of CAR to EGFR Density Limits Recognition of Normal Tissue While Maintaining Potent Antitumor Activity. *Cancer Res.* **75**, 3505–3518 (2015).
42. Hudecek, M. *et al.* Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin. Cancer Res.* **19**, 3153–3164 (2013).
43. Liu, X. *et al.* Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. *Cancer Res.* **75**, 3596–3607 (2015).
44. James, S. E. *et al.* Antigen Sensitivity of CD22-Specific Chimeric TCR Is Modulated by Target Epitope Distance from the Cell Membrane. *J. Immunol.* **180**, 7028–7038 (2008).
45. Hombach, A. A., Hombach, A. A. & Abken, H. Adoptive immunotherapy with genetically engineered T cells: Modification of the IgG1 Fc spacer domain in the extracellular moiety of chimeric antigen receptors avoids off-target activation and unintended initiation of an innate immune response. *Gene Ther.* **17**, 1206–1213 (2010).
46. Jonnalagadda, M. *et al.* Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy. *Mol. Ther.* **23**, 757–768 (2015).
47. Dotti, G., Gottschalk, S., Savoldo, B. & Brenner, M. K. Design and development of therapies using chimeric antigen receptor-expressing T cells. *Immunol. Rev.* **257**, 107–126 (2014).
48. Savoldo, B. *et al.* CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J. Clin.*

Invest. **121**, 1822–1826 (2011).
49. Tian, Y., Li, Y., Shao, Y. & Zhang, Y. Gene modification strategies for next-generation CAR T cells against solid cancers. *Journal of Hematology and Oncology* **13**, 1–16 (2020).
50. Kalaitsidou, M., Kueberuwa, G., Schütt, A. & Gilham, D. E. CAR T-cell therapy: Toxicity and the relevance of preclinical models. *Immunotherapy* **7**, 487–497 (2015).
51. Kochenderfer, J. N. *et al.* Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* **116**, 4099–4102 (2010).
52. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A. & June, C. H. Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia. *N. Engl. J. Med.* **365**, 725–733 (2011).
53. Kochenderfer, J. N. *et al.* Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *J. Clin. Oncol.* **33**, 540–549 (2015).
54. Park, J. H. *et al.* Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N. Engl. J. Med.* **378**, 449–459 (2018).
55. Hombach, A. A. & Abken, H. Costimulation by chimeric antigen receptors revisited the T cell antitumor response benefits from combined CD28-OX40 signalling. *Int. J. Cancer* **129**, 2935–2944 (2011).
56. Song, D.-G. & Powell, D. J. Pro-survival signaling via CD27 costimulation drives effective CAR T-cell therapy. *Oncoimmunology* **1**, 547–549 (2012).
57. Guedan, S. *et al.* Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation. *JCI insight* **3**, (2018).
58. Chmielewski, M. & Abken, H. TRUCKS, the fourth-generation CAR T cells: Current developments and clinical translation. *Adv. CELL GENE Ther.* **3**, (2020).
59. Liu, Y. *et al.* Armored Inducible Expression of IL-12 Enhances Antitumor Activity of Glypican-3–Targeted Chimeric Antigen Receptor–Engineered T Cells in Hepatocellular Carcinoma. *J. Immunol.* **203**, 198–207 (2019).
60. Kagoya, Y. *et al.* A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects. *Nat. Med.* **24**, 352–359 (2018).
61. Tokarew, N., Ogonek, J., Endres, S., von Bergwelt-Baildon, M. & Kobold, S. Teaching an old dog new tricks: next-generation CAR T cells. *British Journal of Cancer* **120**, 26–37 (2019).
62. Metzinger, M. N. *et al.* Chimeric Antigen Receptor T-Cell Therapy: Reach to Solid Tumor Experience. *Oncol.* **97**, 59–74 (2019).
63. Hackett, P. B., Largaespada, D. A. & Cooper, L. J. N. A transposon and transposase system for human application. *Molecular Therapy* **18**, 674–683 (2010).
64. Ellis, J. Silencing and variegation of gammaretrovirus and lentivirus vectors. *Human Gene Therapy* **16**, 1241–1246 (2005).
65. Haas, A. R. *et al.* Phase I Study of Lentiviral-Transduced Chimeric Antigen Receptor-Modified T Cells Recognizing Mesothelin in Advanced Solid Cancers. *Mol. Ther.* **27**, 1919–1929 (2019).
66. Davila, M. L. *et al.* Chimeric antigen receptors for the adoptive T cell therapy of hematologic malignancies. *International Journal of Hematology* **99**, 361–371 (2014).
67. Benmebarek, M. R. *et al.* Killing mechanisms of chimeric antigen receptor (CAR) T cells. *International Journal of Molecular Sciences* **20**, (2019).
68. George, A. *et al.* Strategies to sensitize cancer cells to immunotherapy. *Hum. Vaccin. Immunother.* 1–7 (2021). doi:10.1080/21645515.2021.1891817
69. Villanueva, L., Álvarez-Errico, D. & Esteller, M. The Contribution of Epigenetics to Cancer Immunotherapy. *Trends in Immunology* **41**, 676–691 (2020).
70. Menzel, J. & Black, J. C. Epigenetic modulation of the tumor immune microenvironment to potentiate immune checkpoint blockade therapy. *Cancer Discov.* **10**, 179–181 (2020).
71. Ozyerli-Goknar, E. & Bagci-Onder, T. Epigenetic Deregulation of Apoptosis in Cancers. *Cancers (Basel).* **13**, 3210 (2021).
72. Gaździcka, J., Gołąbek, K., Strzelczyk, J. K. & Ostrowska, Z. Epigenetic Modifications in Head and Neck Cancer. *Biochemical Genetics* **58**, 213–244 (2020).
73. Kouzarides, T. Chromatin Modifications and Their Function. *Cell* **128**, 693–705 (2007).
74. Lawrence, M., Daujat, S. & Schneider,

