Improving Chimeric Antigen Receptor Design and Therapy for Treatment of Cancer

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„It's the job that's never started as takes longest to finish"

- J. R. R. Tolkien

Für meine Eltern

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Summary

Within the last decade cancer immunotherapy has changed the way cancer is being treated and is nowadays, in addition to surgery, radiotherapy, chemotherapy and molecular targeting therapy, referred to as the 5th pillar of cancer care. Central to this dogmatic shift is chimeric antigen receptor (CAR) T-cell therapy, where the patients T-cells are collected and equipped with CARs before being reinfused into the patient to treat the malignancy. CARs are receptors that enable tumor cell recognition and T-cell activation with subsequent lysis of the tumor cells in a single molecule. Thus, CAR T-cell therapy is a highly personalized therapy, where the patient's own immune cells are employed as the therapeutic agent and where the CAR is adapted to the patient's tumor. The huge success of the therapy culminated ultimately in the clinical approval of six CAR T-cell products for the treatment of B-cell-derived malignancies in the last six years and more are expected in the near future.

Before this form of therapy can be applied more broadly, there are several challenges that need to be overcome. Unfortunately, CAR T-cell therapy is associated with several severe adverse events, which can cause life-threatening symptoms, require intensive care and bring along huge amounts of treatment-associated costs. So far, the success of CAR T-cell therapy for hematological malignances could not be translated to solid tumors, since these lack safe antigens to target, are defined by a high degree of tumor heterogeneity, are for immune cells hard to migrate to and infiltrate into and are characterized by a hostile tumor microenvironment. These factors, make it hard to design CAR T-cell therapy for solid tumors in the first place, but also limit therapy efficacy by impeding T-cell migration and function towards and in the tumor. Additionally, CAR T-cells have to be manufactured individually in a time-consuming and labor-intensive process for every patient, which limits CAR T-cell availability and results in immense therapy costs.

Within this dissertation, some of these issues are tackled to drive CAR therapy forward. The inclusion of novel hinges derived from human CD34 and NGFR, allows to easily detect CAR T-cells via flow cytometry and to enrich them with immunomagnetic reagents before a potential infusion into the patient and thus enables to produce pure and defined CAR T-cell products. In the future this system could be combined with suicide genes, which allow to eliminate CAR T-cells when serious adverse events occur, which is a prerequisite for the development of allogeneic therapies. Importantly, CARs equipped with these hinges were as efficacious *in vitro* as well *in vivo* as CARs that contained hinges from clinically approved constructs.

To improve CAR T-cell therapy for solid tumors, high-affinity CARs against CD44v6 as well as EGFR were developed from clinically approved monoclonal antibodies and afterwards validated regarding efficacy and specificity in various *in vitro* models, where both CARs proved to be highly efficacious and specific for their respective target. Moreover, since malignant cells are often epigenetically dysregulated to withstand apoptosis, solid tumor cell lines were sensitized with epigenetic inhibitors towards CAR T-cell mediated killing.

Lastly, for the development of an allogeneic CAR therapy, NK cells were employed as effector cells. Here, an efficient workflow, including lentiviral transduction with subsequent immunomagnetic enrichment of the transduced cells, was established for the generation of CAR NK cells. Moreover, (CAR) NK cells were modified to express various IL15 constructs to improve NK cell persistence *in vivo*, which proved to be crucial for tumor control.

Taken together, these findings will hopefully help to enable *off-the-shelf* CAR therapies, which reduces treatment cost and time, and to close the gap between the treatment of hematological and solid tumors. Ultimately, both challenges must be overcome in order for CAR therapy to benefit as many patients as possible.

Zusammenfassung

In den letzten zehn Jahren hat die Krebsimmuntherapie die Art und Weise der Krebsbehandlung verändert und wird heute neben Chirurgie, Strahlentherapie, Chemotherapie und molekularer zielgerichteter Therapie als fünfte Säule der Krebsbehandlung bezeichnet. Im Mittelpunkt dieses dogmatischen Wandels steht die chimäre Antigenrezeptor (CAR)-T-Zelltherapie, bei der die T-Zellen des Patienten gesammelt und mit CARs ausgestattet werden, bevor sie dem Patienten zur Behandlung des Krebses reinfundiert werden. CARs sind Rezeptoren, die die Erkennung von Tumorzellen und die Aktivierung von T-Zellen mit anschließender Lyse der Tumorzellen in einem einzigen Molekül ermöglichen. Die CAR-T-Zelltherapie ist demnach eine hochgradig personalisierte Therapie, bei der die eigenen Immunzellen des Patienten als Therapeutikum eingesetzt werden und das CAR an den Tumor des Patienten angepasst wird. Der große Erfolg der Therapie resultierte in der klinischen Zulassung von sechs CAR-T-Zell-Produkten für die Behandlung von B-Zell-Malignomen in den letzten sechs Jahren, und weitere werden in naher Zukunft erwartet.

Bevor diese Therapie jedoch in größerem Umfang eingesetzt werden kann, müssen noch einige Herausforderungen bewältigt werden. Leider ist die CAR-T-Zell-Therapie mit mehreren schwerwiegenden unerwünschten Ereignissen verbunden, welche lebensbedrohliche Symptome hervorrufen können, eine intensivmedizinische Betreuung erfordern und enorme Behandlungskosten verursachen. Bisher konnte der Erfolg der CAR-T-Zell-Therapie bei hämatologischen Malignomen nicht auf solide Tumore übertragen werden, da diese nicht über sichere Zielantigene verfügen, durch ein hohes Maß an Tumorheterogenität definiert sind, für Immunzellen schwer zu erreichen und zu infiltrieren sind und durch eine feindliche Tumormikroumgebung gekennzeichnet sind. Diese Faktoren erschweren nicht nur die Entwicklung einer CAR-T-Zelltherapie für solide Tumore, sondern schränken auch die Wirksamkeit der Therapie ein, da sie die Migration und Funktion der T-Zellen zum und im Tumor behindern. Zusätzlich müssen CAR-T-Zellen für jeden Patienten einzeln in einem zeit- und arbeitsintensiven Prozess hergestellt werden, was die Verfügbarkeit von CAR-T-Zellen einschränkt und zu immensen Therapiekosten führt.

In dieser Dissertation werden einige dieser Probleme angegangen, um die CAR-Therapie voranzutreiben. Die Integration neuartiger *Hinge*-Domänen, die von humanem CD34 und NGFR abgeleitet sind, ermöglicht es, CAR-T-Zellen mittels Durchflusszytometrie leicht zu erkennen und sie vor einer potenziellen Infusion in den Patienten mit immunmagnetischen Reagenzien anzureichern und somit reine und definierte CAR-T-Zellprodukte herzustellen. In Zukunft könnte dieses System mit Suizidgenen kombiniert werden, die es ermöglichen, CAR T-Zellen zu eliminieren, wenn schwerwiegende Nebenwirkungen auftreten, was eine

Voraussetzung für die Entwicklung allogener Therapien ist. Wichtig ist, dass die mit diesen *Hinges* ausgestatteten CARs sowohl *in vitro* als auch *in vivo* genauso wirksam waren wie CARs, die *Hinge*-Domänen von klinisch zugelassenen Konstrukten enthielten.

Zur Verbesserung der CAR-T-Zelltherapie bei soliden Tumoren wurden aus klinisch zugelassenen monoklonalen Antikörpern hochaffine CARs gegen CD44v6 und EGFR entwickelt und anschließend in verschiedenen *in-vitro*-Modellen hinsichtlich ihrer Wirksamkeit und Spezifität validiert. Da Tumorzellen häufig epigenetisch dysreguliert sind, um der Apoptose zu widerstehen, wurden zudem Tumorzelllinien mit epigenetischen Inhibitoren für die CAR-T-Zell-vermittelte Abtötung sensibilisiert.

Schließlich wurden für die Entwicklung einer allogenen CAR-Therapie NK Zellen als Effektorzellen eingesetzt. Hier wurde ein Protokoll, einschließlich lentiviraler Transduktion und immunomagnetischer Anreicherung der transduzierten Zellen, zur effizienten Herstellung von CAR NK Zellen etabliert. Zudem wurden (CAR-) NK-Zellen mit IL-15-Konstrukten modifiziert, um die NK-Zellpersistenz *in vivo* zu verbessern, was sich als entscheidend für die Tumorkontrolle erwies.

Zusammengenommen werden diese Ergebnisse hoffentlich dazu beitragen, *Off-the-Shelf*-CAR-Therapien, zur Reduzierung von Behandlungskosten und -zeit, zu ermöglichen, und die Lücke zwischen der Behandlung von hämatologischen und soliden Tumoren zu schließen. Letztlich müssen beide Herausforderungen überwunden werden, damit die CAR-Therapie möglichst vielen Patienten zugutekommen kann.

1 Introduction

1.1 CAR therapy for hematological malignancies

Leukemias and lymphomas are malignancies of the hematologic system and with about 1.1 million cases per year account for 5.7 % of all cancers and are attributed to 600,000 deaths annually [1]. While leukemias generally arise from immature white blood cells/leukocytes in the bone marrow, lymphomas develop from mature B- or T-cells in lymphoid organs such as the lymph nodes. Depending on the lineage that the leukemia is arising from, there is a general distinction between lymphoid or lymphoblastic and myeloid or myeloblastic leukemias. The acute lymphoblastic leukemia (ALL), the most common pediatric leukemia and 2nd most common adult acute leukemia, is characterized by the predominant presence of lymphoid blasts in the bone marrow and blood, which can lead to bone marrow failure with consequent anemia, thrombocytopenia and immune suppression [2]. Acute myeloid leukemia (AML) blasts arise from myeloid leukemic stem cells in the bone marrow and manifests with similar clinical symptoms. In the pathogenesis of the acute leukemias, consequential somatic hits in immature precursor cells are thought to give rise to uncontrolled proliferation and lacking differentiation in their progeny [3, 4]. Both leukemias are generally treated with chemotherapy as well as stem cell transplantation for high-risk patients and patients that did not respond to or relapsed after previous chemotherapy. For both types of leukemias, remission and survival rates have dramatically improved within the last decades, basically through improved risk group stratification, risk-adapted therapy intensification and strongly improved supportive care. Although promising small molecule inhibitors and also biologics have been tested in clinical trials for both leukemias in the last years, a large number of patients still do not respond to the initial treatment ('refractory') or relapse thereafter, in the following referred to as refractory/relapsed (r/r), novel therapy approaches are direly needed [4, 5, 6]. Here, cellular therapies with genetically modified immune effector cells - especially CAR T-cells - have the potential to still provide curative approaches for a significant proportion of these patients.

T-cells make up an integral part of the adaptive immune system, where they clear the body from infected or transformed cells. They are equipped with T-cell receptors (TCRs), which recognize foreign peptides presented on major histocompatibility (MHC) class I or class II molecules, leading to the elimination of the recognized cell(s). This elimination is mediated via the secretion of lytic molecules such as perforin and granzymes, but also by the upregulation of death receptor ligands, which induce apoptosis when bound to the targeted cell. Importantly, the binding of the TCR to MHC II is supported by CD4, expressed on T helper cells, while CD8, expressed on cytotoxic T-cells, is needed for the binding to MHC I [7, 8]. The TCR itself consist of an α- and a β-chain and aggregates with the

δ/ε- and γ/ε heterodimers as well as ζ-homodimers of the CD3 complex (**Figure 1A**). These are crucial for the signaling of the TCR and subsequent T-cell activation, which is mediated via the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) within CD3δ, CD3ε, CD3γ (one ITAM each) and CD3ζ (three ITAMs per monomer) [9].

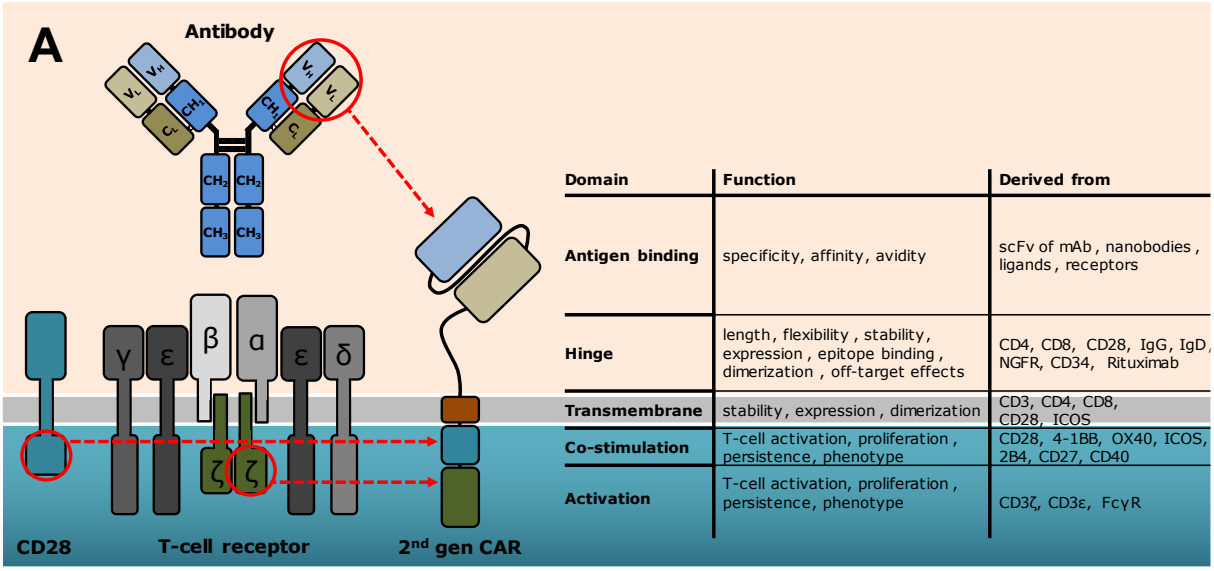
However, before T-cells can use their TCRs to eradicate infected or malignant cells, they initially need to be specifically activated by antigen-presenting cells (APCs) such as macrophages or dendritic cells. Central to this process are three signals the T-cells receives to drive its activation, proliferation and differentiation. Firstly, the APC presents a short peptide on its MHC complex to the TCR of the T-cell, thereby activating the CD3ζ chain. Secondly, the interaction of the surface molecules CD80 or CD86 on the APC with CD28 as co-stimulatory activating receptor on the T-cell delivers crucial co-stimulation. Lastly, cytokines such as IFNγ, IL-2 and IL-6 (pro-inflammatory) or IL-10 (anti-inflammatory) drive proliferation and differentiation or senescence and apoptosis of the T-cell [10, 11].

Tumor manifestation as well as growth is at least partially the result of an impeded immune surveillance that ultimately fails to recognize and eliminate the autologous malignant cells during the growth and development of the tumor [12, 13]. Thus, to redirect T-cells towards tumor cells, chimeric antigen receptors (CARs), which directly link tumor antigen recognition to T-cell activation, have been developed. These receptors are synthetic molecules that extracellularly carry a single chain fragment of the variable region (scFv) – the antigen-binding domain – of a monoclonal antibody [14, 15]. This scFv is linked via a hinge and transmembrane domain to intracellular T-cell activation motifs (Figure 1A). After engagement of the CAR, an immunological synapsis is formed between the T- and its target cell leading to signaling and activation of the T-cell by the cytoplasmic portion of the CAR (Figure 1B), which will be discussed in the next chapter. After activation, the T-cell secretes lytic molecules such as granzyme B and perforin and upregulates FasL, the ligand of the apoptosis-inducing death receptor, FasR, to kill the target cell. Moreover, the secretion of pro-inflammatory and proliferative cytokines modulates the immune response and drive T-cell expansion [16]. Importantly, unlike natural recognition of tumor cells by T-cells, where the TCR binds to an MHC-presented peptide, CARs function without MHC restriction. Consequently, CARs remain functional even if the malignant cells lose MHC expression thereby trying to evade the immune system [17].

1.1.1 CAR design

The **1st generation of CARs** was already described in the 90s and carried only the CD3ζ chain of the TCR as signaling motif [14, 18, 19] (**Figure 2**). While these

cells recognize and eliminate antigen-bearing tumor cells *in vitro*, they failed to induce lasting remissions in patients as proliferation and cytokine production and thus persistence were lacking [20, 21].