R. Lateral Thinking: How Histone Modifications Regulate Gene Expression. *Trends in Genetics* **32**, 42–56 (2016).
75. Li, Y. & Sasaki, H. Genomic imprinting in mammals: Its life cycle, molecular mechanisms and reprogramming. *Cell Research* **21**, 466–473 (2011).
76. Wilson, A. G. Epigenetic Regulation of Gene Expression in the Inflammatory Response and Relevance to Common Diseases. *J. Periodontol.* **79**, 1514–1519 (2008).
77. Gräff, J., Kim, D., Dobbin, M. M. & Li-Huei, T. Epigenetic Regulation of Gene Expression in Physiological and Pathological Brain Processes. *Physiol. Rev.* **91**, 603–649 (2011).
78. Peng, J. C. & Karpen, G. H. Epigenetic regulation of heterochromatic DNA stability. *Current Opinion in Genetics and Development* **18**, 204–211 (2008).
79. Fraga, M. F. *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10604–10609 (2005).
80. Franklin, T. B. & Mansuy, I. M. Epigenetic inheritance in mammals: Evidence for the impact of adverse environmental effects. *Neurobiology of Disease* **39**, 61–65 (2010).
81. Feinberg, A. P., Koldobskiy, M. A. & Göndör, A. Epigenetic modulators, modifiers and mediators in cancer aetiology and progression. *Nature Reviews Genetics* **17**, 284–299 (2016).
82. Zhao, Z. & Shilatifard, A. Epigenetic modifications of histones in cancer. *Genome Biology* **20**, 1–16 (2019).
83. Le, J. Histone modifications: Targeting head and neck cancer stem cells. *World J. Stem Cells* **6**, 511 (2014).
84. Saldaña-Meyer, R. & Recillas-Targa, F. Transcriptional and epigenetic regulation of the p53 tumor suppressor gene. *Epigenetics* **6**, 1068–1077 (2011).
85. Nie, J., Liu, L., Li, X. & Han, W. Decitabine, a new star in epigenetic therapy: The clinical application and biological mechanism in solid tumors. *Cancer Letters* **354**, 12–20 (2014).
86. Grant, C. *et al.* Romidepsin: A new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors. *Expert Review of Anticancer Therapy* **10**, 997–1008 (2010).
87. Park, S. Y. & Kim, J. S. A short guide to histone deacetylases including recent progress on class II enzymes. *Experimental and Molecular Medicine* **52**, 204–212 (2020).
88. Seto, E. & Yoshida, M. Erasers of histone acetylation: The histone deacetylase enzymes. *Cold Spring Harb. Perspect. Biol.* **6**, (2014).
89. Castilho, R. M., Squarize, C. H. & Almeida, L. O. Epigenetic modifications and head and neck cancer: Implications for tumor progression and resistance to therapy. *International Journal of Molecular Sciences* **18**, (2017).
90. Li, Y. & Seto, E. HDACs and HDAC inhibitors in cancer development and therapy. *Cold Spring Harb. Perspect. Med.* **6**, (2016).
91. Falkenberg, K. J. & Johnstone, R. W. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nature Reviews Drug Discovery* **13**, 673–691 (2014).
92. Piekarz, R. L. *et al.* Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: A case report. *Blood* **98**, 2865–2868 (2001).
93. Moore, L. D., Le, T. & Fan, G. DNA methylation and its basic function. *Neuropsychopharmacology* **38**, 23–38 (2013).
94. Saxonov, S., Berg, P. & Brutlag, D. L. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1412–1417 (2006).
95. Gardiner-Garden, M. & Frommer, M. CpG Islands in vertebrate genomes. *J. Mol. Biol.* **196**, 261–282 (1987).
96. Stephen, J. K., Chen, K. M., Havard, S., Harris, G. & Worsham, M. J. Promoter methylation in head and neck tumorigenesis. *Methods in Molecular Biology* **863**, 187–206 (2012).
97. Derissen, E. J. B., Beijnen, J. H. & Schellens, J. H. M. Concise Drug Review: Azacitidine and Decitabine. *Oncologist* **18**, 619–624 (2013).
98. Dhillon, S. Decitabine/Cedazuridine: First Approval. *Drugs* **80**, 1373–1378 (2020).
99. Feng, S. & De Carvalho, D. D. Clinical advances in targeting epigenetics for cancer therapy. *FEBS J.* febs.15750 (2021). doi:10.1111/febs.15750
100. June, C. H. & Sadelain, M. Chimeric Antigen Receptor Therapy. *N. Engl. J. Med.* **379**, 64–73 (2018).
101. Poorebrahim, M. *et al.* Genetically modified immune cells targeting tumor antigens. *Pharmacology and Therapeutics* **214**, 107603 (2020).