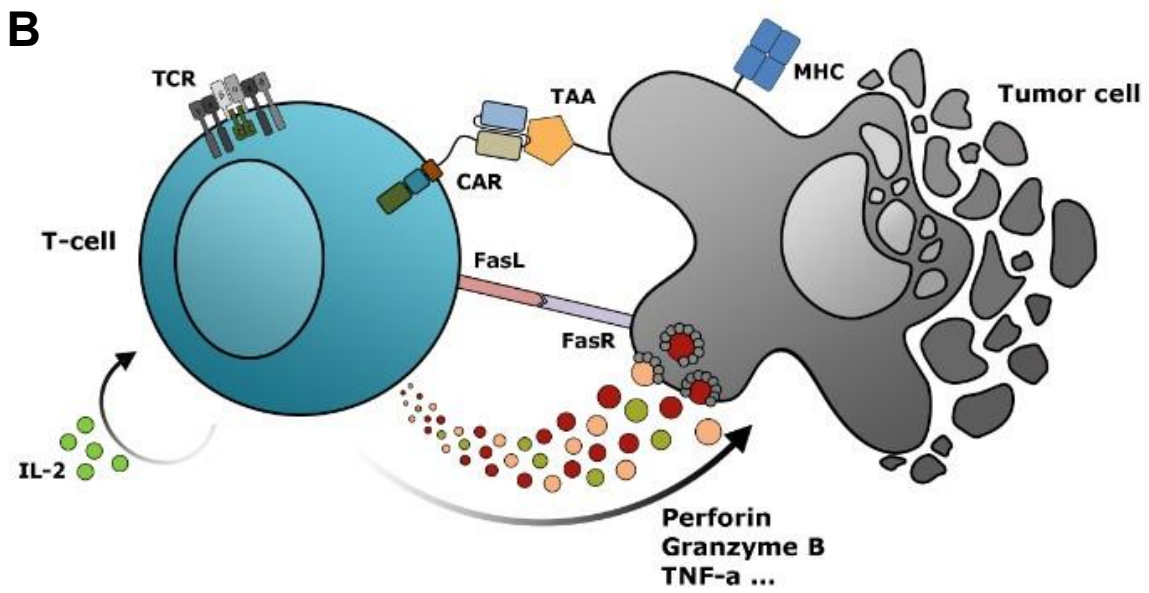


Figure 1: Structure and mechanism of action of CARs. A) CARs are constructed from domains of antibodies as well as T-cell cell surface receptors. **B)** Once the CAR binds a tumor-associated antigen (TAA), the T-cell is activated and induces apoptosis of the tumor cell as well as secretion of proinflammatory cytokines.

Importantly, efficient T-cell activation by APCs normally involves signaling via co-stimulatory molecules such as CD28 or 4-1BB/CD137 to drive its activation, proliferation and cytokine secretion [8]. Consequently, when integrated into the **2nd generation of CARs** (**Figure 2**), the intracellular/cytoplasmic signaling units of co-stimulatory molecules boosted the primary signal from the CD3ζ chain engagement. 2nd generation CAR T-cells are indeed able to control tumor growth not only *in vitro* but also *in vivo*; in addition, numerous clinical trials have demonstrated the effectiveness of 2nd generation CARs in human disease, which

ultimately led to the approval of several CAR therapies by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA; [22, 23]). Here, CD28 and 4-1BB are the most used co-stimulatory domains and cause profound differences on T-cell biology and function. When incorporated in 2nd generation constructs, CD28 signaling induces strong but relatively short-lived T-cell responses with faster exhaustion, while 4-1BB signaling is associated with long-lasting T-cell persistence, but weaker responses [24, 25, 26, 27, 28]. Other frequently used co-stimulatory domains used in preclinical trials are derived from OX40 [29], 2B4 [30], CD27 [31] or ICOS [32]. In attempts to further increase the potential of CARs, additional generation of CARs have been developed (**Figure 2**). While the **3rd generation** includes two instead of only one co-stimulatory signal in combination with the CD3ζ chain [32, 33, 34, 35], the **4th generation**, also known as T-cells redirected for antigen-unrestricted cytokine-initiated killing (**TRUCKs**) induce the expression of other transgenes like IL-7 [36] or IL-18 [37] to further stimulate the immune response. This is mediated via the activation of nuclear factor of activated T-cells (NFAT) through CAR signaling, which binds the minimal IL-2 promoter in these constructs and thus drives the expression of these cytokines. In contrast, the **5th generation** includes intracellular signaling domains of interleukin receptors such as the IL-2 receptor, which signal via Janus kinase/signal transducer and activator of transcription proteins (JAK/STAT) pathways to drive expression of endogenous genes. These CARs showed superior proliferation and tumor control as well as reduced T-cell exhaustion compared to 2nd generation CARs [38]. Apart from CD3ζ, also other primary signaling units have been tested, most notably FcεRIγ [39] and CD3ε [40], however, CD3ζ prevailed as the most commonly used one.

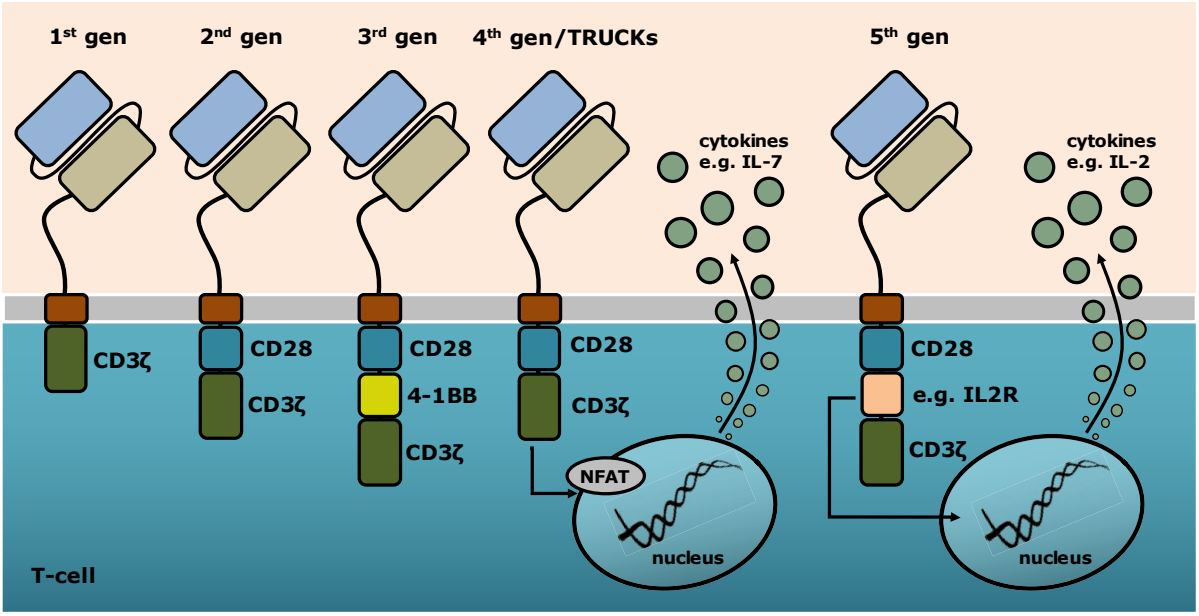


Figure 2: Five generations or CAR T-cells. The 1st generation of CARs solely contained the primary activation signal. In subsequent generations co-stimulatory domains were added. The 4th and 5th generation additionally induce the secretion of additional cytokines.

While the signaling domains are crucial for T-cell activation, the **antigen-binding domain** is – as the name suggests – essential for antigen binding and thus tumor cell recognition. Most commonly, this moiety is derived from the scFv of a monoclonal antibody and provides the antigen specificity for the CAR construct [15], but also ligands [41], cytokines [42] or extracellular domains of receptors [43] have been used as binding moiety. Structurally, the antigen-binding domain is located at the N-terminus of the CAR, where e.g. both the heavy and the light chain of the scFv are joined to one continuous amino acid sequence by a short peptide linker [15]. Here, the best orientation of the variable heavy (V_H) and light chain (V_L) in the constructs (V_H-linker-V_L or V_L-linker-V_H) depends on the monoclonal antibody clone utilized [44]. Besides the target antigen specificity, the affinity of the scFv determines how strongly this antigen is bound. The affinity is determined by the non-covalent bonds that can be formed between the scFv and the antigen and is the result of the association and dissociation equilibrium of the complex [45]. Generally speaking, high-affinity CARs have been shown to confer stronger activation and killing capabilities and are generally more useful for targeting antigens which are expressed at lower levels. However, high affinity scFvs in CARs are also more prone to *off-tumor* toxicities as they also bind healthy tissues with low expression of the antigen in an *on-target off-tumor* reaction; importantly, high affinity CAR constructs can also lead to faster exhaustion of the T-cell [46, 47, 48, 49, 50, 51].

In this dissertation, the antigens CD19, CD5, CD33, CD123 and ROR1 (receptor tyrosine kinase-like orphan receptor 1) are, due to their overexpression on several leukemias and lymphomas, and CD44v6, EGFR (epidermal growth factor receptor) and ErbB2 (erythroblastic oncogene B2), due to their overexpression on solid tumors such as head and neck carcinoma, of special interest as target antigens. **CD19** is a B-cell marker, which is overexpressed on most B-cell derived leukemias or lymphomas. Physiologically it plays a role during B-cell development and is critical for signaling of the B-cell receptor [52]. **CD5** is a pan T-cell marker and plays a role in T-cell development. Although its physiological role is not fully elucidated yet, it appears to negatively regulate T-cell function. Besides T-cells, CD5 is also expressed on a subset of B-cells and on T-cell derived malignancies like T-cell ALL and also on some B-cell cancers such as mantle cell lymphoma (MCL) [53, 54]. **CD33** is a differentiation marker on myeloid cells and is normally expressed on various myeloid cells. Its physiological role is not clearly understood, but evidence suggests that it negatively regulates immune responses. Therapeutically it is an intriguing target, especially for AML, since it is expressed on nearly all cases of AML [55]. **CD123** is the α-chain of the IL-3 receptor and thus plays important roles in the proliferation and differentiation of immature hematopoietic cells in immune homeostasis. Importantly, CD123 is expressed on myeloid as well as lymphoid malignancies, making it an attractive target for immunotherapy [56]. While **ROR1** is highly expressed during embryonic development, expression recedes in adult tissues except for apart from low

expression in adipose tissues [57]. Importantly, ROR1 is temporarily expressed during normal B-cell development, which might explain its expression on MCL and other B-cell malignancies [57, 58, 59]. **CD44v6** is a splice variant of CD44, which functions as hyaluronic acid receptor. While its expression on healthy tissues is restricted to keratinocytes in the skin and mucous membranes as well as some monocytic cells, it is abundantly expressed on cancers of the lung, skin, cervix and head and neck as well as multiple other malignancies including AML [60, 61]. Both **EGFR** as well **ErbB2** belong to the ErbB tyrosine receptor kinase family and are naturally expressed on various tissues including lung and skin. After ligand binding, both receptors stimulate cellular proliferation and survival, which might explain their overexpression on a range of solid malignancies including lung, breast as well as head and neck cancer. Moreover, high expression is associated with therapy resistance as well as metastasis, making them intriguing targets for immunotherapy [62, 63].

The **hinge**, also referred to as **linker** or **spacer**, connects the scFv to the transmembrane domain and thus to intracellular signaling motifs. Although it does not bind the targeted antigen directly, it is still important for robust CAR function. The hinge gives the CAR the flexibility and length that is needed for the scFv to reach its target epitope. Hence, epitopes that are proximal to the membrane or embedded within areas with heavy glycosylation are often better targeted with longer hinges. In contrast, short hinges can be preferable, if the epitope on the target antigen is located distally from the membrane [39, 47, 64, 65, 66, 67, 68]. Moreover, the incorporation of a hinge can improve CAR expression, T-cell expansion and T-cell persistence [69, 70, 71, 72]. While in most cases, the hinge is simply used as a linking motif between scFv and transmembrane domain, the integration of specific peptide or protein sequences confers additional properties to the CAR and the cell. For example, integration of parts of CD20 allows eradication of CAR T-cells by the administration of rituximab, a widely used and clinically approved monoclonal antibody against CD20, as a safety measure [73]. Similarly, integration of nerve factor growth factor receptor (NGFR) sequences enables detection during as well as enrichment of CAR T-cells prior to therapy [74]. In contrast, incorporation of IL-15, a potent pro-inflammatory cytokine, into the hinge region improves CAR T-cell proliferation as well as persistence (patent EP3184546A1).

Lastly, the **transmembrane domain** anchors the CAR in the cell membrane of the cell and provides the link between extracellular and intracellular domains. Frequently used are domains from proteins that are naturally expressed on T-cells such as CD3, CD8 or CD28 to ensure high and stable expression as well as long CAR T-cell persistence *in vivo* [75, 76]. Here, CD8- and CD28-derived transmembrane domains proved to be superior to CD3-derived ones regarding CAR expression and effector functions [75, 76, 77]. Notably, the transmembrane domain can lead to the homodimerization of CARs or heterodimerization with the molecules they are

derived from. While this can improve CAR function by trans-signaling of the innate receptor, this can also lead to tonic signaling of the CAR irrespective of antigen binding, which can cause unwanted adverse events in the patient [78, 79, 80, 81].

1.1.2 Clinical treatment with autologous CAR T-cells

During autologous CAR T-cell therapy, the patients white blood cells are collected, followed by activation and expansion of the T-cell population *ex vivo* via the stimulation of TCR and CD28 as well as cytokines such as IL-2 or IL-15 [82, 83]. After introduction of the CAR gene into the autologous activated T-cells, most frequently achieved via retroviral [84, 85, 86, 87, 88] or lentiviral vectors [89, 90, 91, 92, 93], which enable stable transduction of the target cells with high gene transfer efficiency [94], CAR T-cells are expanded, undergo quality controls and are finally re-infused back into the patient (**Figure 3**). Prior to infusion of the autologous CAR T-cell products, the patients are commonly treated with fludarabine and cyclophosphamide as immunosuppressive conditioning, whereby autologous T- and NK cells are largely depleted and homeostatic cytokines induced. If the tumor cells are also sensitized for by the conditioning therapy, this can be an added benefit [90, 92, 95, 96]. Depending on the protocol, typically between 1 x 10^6 to 1 x 10^7 CAR T-cells per kg body weight of the patient are intravenously transferred [85, 88, 89, 96], albeit higher [91, 97] and lower doses [92, 98] have also been used in clinical trials. After successful engraftment, the CAR T-cells ultimately migrate into all tissues of the body where they eliminate antigen-bearing normal and malignant cells. With a memory phenotype that promotes T-cell longevity, the CAR T-cells are considered to be a living drug, that in a high percentage of patients persists for years and can still mediate cancer clearance and keep the patient in remission [15, 91, 99]. Indeed, it has been shown that CAR T-cell products with profound central memory phenotype mediate a greater CAR T-cell persistence and thus therapy success [100, 101]. Hence, the CAR T-cell therapy is a highly personalized treatment with the potential to mediate life-long protection. Interestingly, a recent study demonstrated that 10 years after the first application of CD19-directed CAR T-cells for leukemia patients, some patients remain in remission, probably as their CAR memory T-cells still persist [99].

1.1.3 CD19 fuels CAR T-cell therapy

Clinical breakthrough has been achieved with 2nd generation CARs against hematologic malignancies of the B-cell lineage by targeting CD19. First clinical trials with these CARs for patients with advanced CD19+ leukemias and lymphomas showed great promise with response rates of up to 90 % and the CAR T-cells were detected for several months or even years in the patients [89, 91, 92, 93].

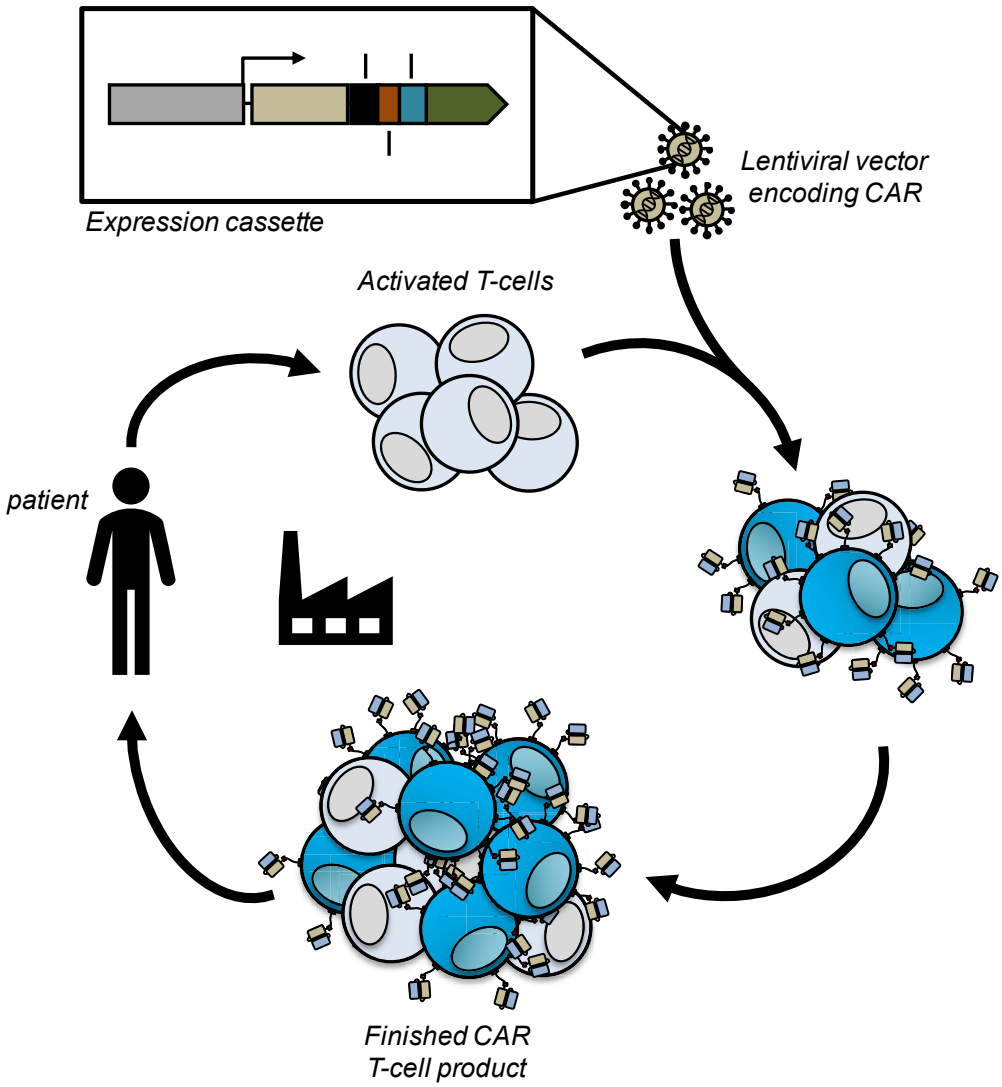


Figure 3: Clinical application of autologous CAR T-cells. T-cells are isolated from a patient and sent to a centralized manufacture site, where they are lentivirally modified to express the CAR gene and expanded to reach treatment-relevant cell numbers. Afterwards, they are sent back to the treatment center, where they are reinfused into the patient.

Within the last six years, six CAR T-cell therapies were clinically approved (**Table 1**). In 2017, after highly successful clinical trials, the first two CD19-targeted CAR T-cell products, namely Tisagenlecleucel/Kymriah by Novartis[1] and Axicabtagene Ciloleucel/Yescarta by Kite/Gilead[2], were approved by the United States FDA for the treatment of r/r diffuse large B-cell lymphoma (DLBCL), r/r B-cell ALL and r/r primary mediastinal B-cell lymphoma (PMBCL). In 2018, market approval for Europe was also given by the EMA [22, 102]. Both CARs utilize the same CD19-recognizing scFv that is derived from the monoclonal antibody clone FMC63 [103]. However, Kymriah is equipped with a 4-1BB co-stimulation along with a CD8-derived hinge and transmembrane domain, while Yescartas hinge, transmembrane and co-stimulatory domains are derived from CD28. In July 2020, Kite/Gilead

[1] https://www.fda.gov/news-events/press-announcements/fda-approval-brings-first-gene-therapy-united-states
[2] https://www.fda.gov/news-events/press-announcements/fda-approves-car-t-cell-therapy-treat-adults-certain-types-large-b-cell-lymphoma

received the approval of Tecartus/brexucabtagene autoleucel also for the treatment of r/r MCL[3]. In February 2021, Juno Therapeutics Inc./Bristol Myers Squibb obtained FDA approval for another CD19-targeted CAR T-cell therapy, Breyanzi/lisocatbagene maraleucel, for the 3rd line treatment of r/r DLBCL[4]. As shown in Table 1, Celgene/Bristol Meyer Squibb received FDA approval in March 2020 for the first B-cell maturation antigen (BCMA)-targeted CAR T-cell therapy, Abecma/idecabtagene vicleucel, for r/r multiple myeloma[5]. Most recently, Janssen were granted FDA approval for their BCMA-targeted CAR T-cell therapy Carvytki/ciltacabtagene autoleucel for the treatment of r/r multiple myeloma[6]. Thus, the field is rapidly gaining traction and more approvals are expected within the upcoming months and years.

Table 1: Summary of clinically approved CAR T-cell products [104].

	Kymriah	Yescarta	Tecartus	Breyanzi	Abecma	Carvykti
Company	Novartis	Kite/Gilead	Kite/Gilead	Juno/BMS	Celgene/BMS	Janssen
Indication	B-ALL, DLBCL	DLBCL, PMBCL	MCL	DLBCL, PBMCL	MM	MM
Gene transfer	Lentiviral	Lentiviral	Lentiviral	Lentiviral	Lentiviral	Lentiviral
Target	CD19	CD19	CD19	CD19	BCMA	BCMA
scFv clone	FMC63	FMC63	FMC63	FMC63	C11D5.3	VHH1/2*
Hinge	CD8	CD28	CD28	IgG4	CD8	CD8
Transmembrane	CD8	CD28	CD28	CD28	CD8	CD8
Co-stimulation	4-1BB	CD28	CD28	4-1BB	4-1BB	4-1BB
Primary signal	CD3ζ	CD3ζ	CD3ζ	CD3ζ	CD3ζ	CD3ζ

B-ALL, B-cell acute lymphoblastic leukemia; BCMA, B-cell maturation antigen; BMS, Bristol Meyer Squibb; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; PMBCL, Primary mediastinal B-cell lymphoma
* uses a nanobody instead of a monoclonal antibody-derived scFv

ZUMA-1 and JULIET are the pivotal clinical trials that led to the rapid approval of Yescarta and Kymriah, respectively. In ZUMA-1 [105, 106], 101 heavily-pretreated r/r DLBCL patients with a median age of 58 years were treated with Yescarta, after having failed multiple lines of therapy including autologous stem-cell transplantation and/or CD20-targeted antibody immunotherapy. Initially, 83 % of patients responded to the treatment and 58 % showed a complete response. However, 95 % of patients suffered severe adverse events including cytokine release syndrome and neurological toxicities and two patients died due to treatment-related complications. In JULIET [90], 93 patients were treated with Kymriah after they have failed first and second line therapies or relapsed after a stem cell transplant for DLBCL. Here initially, 52 % of the treated patients showed

[3] https://www.fda.gov/news-events/press-announcements/fda-approves-first-cell-based-gene-therapy-adult-patients-relapsed-or-refractory-mcl
[4] https://www.fda.gov/news-events/press-announcements/fda-approves-new-treatment-adults-relapsed-or-refractory-large-b-cell-lymphoma
[5] https://www.fda.gov/news-events/press-announcements/fda-approves-first-cell-based-gene-therapy-adult-patients-multiple-myeloma
[6] https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-ciltacabtagene-autoleucel-relapsed-or-refractory-multiple-myeloma

a response, including 40 % complete responses. Every treated patient had adverse events associated with the disease and 85 % experienced severe adverse events. In a similar study for pediatric B-cell lymphoblastic leukemia (ELIANA; [107]), Kymriah was administered to 75 children and young adults with an 81 % remission rate at 3 months after treatment. Overall survival was 90 % and 76 % at 6 and 12 months, respectively. Also here, every patient suffered from adverse events and 88 % of the patients experienced grade III/IV severe side effects. Importantly, the response rates in the clinical studies of Kymriah and Yescarta carried over to the real-world, as 59.6 % of patients with r/r DLBCL [108] and 88 % of patients with r/r ALL [109] responded to Kymriah and 82 % of r/r DLBCL patients responded after Yescarta treatment [110].

1.2 Clinical challenges of CAR T-cell therapy

1.2.1 Toxicities and adverse events

In general, infusion of *in vitro* expanded autologous T-cells transduced with a large variety of CAR constructs was not associated with any autoimmunity of the re-infused T-cells, despite the presence of the endogenous TCRs on the CAR T-cells. Nonetheless, CAR T-cell therapy is associated with severe/profound toxicities: Cytokine release syndrome (CRS), neurotoxicity and reactivity against non-malignant cells (*on-target off-tumor* cross-reactions).

The **cytokine release syndrome** is the most common CAR-associated toxicity and generally describes a rapid inflammatory response that is caused by the massive parallel CAR T-cell activation following infusion. Mechanistically, the CAR T-cells react against target antigen-positive cells and produce, as do other cells that are recruited by the CAR T-cells, high levels of cytokines, most notably IL-6, TNF-α, IFN-γ, IL-10 and IL-2 [111]. Therefore, patients after infusion of the CAR T-cell products frequently experience a wide spectrum of symptoms, from relatively mild symptoms like fever, malaise and fatigue to severe organ toxicities like cardiac dysfunction and hypertension and also be potentially life-threatening liver and kidney failure [112]. Here, the severity, duration and onset of CRS can vary from patient to patient, but there seems to be a direct connection to disease burden prior lymphodepletion and the CAR T-cell dose [113]. Moreover, the CAR design seems to have a role in the development and course of CRS. Yescarta, which harbors a CD28 co-stimulation, shows a median time of two days (range 1 to 12 days) for the onset of CRS [88], while the 4-1BB-equipped Kymriah shows a median time of three days (range 1 to 22 days) for the onset of CRS [107]. These findings support the hypothesis that CD28 induces very strong initial responses, while CARs equipped with 4-1BB induce weaker and more long-lived T-cell reactions [114]. Therapeutically, the treatment of CRS is a balancing act between reducing CRS-mediated symptoms and upholding the anti-tumor effect of the CAR T-cells. Corticosteroids can be used to reduce systemic inflammation and thus resolve CRS, however, the use in CAR T-cell therapy is controversial. While some

groups reported that corticosteroid treatment can dampen CAR T-cell proliferation and thus therapy efficacy [115], others have shown that glucocorticoids do not impede the effects of the CAR T-cells [116]. Alternatively, inhibition of the IL-6 receptor with tocilizumab, a clinically approved immunosuppressive monoclonal antibody, has proven to be very effective at reducing CRS symptoms without hampering CAR T-cell activity [115, 117]. Thus, IL-6 receptor blockade is currently the gold standard of treatment of (severe) CRS. Most CRS-related symptoms resolve within the two weeks following CAR T-cell administration [112].

Neurotoxicities are the 2nd most common adverse event during CAR T-cell therapy. As for CRS, the symptoms and severity can vary greatly from patient to patient. Mild symptoms include general headaches and mild dizziness, while very severe cases suffer from seizures and encephalopathies [112]. Less frequently, neurotoxic complications lead to the death of the patient [118, 119]. Neurotoxicities usually occur after CRS, suggesting that the immune activation with subsequent cytokine secretion during CRS plays a role in the onset of neurological toxicities [120, 121, 122]. Recently, it became evident that CD19 is also expressed on mural cells, which line endothelial cells of the blood-brain-barrier and thus are integral to its barrier function in the brain [123]. Upon lysis of these cells by CD19 CAR T-cells, the blood-brain-barrier is compromised, thereby leading to a leakage of high levels of cytokines from the blood into the cerebrospinal fluid with activation of endothelial cells [119]. Importantly, this neurotoxicity of the treatment with CD19 CAR T-cells could be reproduced in a murine system [123] and similar effects were also observed with bispecific T-cell engagers, which link CD19 and CD3, thereby crosslinking tumor and T-cells [124]. However, since neurotoxicities also occur when other B-cell antigens on malignant cells such as CD22 or BCMA are targeted [125, 126], toxicity against CD19+ mural cells is not sufficient to fully explain the mechanisms leading to neurotoxicities.

On-target off-tumor **toxicities** might be the most serious concern during CAR T-cell therapy but often can be anticipated as the expression profiles for the target antigens on normal cells are generally known. Most antigens are not truly tumor-specific but rather tumor-associated, as they are also expressed to some extent on healthy tissues. Consequently, treatment with CAR T-cells not only leads to clearance of malignant cells, but also healthy cells that express the antigen. Here, CD19 is the most prominent example, as CD19 is expressed on all B-cells and hence, CD19 CAR T-cell therapy leads to B-cell aplasia and consequently hypogammaglobulinemia, which can partially be treated with immunoglobulin infusions [127]. Thus, while CD19 CAR-mediated *on-target off-tumor* toxicities can be managed, targeting other antigens with CARs can cause more severe outcomes. EGFR and ErbB2 are frequently overexpressed on various solid tumors and thus are considered promising CAR targets. However, EGFR- and ErbB2-redirected CAR T-cells commonly cause toxicities of the skin or lung distress, due to the expression of these antigens on cells in skin and lung tissue [128, 129]. More dramatically,

targeting EGFR with the scFv of the commonly used high-affinity EGFR antibody, cetuximab, as targeting moiety in the CAR, resulted in the death of a colon cancer patient [130]. Similarly, also ErbB2-targeted CAR T-cells caused the death of a patient with colon carcinoma [131]. In both cases, the expression of EGFR and ErbB2, respectively, caused severe lung distress and pulmonary failure, which was thought to be responsible for the death of the patients.

Off-target off-tumor **reactions** can be caused by scFv-unrelated reactions of the CAR constructs with other antigens and tissues. In the past, hinges that consist of the second and third constant heavy domain (CH_2CH_3) of human IgG1, IgG2 or IgG4 have widely been used in preclinical studies. These hinges lead to efficient receptor dimerization and increased expression of the CAR. Moreover, they provide the necessary length and flexibility for a broad band of CARs and hence can be used in CARs directed against a variety of target antigens. However, some of these hinges also bind Fc receptors (FcRs) on other immune cells such as NK cell or macrophages/monocytes, as they are derived from the FcR-binding fragment of antibodies. Consequently, CAR T-cells equipped with these hinges bind, activate and ultimately eliminate FcR-bearing cells, which can be abolished by mutating amino acids that are crucial for FcR-binding [132]. *In vivo* studies clearly demonstrated that CH_2CH_3-hinged CARs were ineffective to clear the malignant cells in immunodeficient mouse models - in contrast to hinges with missing or mutated CH_3 motifs [68, 72]. Here, CAR T-cells sequestered in the lungs of the mice, where they interacted with macrophages and hence were not able to control tumor progression [68].

1.2.2 CAR T-cell therapy for solid tumors

In recent years, CAR T-cell therapy has also been employed as an experimental last line treatment for several solid malignancies including glioblastoma, head and neck cancer, breast cancer, lung cancer and colon cancer [133]. With just over 18 million new cases and about 9.3 million cancer-related deaths, solid cancers make up the vast majority of cancer patients and cancer-related deaths. Here, head and neck squamous cell carcinoma, which are a focus of this dissertation, account for about 700,000 new cases with a mortality rate of up to 50 % [134]. However, while CAR T-cell therapy has been quite the success story for hematological cancers, the translation to solid malignancies is lagging far behind for several reasons [15, 135, 136]. The first obstacle is the absence of tumor-specific and homogenously expressed antigens. Solid cancer CAR therapy therefore must rely on the expression of antigens that are relatively safe to target; however, the most prominent CAR targets for e.g. head and neck and bladder cancers include EGFR and ErbB2, which are also expressed on lung, gastrointestinal, skin and other epithelia and thus can cause severe toxicities in these tissues after CAR T-cell infusion [135, 136]. Another major challenge is the lack of trafficking of the CAR T-cells

into the solid tumor. While CAR T-cells can easily traffic within the blood and lymphatic system to target hematological malignancies, they cannot do so in the case of solid tumors, where fibroblasts and the densely packed extracellular matrix (ECM), along with a poor vascularization, presents a physical barrier [135, 136]. Moreover, solid tumors secrete chemokines that prevent migration and infiltration of T-cells towards and into the tumor, as the corresponding receptors are lacking on T-cells [136, 137]. Arguably the biggest barrier that prevents/impedes successful CAR therapy application in solid malignancies is the hostile **tumor microenvironment** (TME), which malignant cells form to promote their growth and mediate immune escape. On the cellular level, the TME is characterized by the presence of fibroblasts, regulatory T-cells (T_{reg} cells), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages, which create an immunoinhibitory milieu via the secretion of TGF-β, IL-4, IL-10 and other cytokines/chemokines [138]. Together with the expression of inhibitory cell surface molecules such as PD-L1 and CTLA-4 by tumor and immunosuppressive cells, this environment limits T-cell proliferation and drives T-cell inhibition, anergy and exhaustion leading ultimately to CAR T-cell therapy failure [15, 136]. Also, the absence of sufficient nutrients along with a poor oxygen supply greatly impedes T-cell function. The presence of adenosine and the lack of essential amino acids, e.g. degradation of arginine by arginase-1, further diminishes T-cell survival in this hostile environment [136, 139]. Taken together, CAR T-cell therapy encounters various challenges when targeting solid tumors and appropriate solutions need to be adopted to overcome these and reach success of CAR T-cell therapy for hematological malignancies.

1.2.3 Need for autologous therapy

In the six approved clinical CAR T-cell products, autologous T-cells from the patients are used to generate the cellular product. This procedure generates vast **economical and logistical problems** as the CAR T-cells need to be individually produced for every patient in order to prevent life-threatening **graft-versus-host-disease** (GvHD). Since T-cells efficiently recognize MHC molecules, this can be caused by the infusion of allogeneic (CAR) T-cells from a donor to a non-completely human leukocyte antigen (HLA)-matched patient. Here, the donor T-cells recognize the recipient's cells due to a different MHC composition/signature as foreign and start to strongly attack every organ system in the recipient. The resulting symptoms initially manifest at the liver, gut and skin and can range from local inflammations/immune reactions to multi-organ failure [140]. Often, these strong autoimmune reactions are extremely difficult to control, as has been learned when peripheral blood-derived donor T-cells from HLA-matched stem cell donors were used in patients experiencing disease recurrence after allogeneic stem cell transplantation for leukemias [141].

Currently, CAR T-cell products are manufactured at centralized sites, e.g. the Fraunhofer Institute for Cell Therapy and Immunology in Leipzig, Germany, as one of three sites where Kymriah is being manufactured in Europe [142]. This means that the patients' blood cells have to be harvested by apheresis and sent to these centers, where they are genetically modified and expanded to reach CAR T-cell numbers that are needed for the therapy of each patient. Afterwards, they are frozen and sent back to one of the 150 treatment sites in Europe, where they are finally infused into the patient. The whole process takes about three to four weeks and requires immense resources, which in the U.S. results in therapy costs of $ 373,000 to 475,000 - in addition to treatment and care costs of close to half a million US dollar per patient [143]. Moreover, since the process takes several weeks, the cancer can progress too quickly in this time, thereby rendering the patients ineligible for CAR T-cell therapy or even resulting in the death of the patient. Here, an *off-the-shelf* CAR therapy would drastically reduce the treatment time as well as costs. Additionally, cancer and especially pediatric patients have, due to disease progression and previous rounds of chemotherapy, often a damaged immune system and hence cannot provide sufficient T-cells to generate CAR T-cells from [144]. Thus, a donor-derived *off-the-shelf* therapy would provide a solution for these patients.