102. Von Witzleben, A., Wang, C., Laban, S., Savelyeva, N. & Ottensmeier, C. H. cells HNSCC: Tumour Antigens and Their Targeting by Immunotherapy. *Cells* **9**, (2020).
103. Leko, V. & Rosenberg, S. A. Identifying and Targeting Human Tumor Antigens for T Cell-Based Immunotherapy of Solid Tumors. *Cancer Cell* **38**, 454–472 (2020).
104. Gan, H. K., Burgess, A. W., Clayton, A. H. A. & Scott, A. M. Targeting of a conformationally exposed, tumor-specific epitope of EGFR as a strategy for cancer therapy. *Cancer Research* **72**, 2924–2930 (2012).
105. O'Rourke, D. M. *et al.* A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci. Transl. Med.* **9**, (2017).
106. Miao, H. *et al.* EGFRvIII-specific chimeric antigen receptor T cells migrate to and kill tumor deposits infiltrating the brain parenchyma in an invasive xenograft model of glioblastoma. *PLoS One* **9**, (2014).
107. Choi, B. D. *et al.* CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. *J. Immunother. Cancer* **7**, 304 (2019).
108. Wheeler, S. E. *et al.* Challenges in EGFRvIII detection in head and neck squamous cell carcinoma. *PLoS One* **10**, (2015).
109. Jakobsen, M. K. & Gjerstorff, M. F. CAR T-Cell Cancer Therapy Targeting Surface Cancer/Testis Antigens. *Front. Immunol.* **11**, 1568 (2020).
110. Atanackovic, D. *et al.* Expression of cancer-testis antigens as possible targets for antigen-specific immunotherapy in head and neck squamous cell carcinoma. *Cancer Biol. Ther.* **5**, 1218–1225 (2006).
111. Concu, R. & Cordeiro, M. N. D. S. Cetuximab and the Head and Neck Squamous Cell Cancer. *Curr. Top. Med. Chem.* **18**, 192–198 (2018).
112. Spiegelberg, D. & Nilvebrant, J. CD44v6-Targeted Imaging of Head and Neck Squamous Cell Carcinoma: Antibody-Based Approaches. *Contrast Media Mol. Imaging* **2017**, 2709547 (2017).
113. Mack, B. & Gires, O. CD44s and CD44v6 Expression in Head and Neck Epithelia. *PLoS One* **3**, e3360 (2008).
114. Caruso, H. G. *et al.* Tuning sensitivity of CAR to EGFR density limits recognition of normal tissue while maintaining potent antitumor activity. *Cancer Res.* **75**, 3505–3518 (2015).
115. Ocvirk, J. & Cencelj, S. Management of cutaneous side-effects of cetuximab therapy in patients with metastatic colorectal cancer. *Journal of the European Academy of Dermatology and Venereology* **24**, 453–459 (2010).
116. Börjesson, P. K. E. *et al.* Radioimmunodetection and radioimmunotherapy of head and neck cancer. *Oral Oncology* **40**, 761–772 (2004).
117. Jayaraman, J. *et al.* CAR-T design: Elements and their synergistic function. *EBioMedicine* **58**, (2020).
118. Lynn, R. C. *et al.* High-affinity FRβ-specific CAR T cells eradicate AML and normal myeloid lineage without HSC toxicity. *Leukemia* **30**, 1355–1364 (2016).
119. Richman, S. A. *et al.* High-Affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. *Cancer Immunol. Res.* **6**, 36–46 (2018).
120. Kloss, C. C., Condomines, M., Cartellieri, M., Bachmann, M. & Sadelain, M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat. Biotechnol.* **31**, 71–75 (2013).
121. Wilkie, S. *et al.* Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J. Clin. Immunol.* **32**, 1059–1070 (2012).
122. Sun, S., Hao, H., Yang, G., Zhang, Y. & Fu, Y. Immunotherapy with CAR-modified T cells: Toxicities and overcoming strategies. *Journal of Immunology Research* **2018**, (2018).
123. Di Stasi, A. *et al.* Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy. *N. Engl. J. Med.* **365**, 1673–1683 (2011).
124. Ciceri, F. *et al.* Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol.* **10**, 489–500 (2009).
125. Kalinin, R. S. *et al.* Molecular approaches to safe and controlled engineered T-cell therapy. *Acta Naturae* **10**, 16–23 (2018).

126. Caulier, B., Enserink, J. M. & Wälchli, S. Pharmacologic control of car t cells. *International Journal of Molecular Sciences* **22**, 4320 (2021).
127. Gargett, T. & Brown, M. P. The inducible caspase-9 suicide gene system as a 'safety switch' to limit on-target, off-tumor toxicities of chimeric antigen receptor T-cells. *Front. Pharmacol.* **5**, 235 (2014).
128. Sakemura, R. *et al.* A Tet-On Inducible System for Controlling CD19-Chimeric Antigen Receptor Expression upon Drug Administration. *Cancer Immunol. Res.* **4**, 658–68 (2016).
129. Zhang, R.-Y. *et al.* Doxycycline Inducible Chimeric Antigen Receptor T Cells Targeting CD147 for Hepatocellular Carcinoma Therapy. *Front. Cell Dev. Biol.* **7**, 233 (2019).
130. Bonifant, C. L., Jackson, H. J., Brentjens, R. J. & Curran, K. J. Toxicity and management in CAR T-cell therapy. *Molecular Therapy - Oncolytics* **3**, 16011 (2016).
131. Larcombe-Young, D., Papa, S. & Maher, J. PanErbB-targeted CAR T-cell immunotherapy of head and neck cancer. *Expert Opin. Biol. Ther.* **20**, 965–970 (2020).
132. Tchou, J. *et al.* Safety and efficacy of intratumoral injections of chimeric antigen receptor (CAR) T cells in metastatic breast cancer. *Cancer Immunol. Res.* **5**, 1152–1161 (2017).
133. Agliardi, G. *et al.* Intratumoral IL-12 delivery empowers CAR-T cell immunotherapy in a pre-clinical model of glioblastoma. *Nat. Commun.* **12**, 1–11 (2021).
134. Alwan, L. M. *et al.* Comparison of acute toxicity and mortality after two different dosing regimens of high-dose interleukin-2 for patients with metastatic melanoma. *Target. Oncol.* **9**, 63–71 (2014).
135. Jin, J., Cheng, J., Huang, M., Luo, H. & Zhou, J. Fueling chimeric antigen receptor T cells with cytokines. *Am. J. Cancer Res.* **10**, 4038–4055 (2020).
136. Sanz-Ortega, L. *et al.* T cells loaded with magnetic nanoparticles are retained in peripheral lymph nodes by the application of a magnetic field. *J. Nanobiotechnology* **17**, 14 (2019).
137. Slovak, M. L. *et al.* Tumourigenic multidrug-resistant HT1080 cells do not overexpress receptors for epidermal growth factor. *Br. J. Cancer* **64**, 296–298 (1991).
138. Kriegs, M. *et al.* Analyzing expression and phosphorylation of the EGF receptor in HNSCC. *Sci. Rep.* **9**, 1–8 (2019).
139. Rehmani, H. S. & Issaeva, N. EGFR in head and neck squamous cell carcinoma: exploring possibilities of novel drug combinations. *Ann. Transl. Med.* **8**, 813–813 (2020).
140. Sheu, J. J. C. *et al.* Functional genomic analysis identified epidermal growth factor receptor activation as the most common genetic event in oral squamous cell carcinoma. *Cancer Res.* **69**, 2568–2576 (2009).
141. Gröbe, A. *et al.* Immunohistochemical and FISH analysis of EGFR and its prognostic value in patients with oral squamous cell carcinoma. *J. Oral Pathol. Med.* **43**, 205–210 (2014).
142. Salmi, M. *et al.* Regulated expression of exon v6 containing isoforms of CD44 in man: Downregulation during malignant transformation of tumors of squamocellular origin. *J. Cell Biol.* **122**, 431–442 (1993).
143. Soukka, T. *et al.* Regulation of CD44v6-containing Isoforms during Proliferation of Normal and Malignant Epithelial Cells. *Cancer Res.* **57**, (1997).
144. Odenthal, J. *et al.* Targeting CD44v6 for fluorescence-guided surgery in head and neck squamous cell carcinoma. *Sci Rep.* **11**, 10467 (2018).
145. Economopoulou, P., Kotsantis, I. & Psyrri, A. Tumor Microenvironment and Immunotherapy Response in Head and Neck Cancer. *Cancers (Basel)*. **12**, 3377 (2020).
146. Rodriguez-Garcia, A., Palazon, A., Noguera-Ortega, E., Powell, D. J. & Guedan, S. CAR-T Cells Hit the Tumor Microenvironment: Strategies to Overcome Tumor Escape. *Frontiers in Immunology* **11**, 1109 (2020).
147. Mack, B. & Gires, O. CD44s and CD44v6 expression in head and neck epithelia. *PLoS One* **3**, e3360 (2008).
148. Canning, M. *et al.* Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. *Frontiers in Cell and Developmental Biology* **9**, 52 (2019).
149. Rafiq, S., Hackett, C. S. & Brentjens, R. J. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nature Reviews Clinical Oncology* **17**, 147–167 (2020).
150. Cho, J. H., Collins, J. J. & Wong, W. W. Universal Chimeric Antigen Receptors for Multiplexed and Logical Control of T