1.3 Alternative immune effector cells for CAR therapy

Natural killer (NK) cells are professional immune effector cells of the innate immune system whose main function is the immunosurveillance and subsequent clearance of infected as well as malignant cells [145]. Similar to T-cells, NK cells induce lysis of infected or malignant cells via the release of cytotoxic granules and through death receptor signaling. However, the mode of activation is completely different, as NK cells, unlike T-cells, are not MHC-restricted and as such do not recognize intracellularly processed peptides on MHC molecules. Rather, they harbor an array of cell surface receptors by which they survey the environment for cells that have lost so-called self-signals or which express stress or danger signals (**Figure 4**) [146]. Importantly, NK cell activation depends on the net activation/inhibition signaling that is conveyed via these cell surface receptors, e.g. if the NK cell detects self-signals, most prominently HLA class I, and/or does not detect danger signals, the NK cell stays inactive. However, once self-signals are missing on cells, the corresponding inhibitory receptors on the NK cell are no longer triggered and the net activation/inhibition signal shifts towards NK cell activation. Similarly, the net signal also shifts towards NK cell activation when stimulatory receptors recognize danger or stress signals. Both, the loss of self-signals as well as the expression of danger or stress signatures are hallmark events in cancer cells, thus making NK cells a key player in tumor immunosurveillance [146, 147].

NK cells have clinically already been used for more than 20 years as a form of adoptive cellular therapy for various cancer entities [148]. They also play a major role during hematopoietic stem cell transfer since they are the first lymphocytic population to be reconstituted after engraftment [149]. Importantly, even the administration of large number of allogeneic NK cells proved to be well tolerated and safe, since NK cells do not cause GvHD [150]. However, even repeated infusions of unmodified allogeneic NK cells were not sufficient to induce/uphold long-term clearance/remission in cancer patients [151]. Since NK cells are able to mediate antibody-dependent cellular cytotoxicity (ADCC), they are prime candidates for combination therapies with monoclonal antibodies, which are recognized by the FcR CD16 on the NK cells [152]. More sophisticatedly, bi- and trispecific killer engagers (BiKEs and TriKEs), which consist of two to three linked scFvs, allow the crosslinking of up to two antigens to CD16. Preclinically, this platform has been used to target CD19, CD20, CD33 and/or CD123 for hematologic cancers and ErbB2, EGFR and EpCAM for solid tumors [153]. Currently, a TriKE, which crosslinks CD16 and CD33 and stimulates the IL-15 receptor [154] is under clinical investigation (NCT03214666). Another approach is to equip NK cells with CARs, thereby redirecting NK cells towards malignant cells and hence improving tumor control. Indeed, NK cells can be transduced with lentiviral CAR vectors and CAR NK cells show disease control in preclinical models as well as in a first clinical trial for the treatment of CD19+ malignancies [155]. Since NK cells are not MHC-restricted, NK cell products can be manufactured from healthy donors, which is paramount to drive cellular therapies from an autologous towards an *off-the-shelf* approach, where (CAR) NK cells can be produced from one donor and then given to multiple patients (**Figure 4**) [155]. Moreover, NK cells seem to cause less severe CRS and neurotoxicities, possibly because a narrower array of cytokines is secreted by NK cells compared to T-cells [156]. Besides reducing costs and time for manufacturing of adoptive cellular products, this also enables to treat patients, whose immune systems are already impaired from previous rounds of chemotherapies or disease progression.

However, while NK cells are a highly promising effector cell type for CAR therapy, there are still hurdles to be overcome before CAR NK cell therapy reaches or even surpasses the success of CAR T-cell therapy (**Figure 4**). First, while transduction and manufacturing protocols are already established for CAR T-cells, the widely used lentiviral envelope pseudotype, vesicular stomatitis virus glycoprotein (VSV-G), does not suffice to reach desirable transduction efficiencies in NK cells [157, 158]. Moreover, unlike T-cells, which can stimulate themselves by autocrine feedback loops for proliferation and survival (e.g. IL-2), NK cells do not possess comparable self-stimulation and thus need extrinsic growth signals for longer-term persistence that appears necessary for successful tumor control [159]. Historically, NK cells have been cultured on (genetically-modified) feeder cells such as K562 [160] to promote their growth *in vitro*. However, from a regulatory point of

view in Europe, the use of malignant or at least immortalized feeder cells is a major concern and thus will be a hurdle in clinical approval of NK cell therapies.

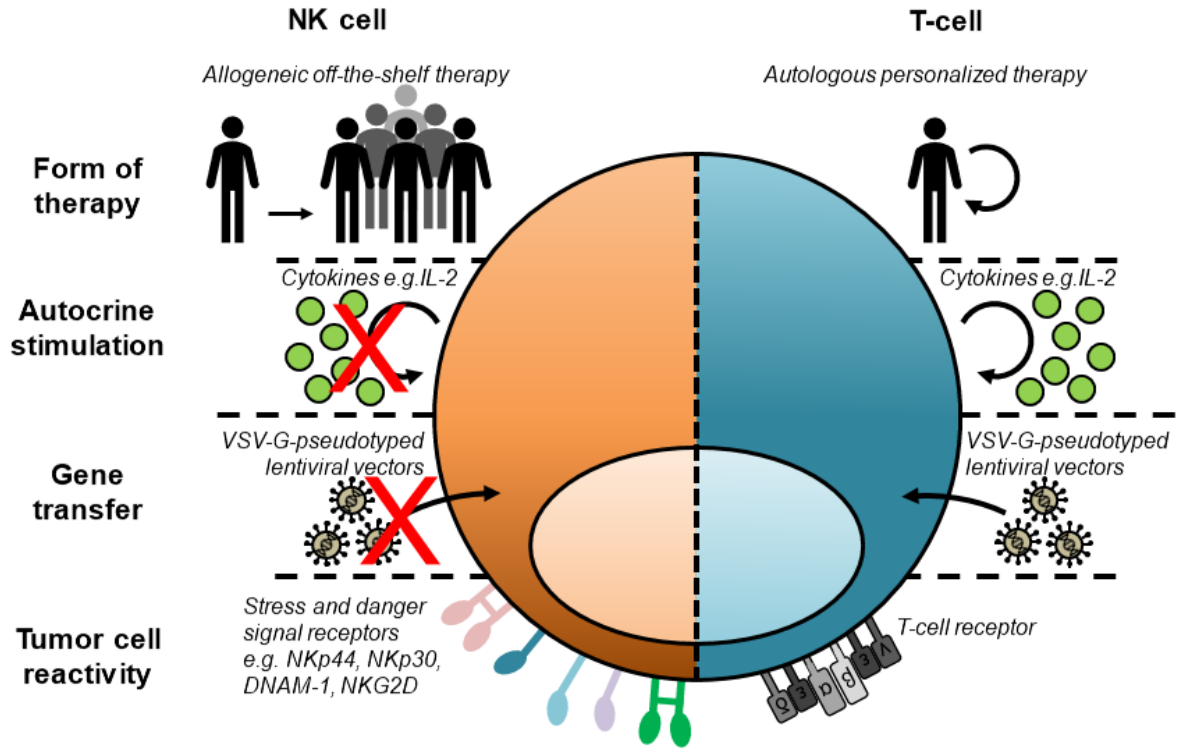


Figure 4: Key points about using NK or T-cells for CAR therapy. NK cells recognize tumors with an array of cell surface receptors and not the T-cell receptor complex. NK cells are MHC-unrestricted and can be given allogeneically to multiple donors. However, NK cells are unable to stimulate their proliferation via cytokines and the widely used VSVG envelope is not sufficient to transduce NK cells.

As CAR NK cell therapy is gaining traction in recent years, there is an increasing focus on the merits of their use. Consequently, important milestones for producing allogeneic CAR NK cells for clinical use have been reached in recent years, including alternative lentiviral envelopes [161, 162], feeder cell-free good manufacturing practice (GMP)-compliant NK cell media and expansion protocols [163, 164] and approaches to improve NK cell persistence *in vivo* [165]. As shown below, a part of this dissertation is the generation of a GMP-compliant workflow for the efficient generation of CAR NK cells using lentiviral vectors and a newly developed CD34-hinge, which enables immunomagnetic purification of CAR NK cell populations that can be used for allogeneic therapies in the future.

1.4 Aims of this dissertation

The aim of this dissertation is to optimize CAR designs for improved cellular therapy of various cancer entities. To achieve this aim, three aspects of CAR therapy were addressed.

1 Development of new hinges for CARs

The first part of this thesis focuses on the development of novel CAR hinges derived from CD34 and NGFR that allow detection and enrichment of CAR T- or NK cells with clinical-grade immunomagnetic reagents. These hinges were also tested with regard to safety and CAR efficacy in *in vitro* and in *in vivo* models.

2 Improving CAR T-cell therapy for solid tumors

To further develop CAR T-cell therapy for solid cancers, high-affinity CAR constructs targeting EGFR and CD44v6 were established, that allow efficacious eradication of head and neck carcinoma cell lines. Moreover, to improve efficacy against urothelial and head and neck cancer cells, the combination therapy with epigenetic drugs was investigated.

3 NK cells as an alternative immune effector cell

To further develop allogeneic NK cells for *off-the-shelf* usage in CAR therapies, the last part of this dissertation established an optimal protocol for the generation of CAR NK cells under GMP-compliant conditions. In addition, our strategies also included engineering of the NK cells to improve their persistence and thus tumor control *in vivo*

2 Results

The following results section comprises six manuscripts, which were done in close cooperation with the Department of Hematology, Oncology and Clinical Immunology of the Heinrich-Heine-University, the Department of Experimental Medical Physics of the Heinrich-Heine-University, the Urological Research Laboratory of the Heinrich-Heine-University, the Institute for Pharmaceutical and Medical Chemistry of the Heinrich-Heine-University, the Biological and Medical Research Center of the Heinrich-Heine-University, the Department of Pediatric III of the University of Duisburg-Essen and the German Research Center for Environmental Health of the Helmholtz Center Munich. For each manuscript the own work was assessed regarding design, execution and analysis of experiments, supervision of students as well as writing and correction of the manuscript. Moreover, for each manuscript the current status (submitted/accepted/published) and a very short summary are given.

2.1 New hinge domains for CAR T-cells

2.1.1 CD34 hinge for CAR T-cell detection & enrichment

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

Authors: **Arthur Bister**, Tabea Ibach, Corinna Haist, Denise Smorra, Katharina Roellecke, Martin Wagenmann, Kathrin Scheckenbach, Norbert Gattermann, Constanze Wiek*, Helmut Hanenberg*
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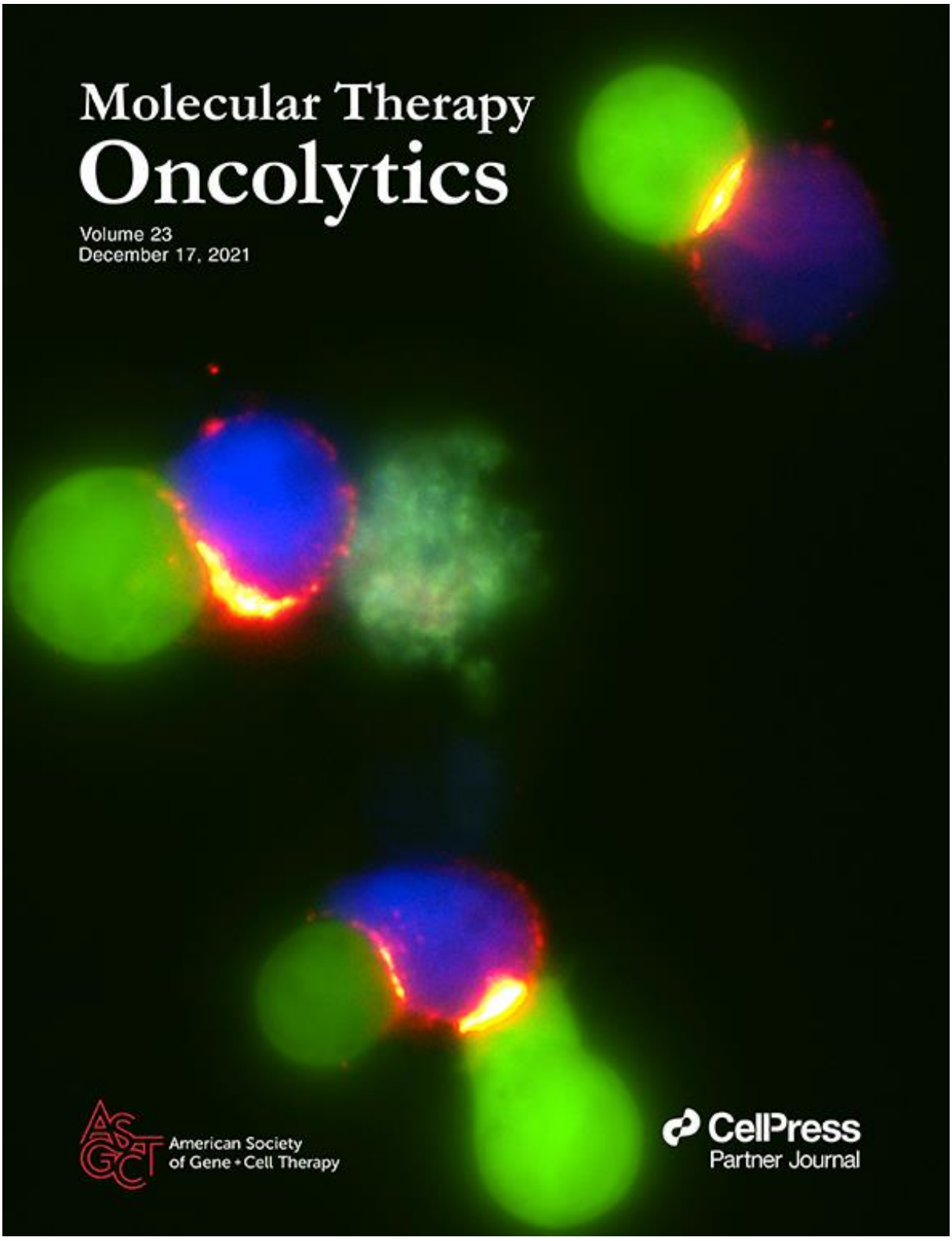
Own work: 50 % - Design, execution and analysis of most experiments; generation of manuscript figures; writing and correction of the manuscript

This manuscript describes the development of a hinge, derived from human CD34, that when incorporated into the CAR backbone allows immunomagnetic enrichment of CAR T-cells and their detection in the blood stream. Since CARs equipped with this hinge are as efficacious *in vitro* as well as *in vivo* as CARs with a clinically approved CD8 hinge, this hinge could be an alternative to the widely used CD8- and CD28-hinges for a clinical application, especially since it allows to generate more defined CAR T-cell products.



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 single (top), two (middle) or three (bottom) REH cells (green cells).

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Original Article

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

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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

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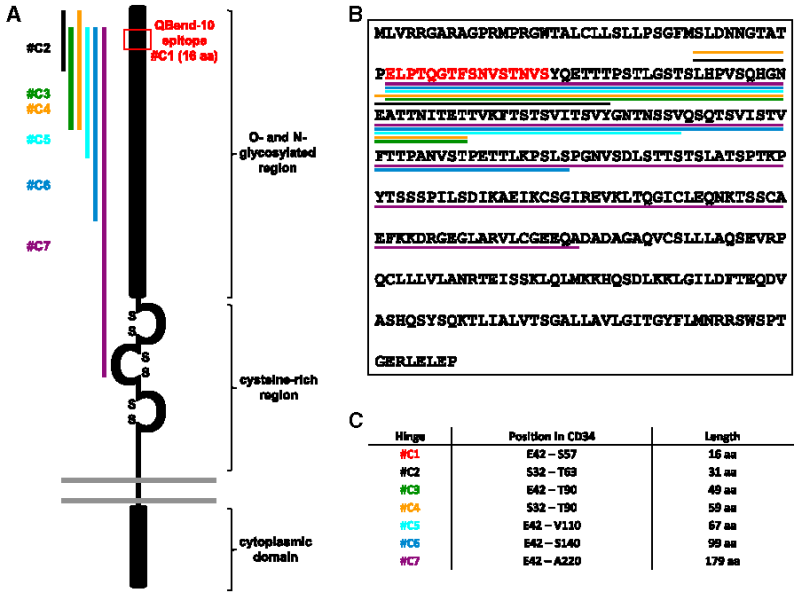

Figure 1. Structure and amino acid sequence of human CD34

(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.

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.

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.

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

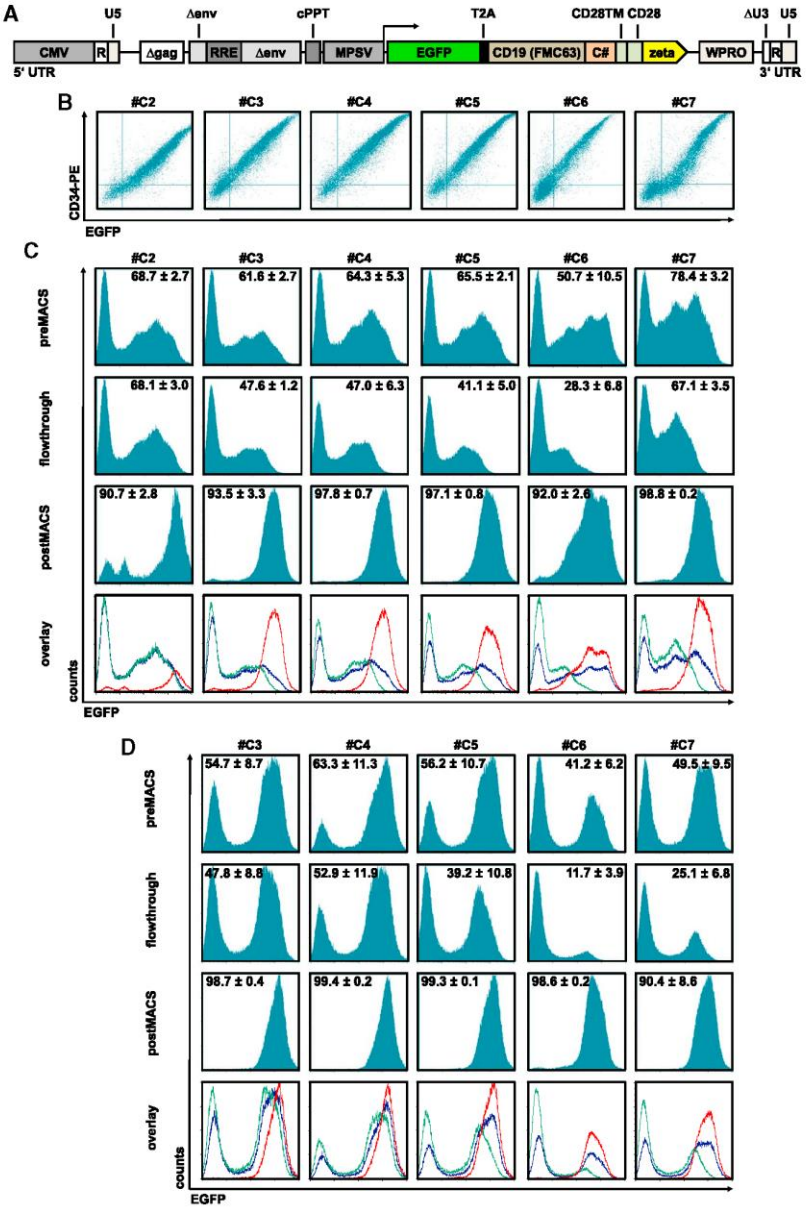

Figure 2. CD34-hinged CAR expression and enrichment

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.

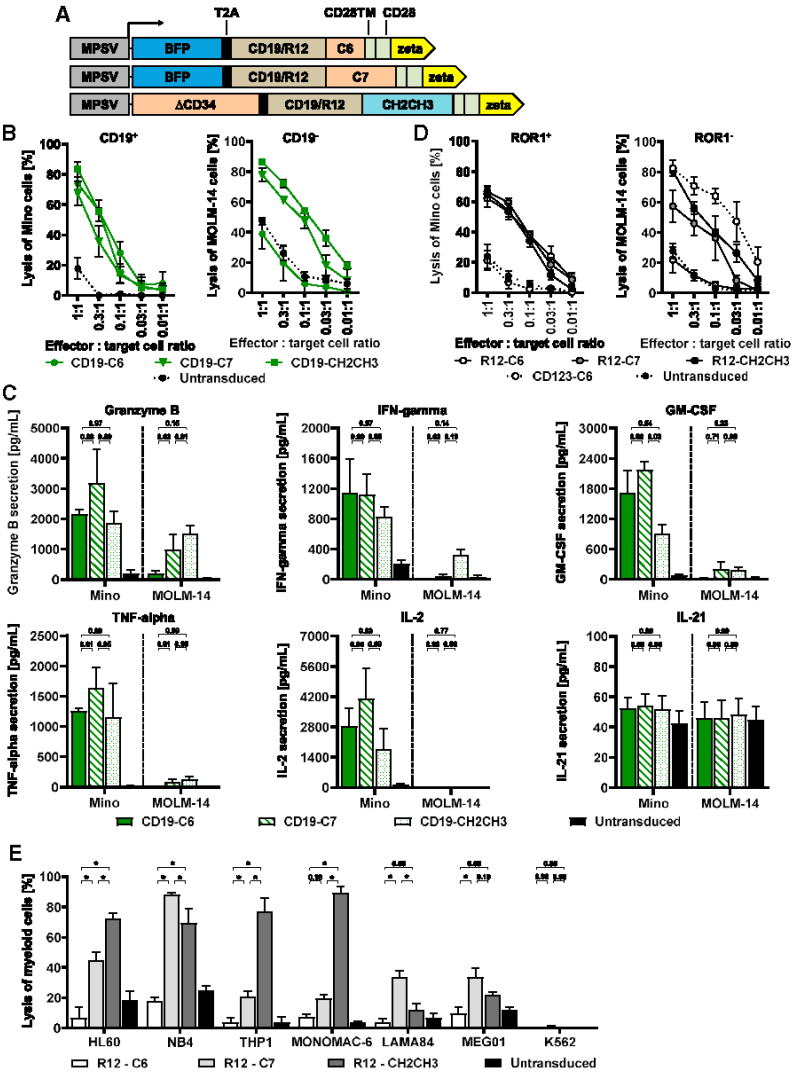

Figure 3. Comparison of C6- and C7-hinged CD19 and R12 CARs with CH_2CH_3-hinged counterparts

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).

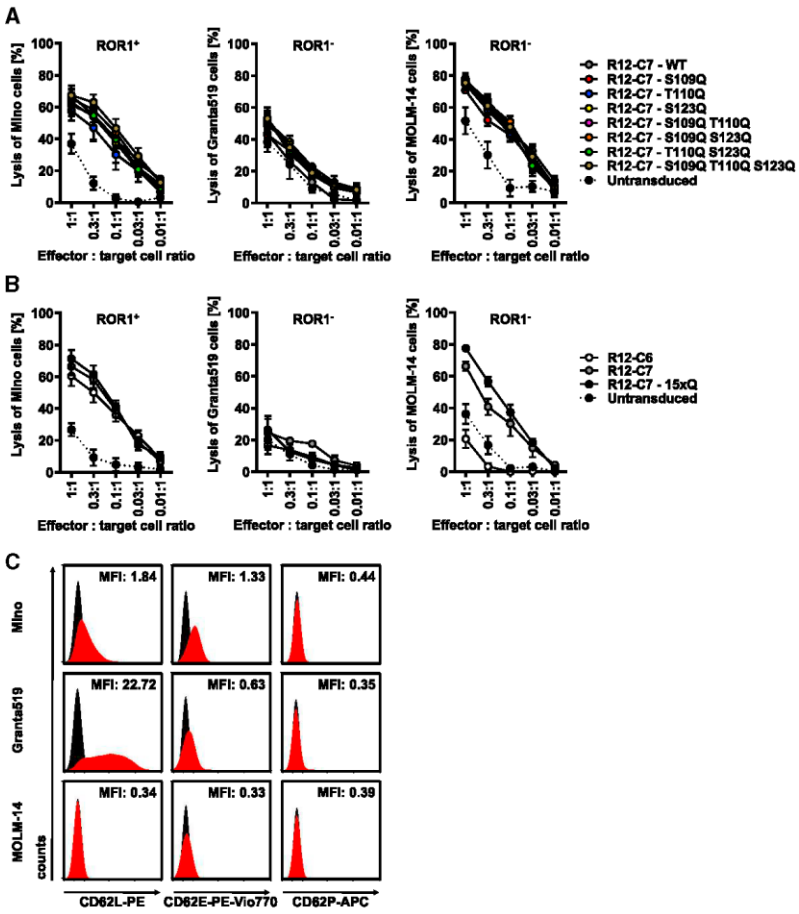

Figure 4. Unspecific lysis by C7-hinged ROR1 CARs does not depend on *O*-glycosylation

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).

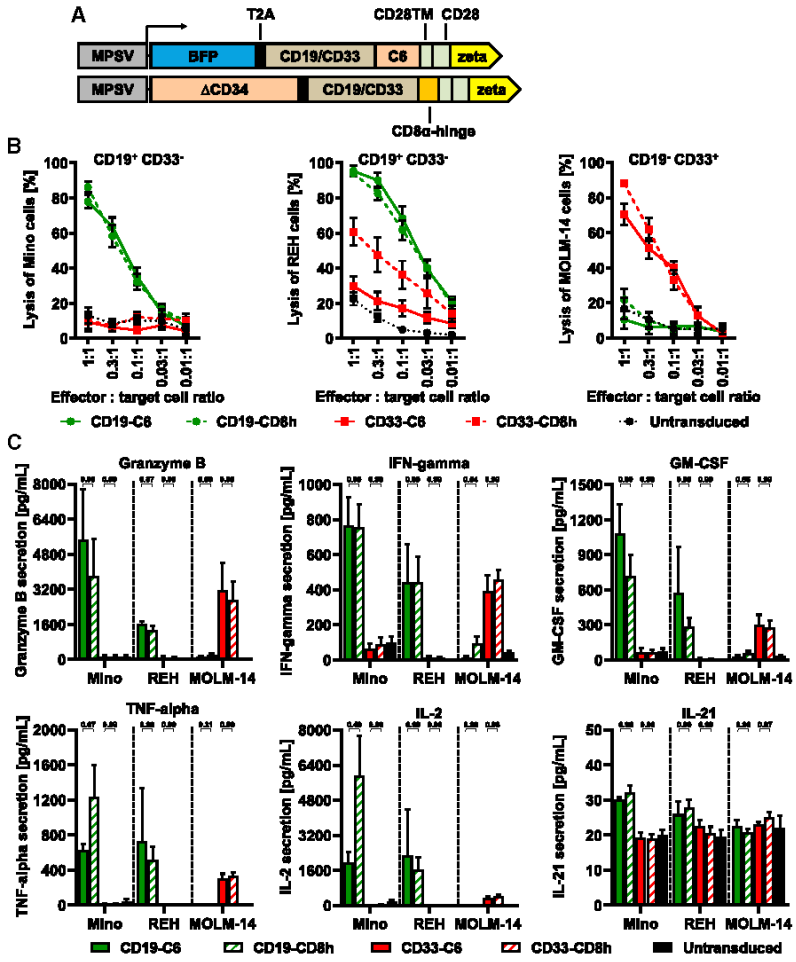

Figure 5. C6-hinged CD19 and CD33 CAR constructs on T cells are as efficient as their CD8-hinged counterparts *in vitro*

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

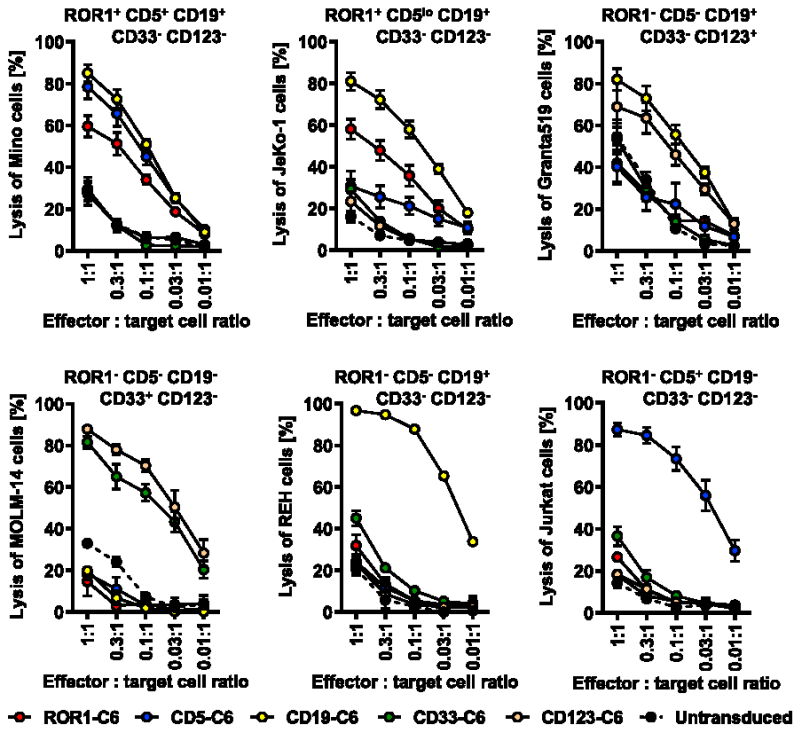

Figure 6. The C6 hinge can be used for a variety of CARs against hematological malignancies

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

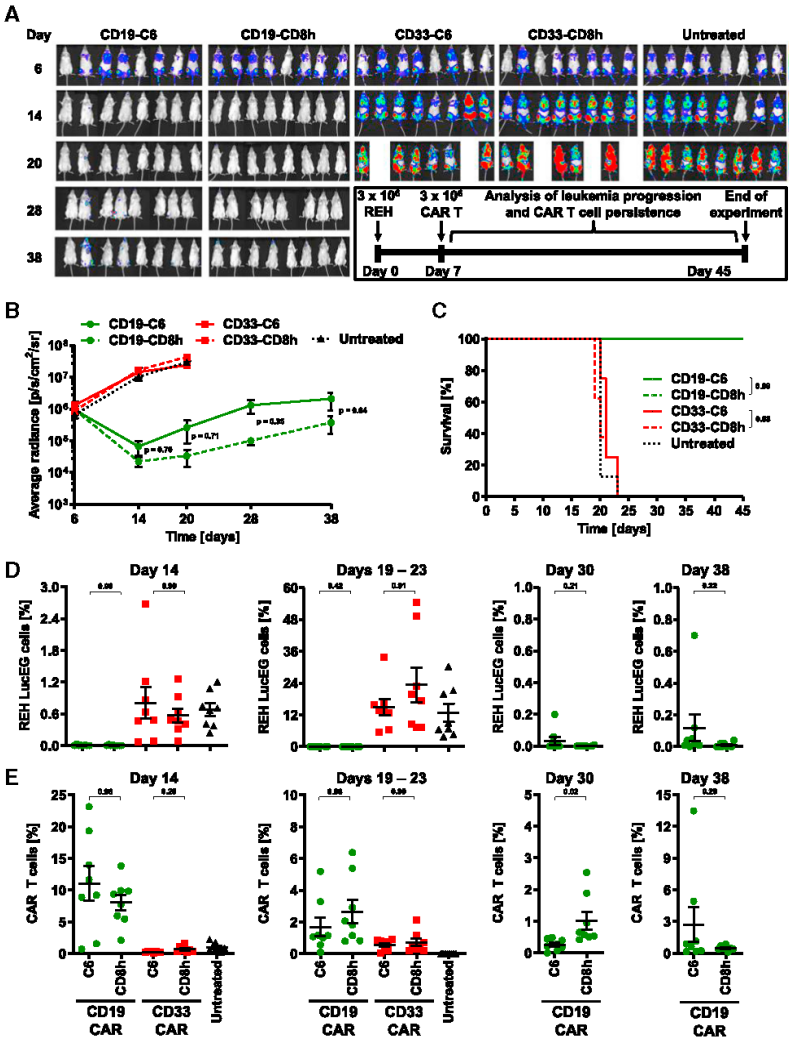

Figure 7. C6-hinged CD19 CARs efficaciously control ALL disease progression *in vivo*

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.

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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.