Cell Responses. *Cell* **173**, 1426-1438.e11 (2018).
151. Chen, Y. Y. Increasing T Cell Versatility with SUPRA CARs. *Cell* **173**, 1316–1317 (2018).
152. Bachmann, M. The UniCAR system: A modular CAR T cell approach to improve the safety of CAR T cells. *Immunology Letters* **211**, 13–22 (2019).
153. Chen, N., Li, X., Chintala, N. K., Tano, Z. E. & Adusumilli, P. S. Driving CARs on the uneven road of antigen heterogeneity in solid tumors. *Current Opinion in Immunology* **51**, 103–110 (2018).
154. Feng, K. chao *et al.* Cocktail treatment with EGFR-specific and CD133-specific chimeric antigen receptor-modified T cells in a patient with advanced cholangiocarcinoma. *J. Hematol. Oncol.* **10**, 1–11 (2017).
155. Genßler, S. *et al.* Dual targeting of glioblastoma with chimeric antigen receptor-engineered natural killer cells overcomes heterogeneity of target antigen expression and enhances antitumor activity and survival. *Oncoimmunology* **5**, e1119354 (2015).
156. Majzner, R. G. & Mackall, C. L. Tumor antigen escape from car t-cell therapy. *Cancer Discovery* **8**, 1219–26 (2018).
157. Wei, J., Han, X., Bo, J. & Han, W. Target selection for CAR-T therapy. *Journal of Hematology and Oncology* **12**, 1–9 (2019).
158. Land, C. A., Musich, P. R., Haydar, D., Krenciute, G. & Xie, Q. Chimeric antigen receptor T-cell therapy in glioblastoma: charging the T cells to fight. *Journal of Translational Medicine* **18**, 428 (2020).
159. Ma, S. *et al.* Current progress in car-t cell therapy for solid tumors. *International Journal of Biological Sciences* **15**, 2548–2560 (2019).
160. Zhang, Y. *et al.* Phase I clinical trial of EGFR-specific CAR-T cells generated by the piggyBac transposon system in advanced relapsed/refractory non-small cell lung cancer patients. *J. Cancer Res. Clin. Oncol.* **147**, 3725–3734 (2021).
161. Liu, Y. *et al.* Anti-EGFR chimeric antigen receptor-modified T cells in metastatic pancreatic carcinoma: A phase I clinical trial. *Cytotherapy* **22**, 573–580 (2020).
162. Porcellini, S. *et al.* CAR T Cells Redirected to CD44v6 Control Tumor Growth in Lung and Ovary Adenocarcinoma Bearing Mice. *Front. Immunol.* **11**, 99 (2020).
163. Perica, K., Varela, J. C., Oelke, M. & Schneck, J. Adoptive T Cell Immunotherapy For Cancer. *Rambam Maimonides Med. J.* **6**, e0004 (2015).
164. Sadelain, M., Rivière, I. & Riddell, S. Therapeutic T cell engineering. *Nature* **545**, 423–431 (2017).
165. Chabannon, C. *et al.* CAR-T cells: The narrow path between hope and bankruptcy? *Bone Marrow Transplantation* **52**, 1588–1589 (2017).
166. Wang, X. & Rivière, I. Clinical manufacturing of CAR T cells: Foundation of a promising therapy. *Molecular Therapy - Oncolytics* **3**, 16015 (2016).
167. Canté-Barrett, K. *et al.* Lentiviral gene transfer into human and murine hematopoietic stem cells: size matters. *BMC Res. Notes* **9**, 312 (2016).
168. Fujiwara, K., Masutani, M., Tachibana, M. & Okada, N. Impact of scFv structure in chimeric antigen receptor on receptor expression efficiency and antigen recognition properties. *Biochem. Biophys. Res. Commun.* **527**, 350–357 (2020).
169. Picanço-Castro, V. *et al.* Establishment of a simple and efficient platform for car-t cell generation and expansion: from lentiviral production to in vivo studies. *Hematol. Transfus. Cell Ther.* **42**, 150–158 (2020).
170. Raes, L., De Smedt, S. C., Raemdonck, K. & Braeckmans, K. Non-viral transfection technologies for next-generation therapeutic T cell engineering. *Biotechnology Advances* **49**, 107760 (2021).
171. Prommersberger, S. *et al.* CARAMBA: a first-in-human clinical trial with SLAMF7 CAR-T cells prepared by virus-free Sleeping Beauty gene transfer to treat multiple myeloma. *Gene Ther.* **28**, 560–571 (2021).
172. Hudecek, M. *et al.* The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer Immunol. Res.* **3**, 125–135 (2015).
173. Guedan, S., Calderon, H., Posey, A. D. & Maus, M. V. Engineering and Design of Chimeric Antigen Receptors. *Mol. Ther. - Methods Clin. Dev.* **12**, 145–156 (2019).
174. Turtle, C. J. *et al.* CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J. Clin. Invest.* **126**, 2123–2138 (2016).
175. Liu, L. *et al.* Inclusion of Strep-tag II in design of antigen receptors for T-cell immunotherapy. *Nat. Biotechnol.* **34**, 430–434 (2016).