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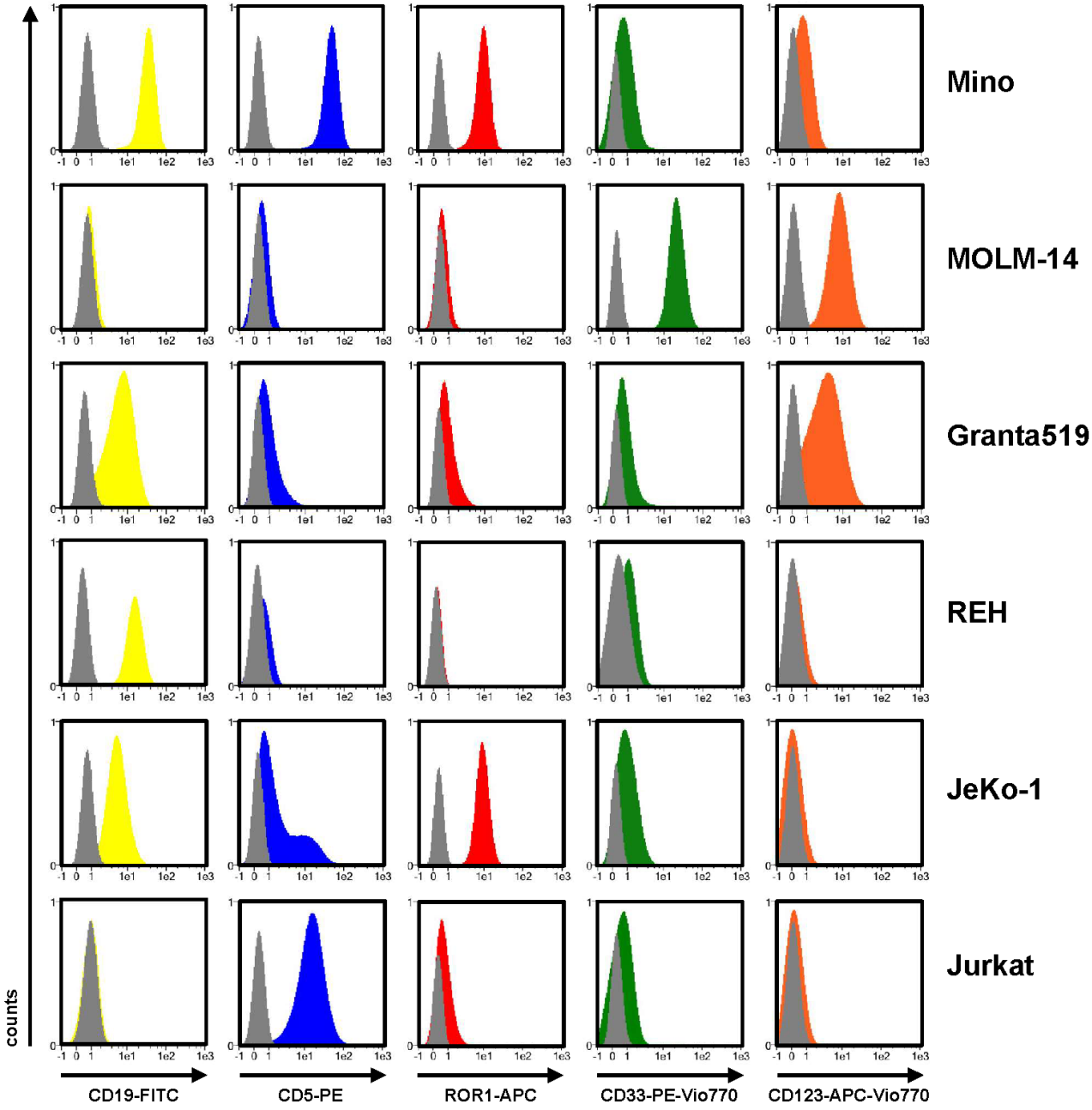
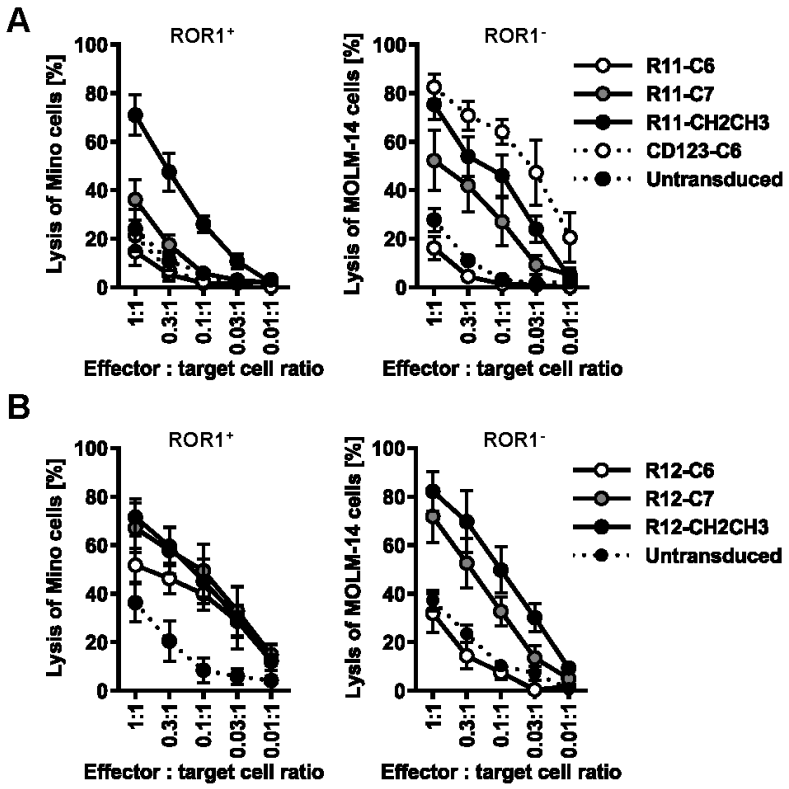
Supplementary Figure 1



Supplementary Figure 1: Target antigen expression profiles of relevant leukemia and lymphoma cell lines. Mino, MOLM-14, Granta519, REH, JeKo-1 and Jurkat cells were stained with CD19-FITC, CD5-PE, ROR1-APC, CD33-PE-Vio770 and CD123-APC-Vio770 and analyzed for antigen expression on a MACSQuant X. Colored bars indicate antibody-stained cells, while grey bars indicate unstained cells.

Supplementary Figure 2



Supplementary Figure 2: Cytotoxicity of C6-, C7- and CH_2CH_3-hinged ROR1 CARs. Primary human T-cells were lentivirally transduced with VSVG-pseudotyped vectors encoding BFP *in cis* with C6- or C7- or **ΔCD**34 *in cis* with CH_2CH_3-hinged ROR1 CARs based on clones **(A)** R11 or **(B)** R12. A C6-hinged CD123 CAR was included as control. Three days after transduction, the CAR T-cells were **(A)** enriched via MACS and subsequently co-cultured with EGFP-transduced Mino or MOLM-14 cells or **(B)** cultured with EGFP-transduced Mino or MOLM-14 cells without prior enrichment via MACS at decreasing effector:target cell ratios. After 16 h, 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 Supplementary Table 1. Data are represented as mean ± SEM of at least three biological replicates.

2.1.2 NGFR hinges for CAR T-cell detection & enrichment

Title: Optimized NGFR-derived hinges for rapid and efficient enrichment and detection of CAR T-cells

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* These authors contributed equally and share last authorship.

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Own work: 50 % - Design, execution and analysis of most experiments; supervision of students; generation of manuscript figures; writing and correction of the manuscript

This manuscript describes the development of two hinges derived from human NGFR. Similar to the CD34 hinge, both hinges allow immunomagnetic enrichment of CAR T-cells to generate pure CAR T-cell products and the detection of CAR T-cells in the blood stream. CARs equipped with the NGFR hinges proved to be as efficacious as counterparts equipped with a CD8 hinge *in vitro* as well as *in vivo*. Together with the CD34-derived hinge, they could be useful for applications where two distinct CARs (e.g. dual or *Split-CARs*) are needed to discriminate expression of the two CARs.

Molecular Therapy
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Original Article

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

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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]

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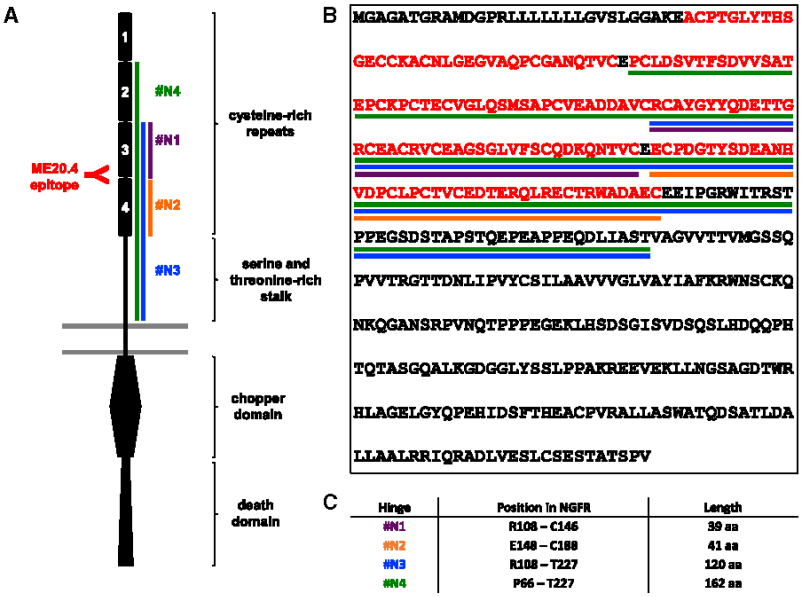

Figure 1. Structure and amino acid sequence of NGFR- and NGFR-derived hinges

(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).

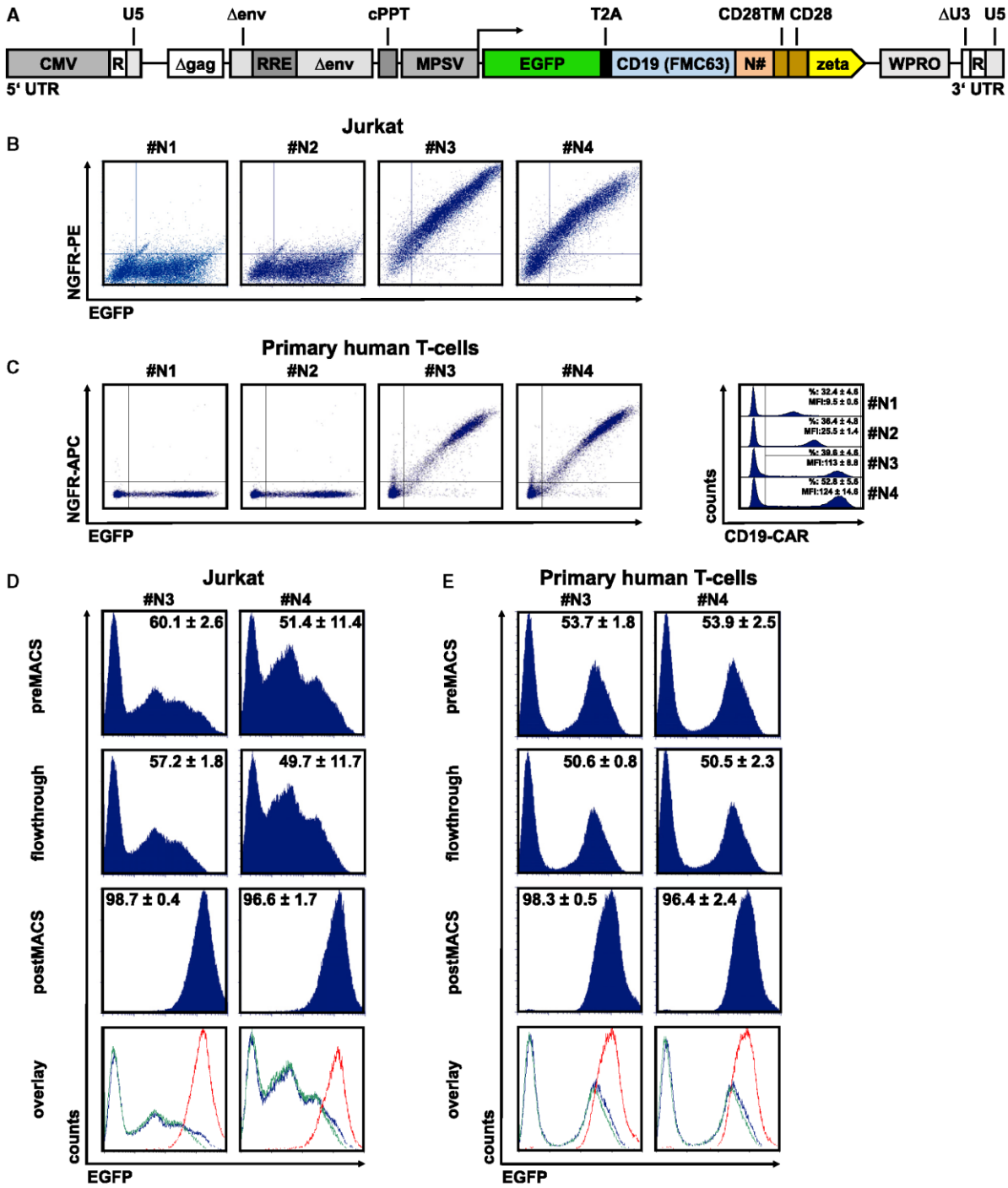

Figure 2. Expression and enrichment of NGFR-hinged CARs in Jurkat and primary human T cells

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.

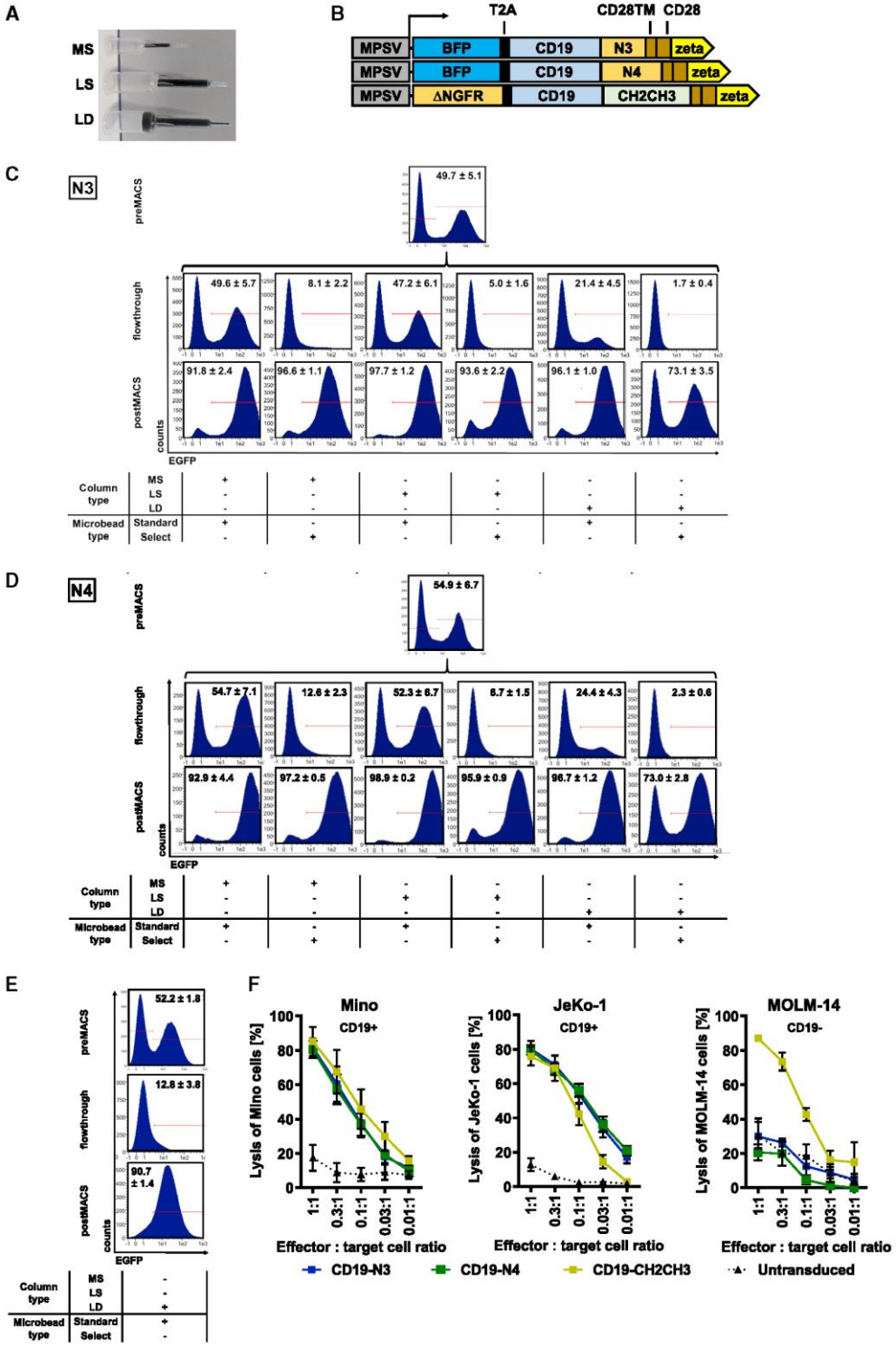
Next, we transduced Jurkat and primary human T cells with VSV-G pseudotyped lentiviral particles introducing the N3- and N4-hinged CD19 CAR bicistronic vectors (Figure 2A) and then used *Standard* CD271 microbeads on MACS MS columns once to enrich for stably transduced cells. To analyze the efficiency 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.