176. Fujiwara, K. *et al.* Hinge and Transmembrane Domains of Chimeric Antigen Receptor Regulate Receptor Expression and Signaling Threshold. *Cells* **9**, 1182 (2020).
177. Qin, L. *et al.* Incorporation of a hinge domain improves the expansion of chimeric antigen receptor T cells. *J. Hematol. Oncol.* **10**, 68 (2017).
178. Mccomb, S. *et al.* Fine Molecular Tuning of Chimeric Antigen Receptors through Hinge Length Optimization. *bioRxiv* (2020).
179. Depil, S., Duchateau, P., Grupp, S. A., Mufti, G. & Poirot, L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nature Reviews Drug Discovery* **19**, 185–199 (2020).
180. Eyquem, J. *et al.* Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543**, 113–117 (2017).
181. Sommer, C. *et al.* Preclinical Evaluation of Allogeneic CAR T Cells Targeting BCMA for the Treatment of Multiple Myeloma. *Mol. Ther.* **27**, 1126–1138 (2019).
182. Provasi, E. *et al.* Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat. Med.* **18**, 807–815 (2012).
183. Xie, G. *et al.* CAR-NK cells: A promising cellular immunotherapy for cancer. *EBioMedicine* **59**, 102975 (2020).
184. Siegler, E. L., Zhu, Y., Wang, P. & Yang, L. Off-the-Shelf CAR-NK Cells for Cancer Immunotherapy. *Cell Stem Cell* **23**, 160–161 (2018).
185. Marofi, F. *et al.* Renaissance of armored immune effector cells, CAR-NK cells, brings the higher hope for successful cancer therapy. *Stem Cell Research and Therapy* **12**, 1–21 (2021).
186. Lamers, C. H. J., Klaver, Y., Gratama, J. W., Sleijfer, S. & Debets, R. Treatment of metastatic renal cell carcinoma (mRCC) with CAIX CAR-engineered T-cells - A completed study overview. *Biochem. Soc. Trans.* **44**, 951–959 (2016).
187. Park, J. R. *et al.* Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol. Ther.* **15**, 825–833 (2007).
188. Kershaw, M. H. *et al.* A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin. Cancer Res.* **12**, 6106–6115 (2006).
189. Morgan, R. A. *et al.* Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol. Ther.* **18**, 843–851 (2010).
190. Xu, J. *et al.* Combination therapy: A feasibility strategy for car-t cell therapy in the treatment of solid tumors (review). *Oncology Letters* **16**, 2063–2070 (2018).
191. Zhao, L. & Cao, Y. J. Engineered T Cell Therapy for Cancer in the Clinic. *Frontiers in Immunology* **10**, 2250 (2019).
192. Kershaw, M. H., Devaud, C., John, L. B., Westwood, J. A. & Darcy, P. K. Enhancing immunotherapy using chemotherapy and radiation to modify the tumor microenvironment. *Oncoimmunology* **2**, e25962 (2013).
193. Motyka, B. *et al.* Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell* **103**, 491–500 (2000).
194. Matsumura, S. *et al.* Radiation-Induced CXCL16 Release by Breast Cancer Cells Attracts Effector T Cells. *J. Immunol.* **181**, 3099–3107 (2008).
195. Lugade, A. A. *et al.* Radiation-Induced IFN-γ Production within the Tumor Microenvironment Influences Antitumor Immunity. *J. Immunol.* **180**, 3132–3139 (2008).
196. Ghanizada, M., Jakobsen, K. K., Grønhøj, C. & von Buchwald, C. The effects of checkpoint inhibition on head and neck squamous cell carcinoma: A systematic review. *Oral Oncology* **90**, 67–73 (2019).
197. Hosseinkhani, N. *et al.* Immune checkpoints and car-t cells: The pioneers in future cancer therapies? *International Journal of Molecular Sciences* **21**, 1–28 (2020).
198. Ramello, M. C., Haura, E. B. & Abate-Daga, D. CAR-T cells and combination therapies: What's next in the immunotherapy revolution? *Pharmacol. Res.* **129**, 194–203 (2018).
199. Bocca, P. *et al.* Bevacizumab-mediated tumor vasculature remodelling improves tumor infiltration and antitumor efficacy of GD2-CAR T cells in a human neuroblastoma preclinical model. *Oncoimmunology* **7**, (2018).
200. Brunelle, J. K. & Letai, A. Control of mitochondrial apoptosis by the Bcl-2 family. *J. Cell Sci.* **122**, 437–441 (2009).
201. Fulda, S. & Debatin, K. M. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* **25**, 4798–4811 (2006).