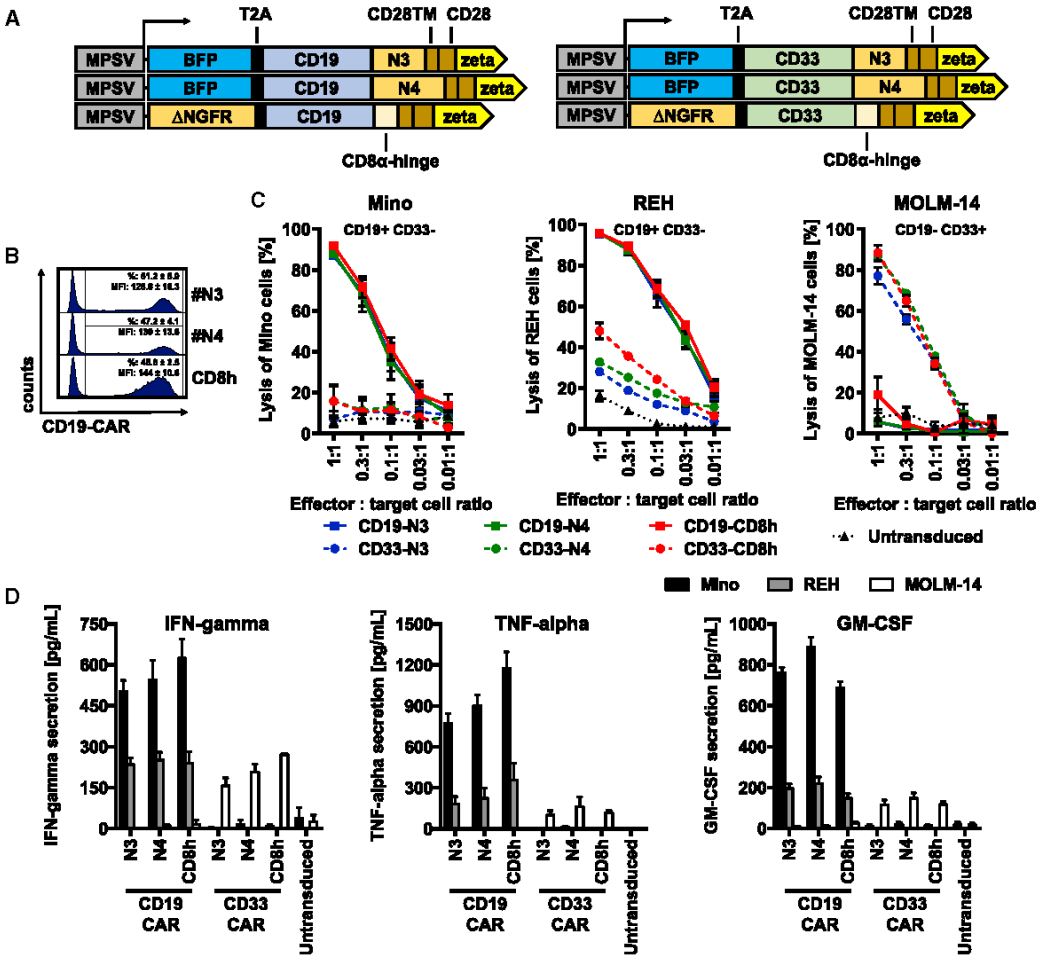

Figure 4. N3- and N4-hinged CD19 and CD33 CARs are as efficacious as CD8-hinged counterparts *in vitro*

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

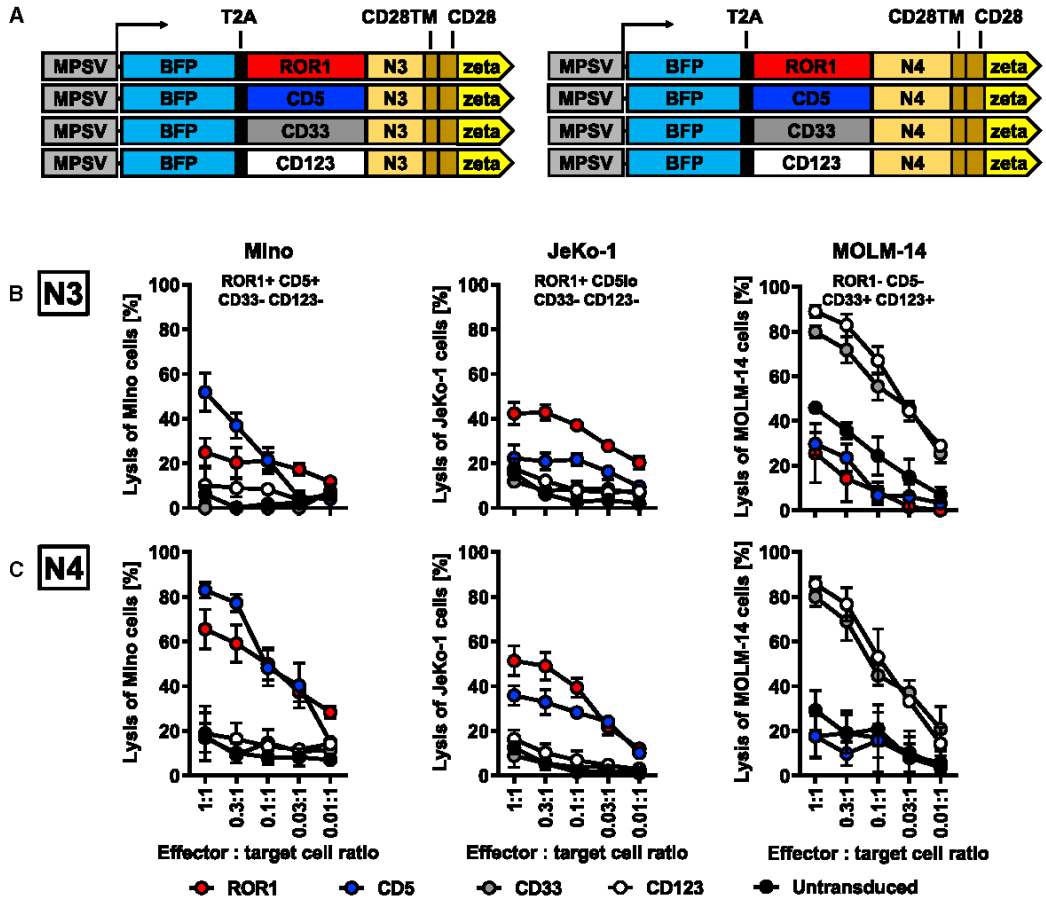

Figure 5. N3- and N4-hinged CARs eliminate malignant cells depending on antigen expression *in vitro*

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

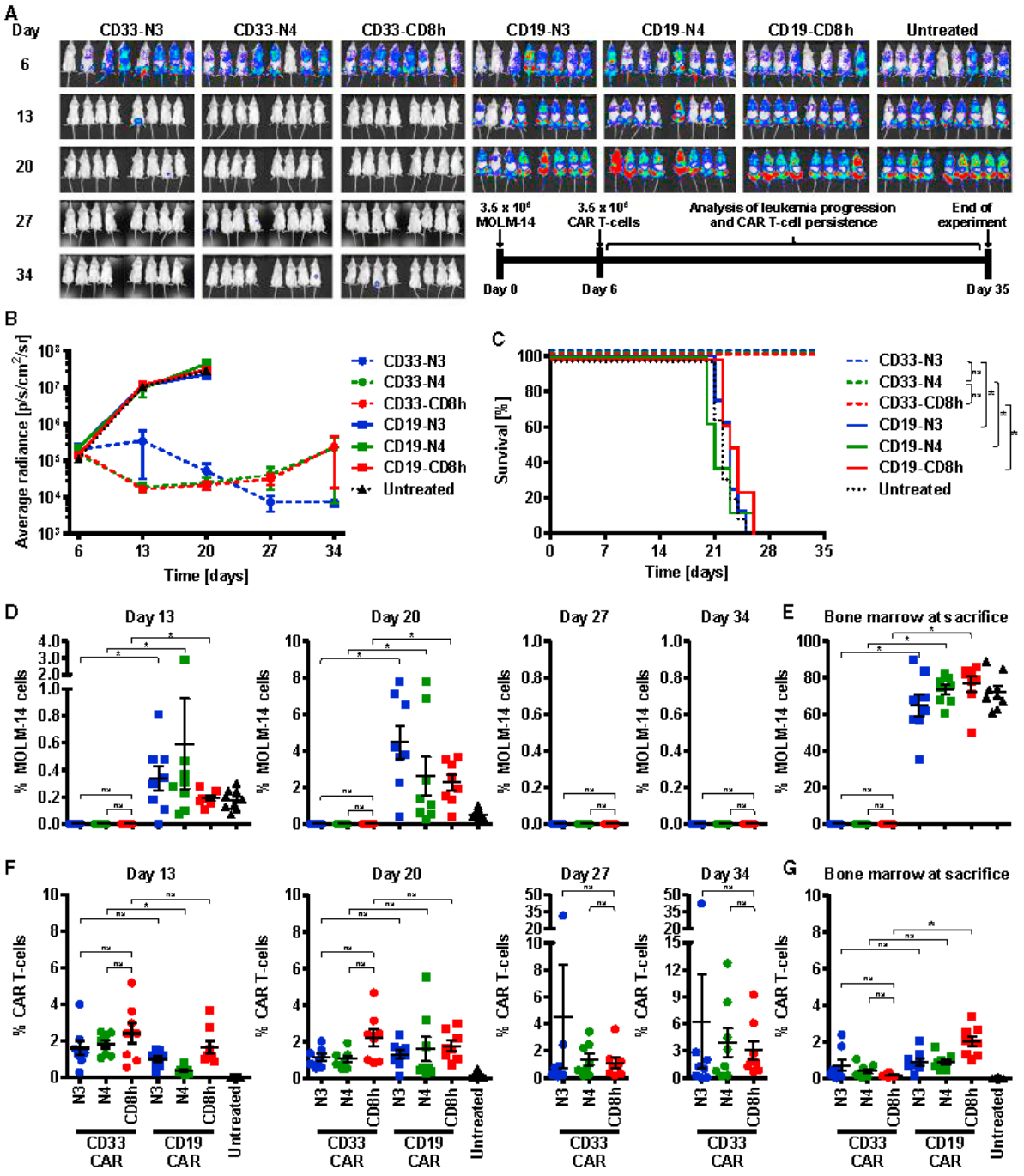

Figure 6. N3- and N4-hinged CD19 and CD33 CARs control AML progression as efficacious as CD8-hinged counterparts

NOD-SCID-gamma mice were xenografted with 3.5 x 10^6 MOLM-14/LucEG cells. After 6 days, mice were xenografted with a single dose of 3.5 x 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

(legend continued on next page)

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.

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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.

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2.2 Improving CAR T-cell therapy for solid malignancies

2.2.1 CD44v6 as target for CAR T-cell therapy for HNSCC

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

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This manuscript describes the generation of a high-affinity CD44v6 CAR, derived from a clinically approved monoclonal antibody against CD44v6 and demonstrates the feasibility to target CD44v6 for the treatment of head and neck carcinoma. CD44v6 is overexpressed on a various cancers, including head and neck squamous cell carcinoma cell lines, which are efficaciously eradicated by T-cells equipped with the newly developed CD44v6 CAR. Importantly, the CAR proved to be specific for CD44v6, since it does induce lysis against cells which express CD44, but not CD44v6.

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

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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

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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

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]

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 (ç) -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 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

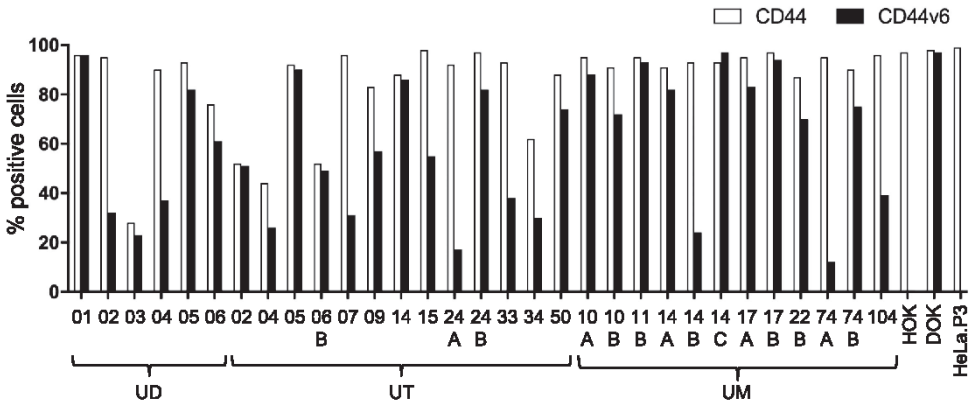

Fig. 1. Screening of CD44 and CD44v6 gene expression in primary HNSCC cell lines. Cells were stained with CD44-APC Vio 770 or CD44v6-PE Vio 770 antibodies according to manufacturer's specifications and analysed by flow cytometry. Percent positive cells (y-axis) are indicated for HNSCC cell lines and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

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

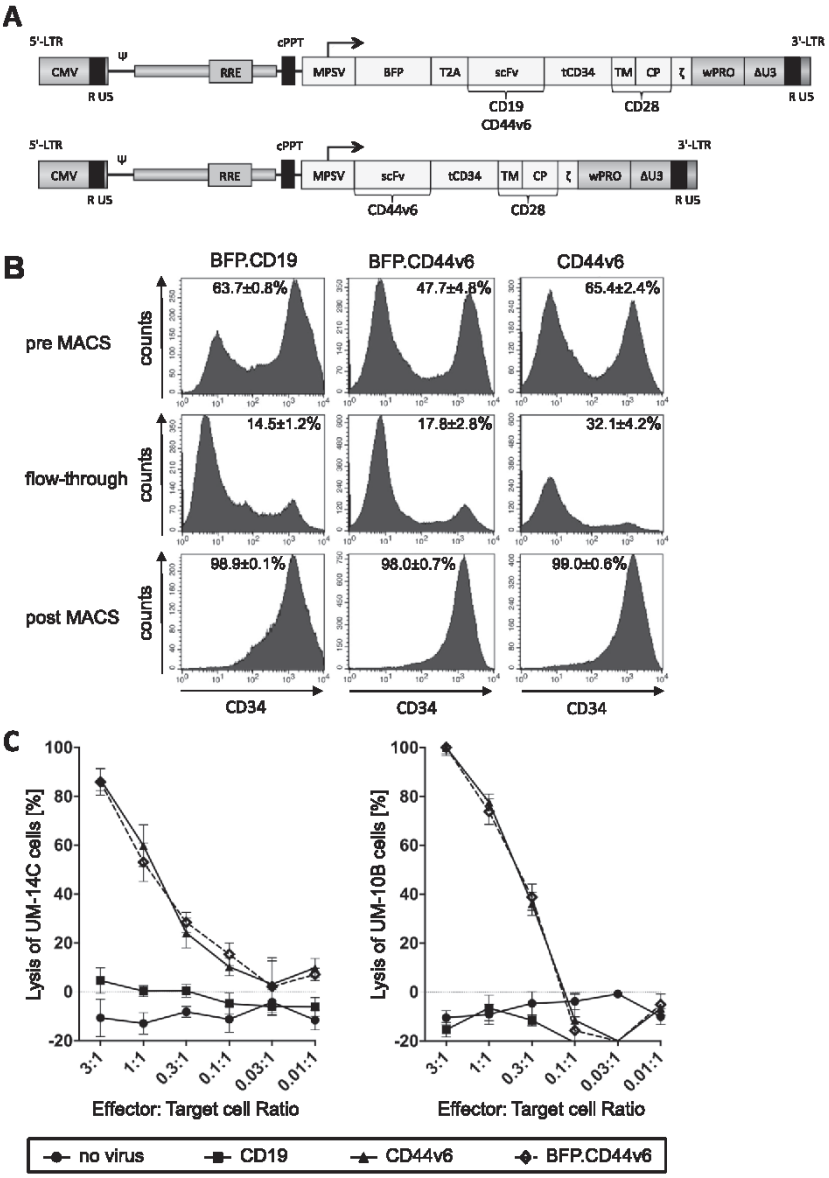

Fig. 2. Design and enrichment of CAR positive T-cells. A) Lentiviral vectors for expression of CARs. The constructs contained CD19 or CD44v6 scFvs, the CD34 hinge domain, the CD28 transmembrane and intracellular domains, and the CD3ζ cytosolic domain. BFP and T2A site were used optionally. B) Flow cytometry 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.

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 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.

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

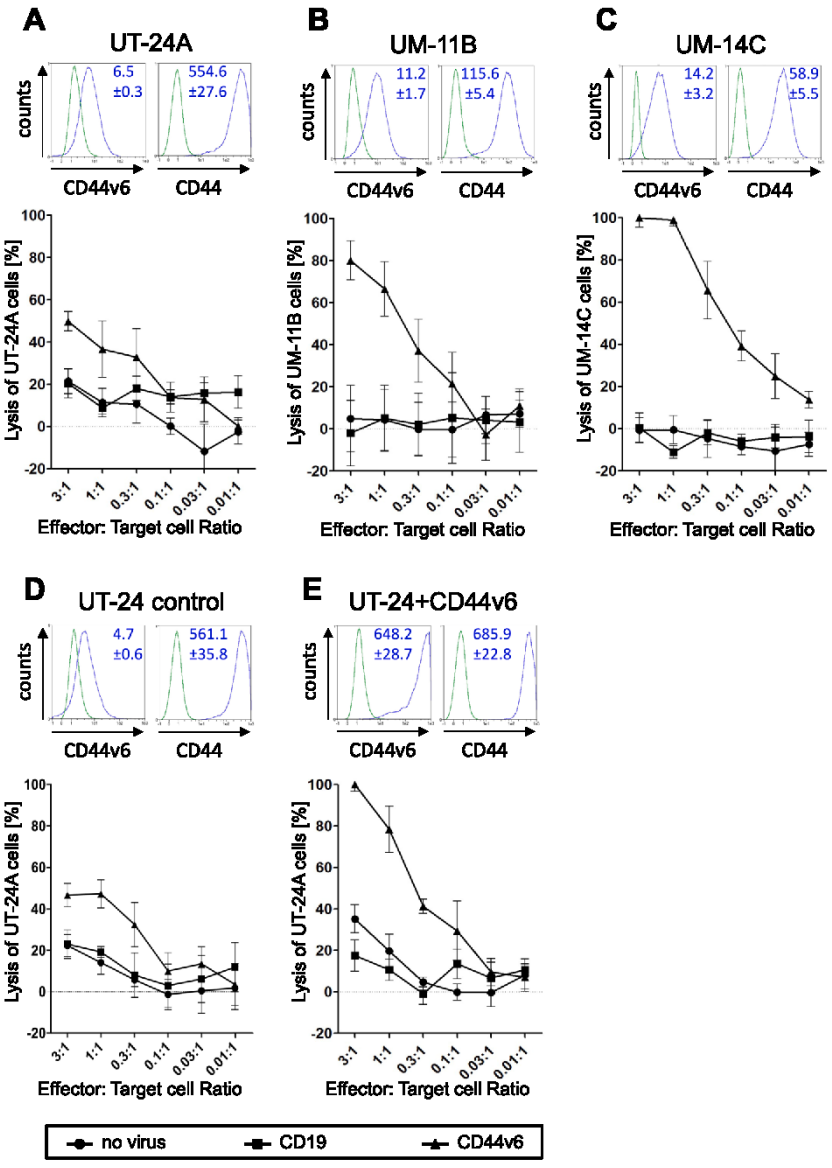

Fig. 3. Correlation between antigen density and killing efficiency. CD44 and CD44v6 expression on the cell surface of primary HNSCC cell lines UT-24A (A), UM-11B (B), UM-14C (C), UT-24A transduced with an empty control vector (D) and UT-24A transduced with an CD44v6 overexpression plasmid (E) were determined 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.