202. Singh, N. *et al.* Impaired death receptor signaling in leukemia causes antigen-independent resistance by inducing CAR T-cell dysfunction. *Cancer Discov.* **10**, 552–567 (2020).
203. Lei, X. Y. *et al.* Knockdown of human bid gene expression enhances survival of CD8+ T cells. *Immunol. Lett.* **122**, 30–36 (2009).
204. Jafarzadeh, L., Masoumi, E., Fallah-Mehrjardi, K., Mirzaei, H. R. & Hadjati, J. Prolonged Persistence of Chimeric Antigen Receptor (CAR) T Cell in Adoptive Cancer Immunotherapy: Challenges and Ways Forward. *Frontiers in Immunology* **11**, 702 (2020).
205. Bogenberger, J. M. *et al.* BCL-2 family proteins as 5-Azacytidine-sensitizing targets and determinants of response in myeloid malignancies. *Leukemia* **28**, 1657–1665 (2014).
206. Trisciuoglio, D. *et al.* BCL-XL overexpression promotes tumor progression-associated properties article. *Cell Death Dis.* **8**, 1–15 (2017).
207. Zhao, B. *et al.* Cooperation between Molecular Targets of Costimulation in Promoting T Cell Persistence and Tumor Regression. *J. Immunol.* **182**, 6744–6752 (2009).
208. Fan, J., Das, J. K., Xiong, X., Chen, H. & Song, J. Development of CAR-T Cell Persistence in Adoptive Immunotherapy of Solid Tumors. *Front. Oncol.* **10**, 2920 (2021).
209. Eaton, D., Gilham, D. E., O°Fneill, A. & Hawkins, R. E. Retroviral transduction of human peripheral blood lymphocytes with bcl-XL promotes in vitro lymphocyte survival in pro-apoptotic conditions. *Gene Ther.* **9**, 527–535 (2002).
210. Chiappinelli, K. B., Zahnow, C. A., Ahuja, N. & Bylin, S. B. Combining epigenetic and immunotherapy to combat cancer. *Cancer Research* **76**, 1683–1689 (2016).
211. Li, S. *et al.* Decitabine enhances cytotoxic effect of T cells with an anti-CD19 chimeric antigen receptor in treatment of lymphoma. *Onco. Targets. Ther.* **Volume 12**, 5627–5638 (2019).
212. Akbari, B. *et al.* Epigenetic strategies to boost CAR T cell therapy. *Molecular Therapy* **29**, 2640–2659 (2021).
213. You, L. *et al.* Decitabine-Mediated Epigenetic Reprograming Enhances Anti-leukemia Efficacy of CD123-Targeted Chimeric Antigen Receptor T-Cells. *Front. Immunol.* **11**, 1787 (2020).
214. Peng, D. *et al.* Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* **527**, 249–253 (2015).
215. Dangaj, D. *et al.* Cooperation between Constitutive and Inducible Chemokines Enables T Cell Engraftment and Immune Attack in Solid Tumors. *Cancer Cell* **35**, 885-900.e10 (2019).
216. Karlsson, S. C. H. *et al.* Combining CAR T cells and the Bcl-2 family apoptosis inhibitor ABT-737 for treating B-cell malignancy. *Cancer Gene Ther.* **20**, 386–393 (2013).
217. Yang, M. *et al.* The Effect of Apoptosis Inhibitor Blockade Agents on the Third Generation CD19 CAR T Cells. *Blood* **134**, 5620–5620 (2019).
218. Ferris, R. L. *et al.* Rationale for combination of therapeutic antibodies targeting tumor cells and immune checkpoint receptors: Harnessing innate and adaptive immunity through IgG1 isotype immune effector stimulation. *Cancer Treatment Reviews* **63**, 48–60 (2018).
219. Whiteside, T. L. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* **27**, 5904–5912 (2008).
220. Mirzaei, H. R., Rodriguez, A., Shepphird, J., Brown, C. E. & Badie, B. Chimeric antigen receptors T cell therapy in solid tumor: Challenges and clinical applications. *Front. Immunol.* **8**, 1850 (2017).
221. Tse, G. M. *et al.* Strong immunohistochemical expression of vascular endothelial growth factor predicts overall survival in head and neck squamous cell carcinoma. *Ann. Surg. Oncol.* **14**, 3558–3565 (2007).
222. Akbari, P., Huijbers, E. J. M., Themeli, M., Griffioen, A. W. & van Beijnum, J. R. The tumor vasculature an attractive CAR T cell target in solid tumors. *Angiogenesis* **22**, 473–475 (2019).
223. Caruana, I. *et al.* Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. *Nat. Med.* **21**, 524–529 (2015).
224. Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S. & Scott, O. C. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br. J. Radiol.* **26**, 638–648 (1953).
225. Augustin, R. C., Delgoffe, G. M. & Najjar, Y. G. Characteristics of the tumor microenvironment that influence immune cell functions: Hypoxia, oxidative stress, metabolic alterations. *Cancers (Basel).* **12**, 1–17 (2020).

226. Rodriguez, C. P. *et al.* A phase II trial of pembrolizumab and vorinostat in recurrent metastatic head and neck squamous cell carcinomas and salivary gland cancer. *Clin. Cancer Res.* **26**, 837–845 (2020).
227. Juillerat, A. *et al.* An oxygen sensitive self-decision making engineered CAR T-cell. *Sci. Rep.* **7**, (2017).
228. Brochez, L., Chevolet, I. & Kruse, V. The rationale of indoleamine 2,3-dioxygenase inhibition for cancer therapy. *European Journal of Cancer* **76**, 167–182 (2017).
229. Ninomiya, S. *et al.* Tumor indoleamine 2,3-dioxygenase (IDO) inhibits CD19-CAR T cells and is downregulated by lymphodepleting drugs. *Blood* **125**, 3905–3916 (2015).
230. Geiger, R. *et al.* L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. *Cell* **167**, 829-842.e13 (2016).
231. Vodnala, S. K. *et al.* T cell stemness and dysfunction in tumors are triggered by a common mechanism. *Science (80-.).* **363**, eaau0135 (2019).
232. de la Cruz-López, K. G., Castro-Muñoz, L. J., Reyes-Hernández, D. O., García-Carrancá, A. & Manzo-Merino, J. Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches. *Frontiers in Oncology* **9**, 1143 (2019).
233. Jo, Y., Ali, L. A., Shim, J. A., Lee, B. H. & Hong, C. Innovative CAR-T cell therapy for solid tumor; current duel between CAR-T spear and tumor shield. *Cancers* **12**, 1–21 (2020).
234. Das, M., Zhu, C. & Kuchroo, V. K. Tim-3 and its role in regulating anti-tumor immunity. *Immunological Reviews* **276**, 97–111 (2017).
235. Buchbinder, E. I. & Desai, A. CTLA-4 and PD-1 pathways similarities, differences, and implications of their inhibition. *American Journal of Clinical Oncology: Cancer Clinical Trials* **39**, 98–106 (2016).
236. Guillerey, C. *et al.* TIGIT immune checkpoint blockade restores CD81 T-cell immunity against multiple myeloma. *Blood* **132**, 1689–1694 (2018).
237. Hu, W. *et al.* CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. *Cancer Immunol. Immunother.* **68**, 365–377 (2019).
238. Ren, J. *et al.* Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin. Cancer Res.* **23**, 2255–2266 (2017).
239. Chen, N., Morello, A., Tano, Z. & Adusumilli, P. S. CAR T-cell intrinsic PD-1 checkpoint blockade: A two-in-one approach for solid tumor immunotherapy. *Oncoimmunology* **6**, e1273302 (2017).
240. Rupp, L. J. *et al.* CRISPR/Cas9-mediated PD-1 disruption enhances anti-Tumor efficacy of human chimeric antigen receptor T cells. *Sci. Rep.* **7**, 1–10 (2017).
241. Chong, E. A. *et al.* Sequential Anti-CD19 Directed Chimeric Antigen Receptor Modified T-Cell Therapy (CART19) and PD-1 Blockade with Pembrolizumab in Patients with Relapsed or Refractory B-Cell Non-Hodgkin Lymphomas. *Blood* **132**, 4198–4198 (2018).
242. Stadtmauer, E. A. *et al.* CRISPR-engineered T cells in patients with refractory cancer. *Science (80-.).* **367**, eaba7365 (2020).
243. Liu, X. *et al.* A chimeric switch-receptor targeting PD1 augments the efficacy of second-generation CAR T cells in advanced solid tumors. *Cancer Res.* **76**, 1578–1590 (2016).
244. Huang, M., Deng, J., Gao, L. & Zhou, J. Innovative strategies to advance CAR T cell therapy for solid tumors. *Am. J. Cancer Res.* **10**, 1979–1992 (2020).
245. Sukumaran, S. *et al.* Enhancing the potency and specificity of engineered T cells for cancer treatment. *Cancer Discov.* **8**, 972–987 (2018).
246. Kloss, C. C. *et al.* Dominant-Negative TGF-β Receptor Enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication. *Mol. Ther.* **26**, 1855–1866 (2018).

5. ABKÜRZUNGEN

A

Ac	Histonacetylierung
AKT	Proteinkinase B
AML	Akute myeloische Leukämie
ATP	Adenosintriphosphat

B

BCL2	B-cell lymphoma 2
BFP	Blau-fluoreszierendes Protein
BID	*BH3 Interacting Domain Death Agonist*
bp	Basenpaar

C

CAR	Chimärer Antigenrezeptor
CD	*Cluster of differentiation*
CD44v6	CD44 Variante 6
CDK	Cyklin-abhängige Kinase
CRISPR	*Clustered Regularly Interspaced Short Repeats*
CRS	Zytokin-Freisetzungssyndrom
CTLA-4	*Cytotoxic T-lymphocyte-associated Protein 4*

D

DEC	Decitabin
DMSO	Dimethylsulfoxid
DNA	Desoxyribonukleinsäure
DNMT	DNA-Methyltransferase
DNMTi	DNA-Methyltransferase Inhibitor

E

EBV	Epstein-Barr-Virus
EGFR	*Epidermal Growth Factor Receptor*
EGFRvIII	EGFR Variante III
EMA	Europäische Arzneimittel-Agentur

F

FDA	*Food and Drug Administration*

G

GDP	Guanosindiphosphat
GMP	*Good Manufacturing Practice*
GTP	Guanosintriphosphat
GvHD	Spender-gegen-Wirt-Krankheit

H

HAT	Histonacetyltransferase
HDAC	Histondeacetylasen
HDACi	Histon-Deacetylase Inhibitor
HIF-1α	Hypoxia-induzierbarer Faktor Alpha
HLA	Humanes Leukozytenantigen
HMTs	Histonmethyltransferasen
HNSCC	*Head and neck squamous cell carcinoma*
HPV	Humanes Papillomavirus
HSV-tk 1	Herpes Simplex Virus Typ 1

I

iCaps9	Induzierbare Caspase 9
IFNγ	Interferon-gamma
IgG1	Immunoglobulin G1
IgG4	Immunoglobulin G4
IL	Interleukin
ITAM	Immunrezeptor-Tyrosin-basierten-Aktivierungsmotive

ITM	*Immunosupressive tumour microenvironment*

M

mAB	Monoklonaler Antikörper
MACS	*Magnetic-activated cell sorting*
MAGE	*Melanoma-associated antigen*
MART-1	*Melanoma antigen recognized by T-cells 1*
Me	DNA-Methylierung
MHC	Haupthistokompatibilitätskomplex
MM	Multiples Myelom
mRNA	Boten-Ribonukleinsäure

N

NFAT	*Nuclear Factor of Activated T-Cells*
NGFR	Nervenwachstumsfaktorrezeptor
NK-Zelle	Natürliche Killerzelle
NY-ESO-1	New York esophageal squamous cell carcinoma-1

O

OS	Gesamtüberleben

P

PD-1	*Programmed cell death protein 1*
PD-L1	*Programmed cell death protein ligand 1*

R

ROM	Romidepsin

S

SCC	*Squamous cell carcinoma*
scFv	*Single chain variable fragment*
SUPRA	*Split, universal and programmable*

T

T_{reg}-Zelle	Regulatorische T-Zelle
TAA	Tumorassoziiertes Antigen
TALEN	*Transcription activator-like effector nuclease*
TCGA	*The Cancer Genome Atlas*
TCR	T-Zellrezeptor
Tet	Tetracyclin
TGF-ß	Transformierender Wachstumsfaktor Beta
TIGIT	T-Zell-Immunrezeptor mit Ig- und ITIM-Domänen
TIL	Tumor infiltrierende Lymphozyten
TM	Transmembran
TME	Tumormikromilieu
TNFα	Tumornekrosefaktor-alpha
TP53	Tumorprotein P53
TRUCK	*T-cells redirected for universal cytokine-mediated killing*
TSA	Tumorspezifisches Antigen

U

UICC	*Union for International Cancer Control*

V

VEGF	Vaskulärer endothelialer Faktor
V_H	*Variable region of the heavy chian*
V_L	*Variable region of the light chain*

Z

z.B.	zum Beispiel

6. KONFERENZBEITRÄGE

POSTER PRÄSENTATIONEN

Ibach, T & Rölleke, K & Wiek, C & Bister, A & **Haist, C** & Altvater, B & Rössig, C & Hanenberg, H (2017). A new spacer domain from human CD34 for detection and selection of CARs. European Society of Gene & Cell Therapy XXV Anniversary Congress, Berlin, Germany

Haist, C & Bister, A & Ibach, T & Schulte, E & Scheckenbach, K & Hanenberg, H & Wiek, C (2019). New hinge domains from human CD34 and CD271 allow detection and selection of CAR T-cells recognizing hematopoietic and solid tumor cells for clinical applications. 1st European CAR T-cell Meeting, Paris, France

Bister, A & **Haist, C** & Schulte, E & Scheckenbach, K & Gattermann, N & Wiek, C (2019). Dual antigen targeting to reduce on-target off-tumour toxicities in CAR T-cell therapy for mantle cell lymphoma. 1st European CAR T-cell Meeting, Paris, France

Haist, C & Bister, A & Ibach, T & Schulte, E & Rölleke, K & Scheckenbach, K & Hanenberg, H & Wiek, C (2019). Developing novel CD34 and CD271 spacer domains for enhanced detection and selection of CAR T-cell. Retreat Düsseldorf School of Oncology, Düsseldorf, Germany

Bister, A & **Haist, C** & Hanenberg, H & Garrermann, N & Wiek, C (2019). Dual antigen targeting to reduce *on-target off-tumour* toxicities in CAR T-cell therapy for mantle cell lymphoma. Retreat Düsseldorf School of Oncology, Düsseldorf, Germany

Schulte, E & **Haist, C** & Bister, A & Scheckenbach, K & Hanenberg, H & Wiek, C (2019). CD44v6-targeted CAR T-cell therapy for head and neck squamous cell carcinoma. Retreat Düsseldorf School of Oncology, Düsseldorf, Germany

Haist, C & Schulte, E & Bister, A & Scheckenbach, K & Hanenberg, H & Wiek, C (2019). Specificity and efficacy of second generation CAR T-cells against CD44v6 antigen in primary HNSCC cells. European Society of Gene & Cell Therapy Congress, Barcelona, Spain

Bister, A & **Haist, C** & Schulte, E & Scheckenbach, K & Hanenberg, H & Gattermann, N & Wiek, C (2019). Combining ROR1, CD5 or CD19 targeted chimeric antigen receptors reduces off-tumour toxicity while maintaining lysis against double.positive mantle cell lymphoma cells. European Society of Gene & Cell Therapy Congress, Barcelona, Spain

Schulte, E & Scheckenbach, K & **Haist, C** & Bister, A & Hanenberg, H & Wiek, C (2020). CD44v6-targeted CAR T-cell therapy for head and neck squamous cell carcinoma. Laryngo-Rhino-Otologie. 99. 10.1055/s-0040-1710995.

Scheckenbach, K & Bister, A & Schulte, E & Hanenberg, H & Wiek, C & Wagenmann, M & Schipper, J & **Haist, C** (2020). Development of an EGFR-targeted CAR T-cell immunotherapy in head and neck cancer. Laryngo-Rhino-Otologie. 99. 10.1055/s-0040-1710993.

Haist, C & Bister, A & Schulte, E & Poschinski, Z & Wiek, C & Scheckenbach, K & Hanenberg, H (2021). EGFR- und CD44v6-basierte CAR T-Zell-Therapie für Kopf-Hals-Plattenepithelkarzinome bei Fanconi-Anämie. Gesellschaft für Pädiatrische Onkologie und Hämatologie, Frankfurt am Main, Germany

Grundewald, C M & **Haist, C** & König, C & Petzsch, P & Nößner, E & Wiek, C & Scheckenbach, K & Köhrer, K & Niegisch, G & Hanenberg, H & Hoffmann, M J (2021). Epigenetic priming to enhance T-cell cytotoxicity in bladder cancer. 72 Kongress der deutschen Gesellschaft für Urologen, Stuttgart, Germany

Haist, C & König, C & Bister, A & Wiek, C & Scheckenbach, K & Niegisch, G & Hanenberg, H & Hoffmann & M J Grundewald, C M (2022). Epigenetic priming of solid tumor cells enhances CAR T-cell cytotoxicity. International Symposium on Tumor-Host Interaction in Head and Neck Cancer, Essen, Germany

VORTRÄGE

Poschinski, Z & **Haist, C** & Hamacher, A & Kassack, M & Hanenberg, H & Scheckenbach, K (2021). CAR-T-Zelltherapie als mögliche neue Therapieoption für Cisplatin-resistente Plattenepithelkarzinome. 92. & 91. Online-Jahresversammlung der Deutschen Gesellschaft für Hals-Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie e.V, online

Schulte, E & Scheckenbach, K & Bister, A & Hanenberg, H & Wiek, C & **Haist, C** (2021). CD44v6-basierte CAR-T-Zell-Therapie für Kopf-Hals-Plattenepithelkarzinome. 92. & 91. Online-Jahresversammlung der Deutschen Gesellschaft für Hals-Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie e.V, online

Scheckenbach, K & Bister, A & Schulte, E & Hanenberg, H & Wiek, C & Wagenmann, M & Schipper, J & **Haist, C** (2021). Entwicklung einer Immuntherapie mit gegen EGFR-gerichteten chimären Antigenrezeptor (CAR) T-Zellen bei Kopf-Hals-Karzinomen. 92. & 91. Online-Jahresversammlung der Deutschen Gesellschaft für Hals-Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie e.V, online

Scheckenbach, K & Bister, A & Schulte, E & Hanenberg, H & Wiek, C & Wagenmann, M & Schipper, J & **Haist, C** (2022). EGFR- und CD44v6-based CAR T-cell therapy for head and neck squamous cell carcinoma in Fanconi anemia. International Symposium on Tumor-Host Interaction in Head and Neck Cancer, Essen, Germany