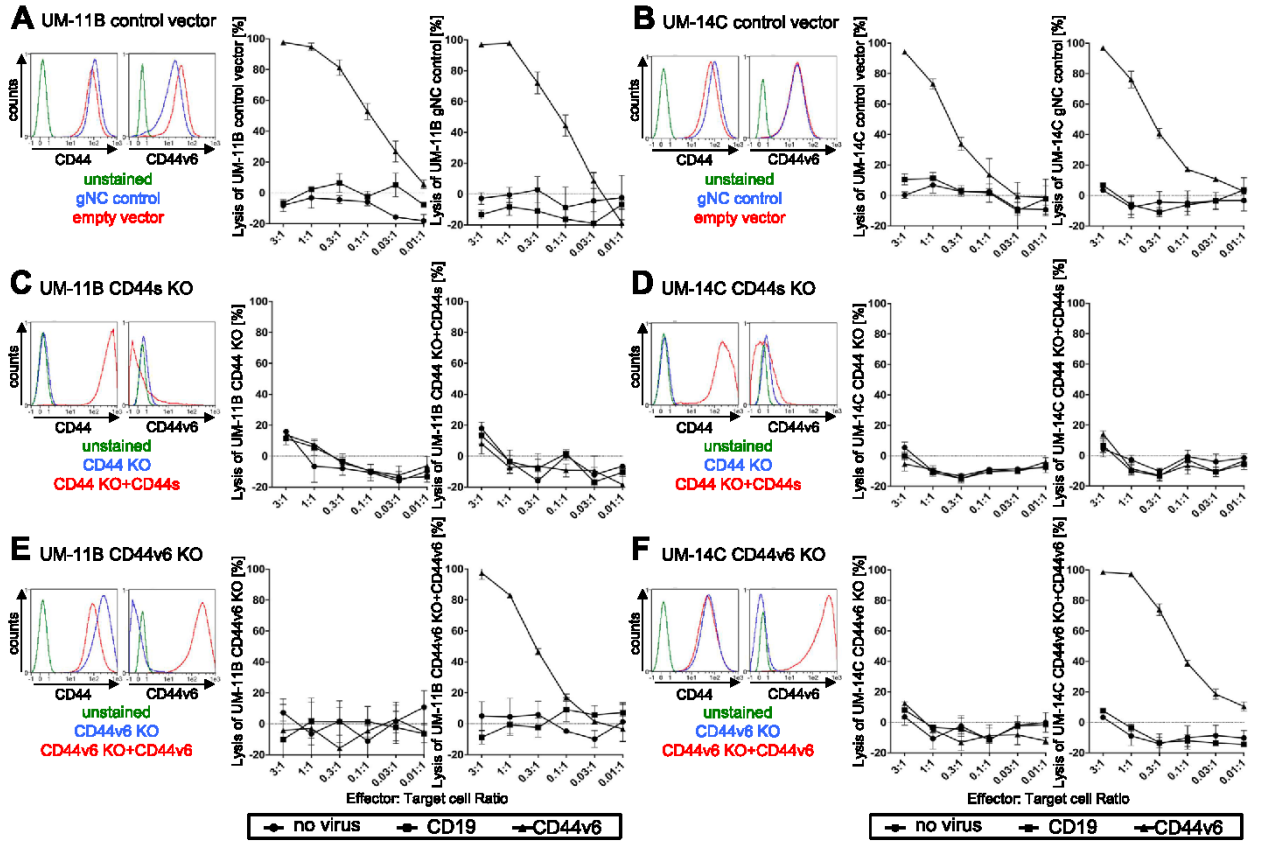

Fig. 4. CD44v6 CAR T-cells specifically recognized CD44v6 on UM-11B and UM-14C cells. Both cell lines were transduced with the CRISPR/Cas9 control vectors (A, B) or contain either CD44s (C,D) or CD44v6 guides (E,F). CD44 and CD44v6 expression on the cell surface of HNSCC cell lines was determined by 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.

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 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],

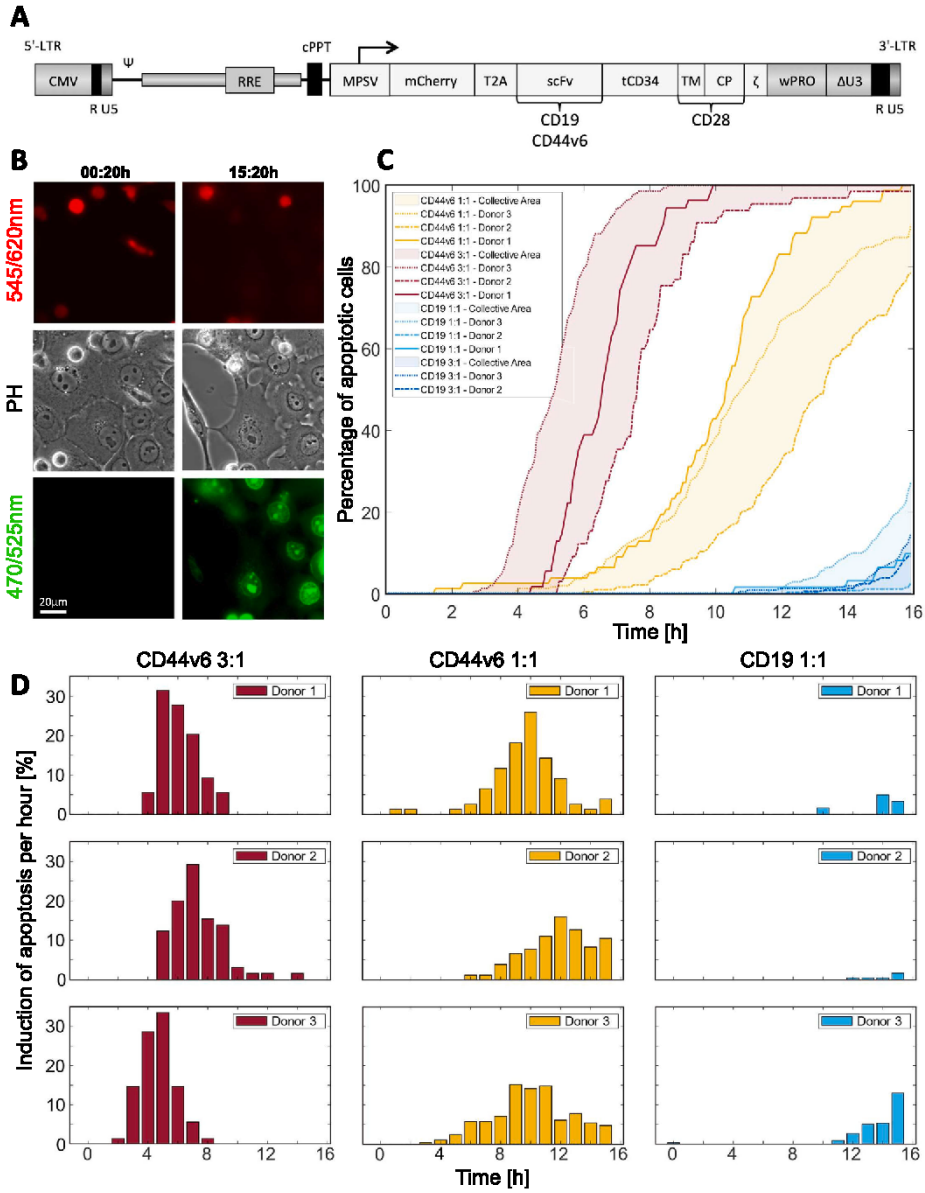

Fig. 5. Killing dynamic of CD44v6 CAR T-cells against $CD19^{neg}$ $CD44v6^{pos}$ UM17-A cells. Primary human T-cells from three healthy 3 donors) were transduced with lentiviral CD44v6 or CD19 CAR vectors. After three days, CAR-positive T-cells were enriched by MACS and co-cultured with UM-17A cells 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.

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.

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.

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

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

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2.2.2 Optimization of CARs for head and neck cancer

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

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Within this study, a high-affinity CAR is engineered from the clinically approved EGFR monoclonal antibody cetuximab. In contrast to a previously described cetuximab-based CAR, the newly engineered CAR construct allows more efficient lentivirus production as well as T-cell transduction, which is crucial for cost-effective production of CAR T-cells. Moreover, the CAR mediates efficacious eradication of EGFR+ head and neck squamous cell carcinoma and other solid tumor cell lines, even when EGFR is only minimally expressed on the cell surface.

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

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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].

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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 ζ-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].

Results

Rational design for a cetuximab-based scFv for CAR T-cell therapy

In principle, the smallest unit with the capability of high-specificity antigen binding consists of a heavy (V_H) and a light (V_L) chain of the variable region of an immunoglobulin, connected through a flexible peptide linker. This scFv is essential for the functionality of the CAR and can influence the expression as well as the stability of the CAR on T-cells [33]. As we intended to create a scFv based on cetuximab as targeting unit for our CAR construct, we used the program PyMOL to create the 3D model for the cetuximab fragment variable (FV) and fragment constant (FC) sequences (Fig. 1A, left) and the surface unit of EGFR (Fig. 1A, right) using the structure of the EGFR extracellular domain in complex with the Fab fragment of cetuximab/Erbitux/IMC-C225 (PDB file 1YY8 [10]). We marked amino acid residues known to be involved or critical for scFv binding of cetuximab and also the 15 most important amino acids mutated in domain III of EGFR in cetuximab resistant tumors [10,34–39]. In parallel, we compared the length of different scFvs that we have used successfully in CAR constructs [25,27,28], and decided to re-engineer the cetuximab scFv for inclusion in an EGFR CAR by systematically removing the amino acids shown in grey (Fig. 1B, middle), but retaining all crucial sequences for cetuximab binding (Fig. 1B, right). Using codon-optimization and overlap-PCR, we generated the four different CAR constructs shown in Fig. 2A, Ctx_{long}, Ctx_{small}, $Ctx_{smallVL}$ and $Ctx_{smallVH}$, with a CD8 signaling peptide, a 3X GGGGS linker, a CD34 hinge, a CD28 transmembrane (TM) and cytoplasmic (CP) domains and a CD3 zeta chain (Fig. 2A/B).

Re-engineering of cetuximab scFv increases lentiviral vector production

Titers of the infectious lentiviral particles for the four cetuximab-derived CAR constructs were determined by serial dilutions on HT1080 cells followed by staining the transduced cells with a CD34-PE monoclonal antibody against the CD34-derived hinge region and analysis by flow cytometry.

The results in Fig. 3A revealed that the lentivirus titers (infectious particles/mL) of the Ctx_{long} CAR with or without BFP were rather low ($1.4x10^7$ U/mL for Ctx_{long}+BFP; $1.6x10^7$ U/mL for Ctx_{long}), but could be improved by 15-fold low-speed concentration [27] of the virus supernatant (Ctx_{long}+BFP* (conc)) to levels achieved by the Ctx_{small} constructs without concentration ($1.6x10^8$ U/mL for Ctx_{small}+BFP, $3.5x10^8$ U/mL for Ctx_{small}). Importantly, the presence of a second transgene did not have a detrimental effect on the titers. Constructs with either shortened V_H or V_L alone also led to increased titers ($5.7x10^7$ U/mL for $Ctx_{smallVH}$ and $1.8x10^8$ U/mL for $Ctx_{smallVL}$).

To test the functionality of the different cetuximab CAR lentiviral vectors, peripheral blood-derived T-cells were transduced with supernatants of the CD34-hinged CAR constructs. The percentages of CAR-positive T-cells in the three fractions (PreMACS, Flow-through, PostMACS) were flow cytometrically determined after staining with CD34-PE antibodies [26]. Transduction efficiencies (PreMACS) of Ctx_{long} constructs (Ctx_{long}+BFP 18.7 ± 1.2%, Ctx_{long}+BFP*(conc) 63.6 ± 2.7%, Ctx_{long} 20.1 ± 6.2%) are compatible with the low virus titers of Ctx_{long}+BFP and the high titers of Ctx_{long}*(conc) (Fig. 3B). For the Ctx_{small} CAR, the presence of BFP in the expression cassette had no effect on the CAR expression levels, as 61.6 ± 5.5% and 64.2 ± 6% of the T-cells expressed the CAR without or with BFP, respectively. The shortening of the V_H alone ($Ctx_{smallVH}$+BFP) led to a lower transduction efficiency (35.1 ± 3.8%), while the $Ctx_{smallVL}$+BFP could not be expressed on the T-cells surface at all (not shown), presumably caused by steric folding problems. After enrichment, the purity of CAR-positive T-cells in the eluates (PostMACS) consistently reached ≥ 97%, while only between 4.6 ± 0.3% and 33.4 ± 6.6% lower CAR-positive T-cells were present in the flow-through fraction

Ctx_{long} and Ctx_{small} CAR T-cells both eliminate low EGFR expressing cells

In order to functionally assess the cytotoxicity of Ctx_{long} and Ctx_{small} CARs, human T-cells were transduced with the cetuximab CAR vectors at comparable titers (with or without BFP) or CD19+BFP CAR construct as control, selected via MACS and then co-incubated with the $EGFR^{high}$ HNSCC cell lines UM–10B (MFI 259 ± 8), UM-14C (MFI 208 ± 17) and UT-24A (MFI 165 ± 16) (Fig. 4A) at different effector to target cell ratios in 96-well plates. After 16 h, T-cells and dead target cells were removed and the remaining living HNSCC cells measured as described [25].

While untransduced (no virus) and the negative control CD19 CAR T-cells did not eliminate the target cells, all cetuximab CAR variants showed identical lysis against the three tested cell lines (Fig. 4A). However, due to the relatively low transduction efficiency, the $Ctx_{smallVH}$+BFP construct was excluded from further experiments. In addition, the lysis obtained with the Ctx_{small} and Ctx_{long} CAR T-cells was not dependent on the presence of BFP as a second transgene; thus we also excluded the Ctx constructs with BFP co-expression from further experiments.

To correlate the EGFR expression level with the killing efficacy, three cell lines with different EGFR expression patterns were chosen (Fig. 4B). Cells from the high EGFR expressing cell line UM-11B (MFI 112 ± 5) and the moderate EGFR expressing cell line UM-17A (MFI 50 ± 9) were efficiently eliminated between 94.2 and 82.1% at effector to target cell ratios of 3:1 by MACS-purified Ctx_{long} and Ctx_{small} CAR T-cells (Fig. 4B), respectively. To study the CAR killing efficacy on a low EGFR expressing cell line, we used MCF-7 cells, described in the literature as having low or no expression of EGFR [40–42]. Although our flow cytometry analysis did not clearly demonstrate that MCF-7 cells were EGFR-positive, both cetux CAR T–cells recognized the minute amounts of EGFR on MCF–7 cells and thereby were able to efficiently eradicate also these cells (between 73.8 and 67.9% lysis at 3:1) (Fig. 4B).

Cetuximab CAR T-cells specifically target EGFR on HNSCC cells

As there was no clear correlation between EGFR expression and killing efficacy of Ctx_{long} and Ctx_{small} CAR T-cells (Fig. 4B), we decided to prove that the Ctx_{long} and Ctx_{small} CAR constructs did not cause unspecific *off-target* cytotoxicity. To this end, two 20-nucleotide guide RNAs (gRNA), both targeting the extracellular domain IV of EGFR (Fig. S1A), were cloned into the LentiCRISPR V2 vector (Fig. S1B) as previously described [30]. To generate the EGFR knockout, cells from the three EGFR highly expressing cell lines UM-10B, UM-14C and UT–24A were transduced with the EGFR gRNAs (EGFR KO#2, EGFR KO#4) lentiviral vector, a vector with a gRNA binding to the chicken actin promotor (gNC) [43] or the empty LentiCRISPR V2 vector (vector control). After selection with puromycin, single-cell clones were generated and expanded. Flow cytometric analysis of EGFR expression revealed, that the EGFR KO#2 and EGFR KO#4 clones lost their EGFR expression, whereas EGFR expression on cells of the control groups (gNC, control vector) was unaffected (Fig. 5A).

Single cell clones of the EGFR knockout cell lines and of the control CRISPR/Cas9 constructs were incubated overnight with MACS-enriched Ctx_{small}, Ctx_{long} or CD19 CAR T-cells and also untransduced T-cells. A previously published CD44v6 CAR lentiviral vector [25] served as a positive control. As shown in Fig. 5B and C, the strong killing of cells from the three HNSCC cell lines UM-10B, UM-14C and UT-24A, transduced with control vectors, by Ctx_{small} and Ctx_{long} CAR T-cells was identical to those observed for the parental cells. Importantly, cells from the knockout cell lines without EGFR expression were not eliminated by the Ctx CAR T-cells. However, it showed to be noted that background cytotoxicity occurred for some of the EGFR KO clones, comparable to the nontransduced T-cells (no virus) (Fig. 5D/E). Finally, the genetic engineering of these three HNSCC cells did not influence neither the killing patterns of CD44v6 CAR T-cells as a positive control nor the unresponsiveness of CD19 CAR T-cells and untransduced T-cells as negative

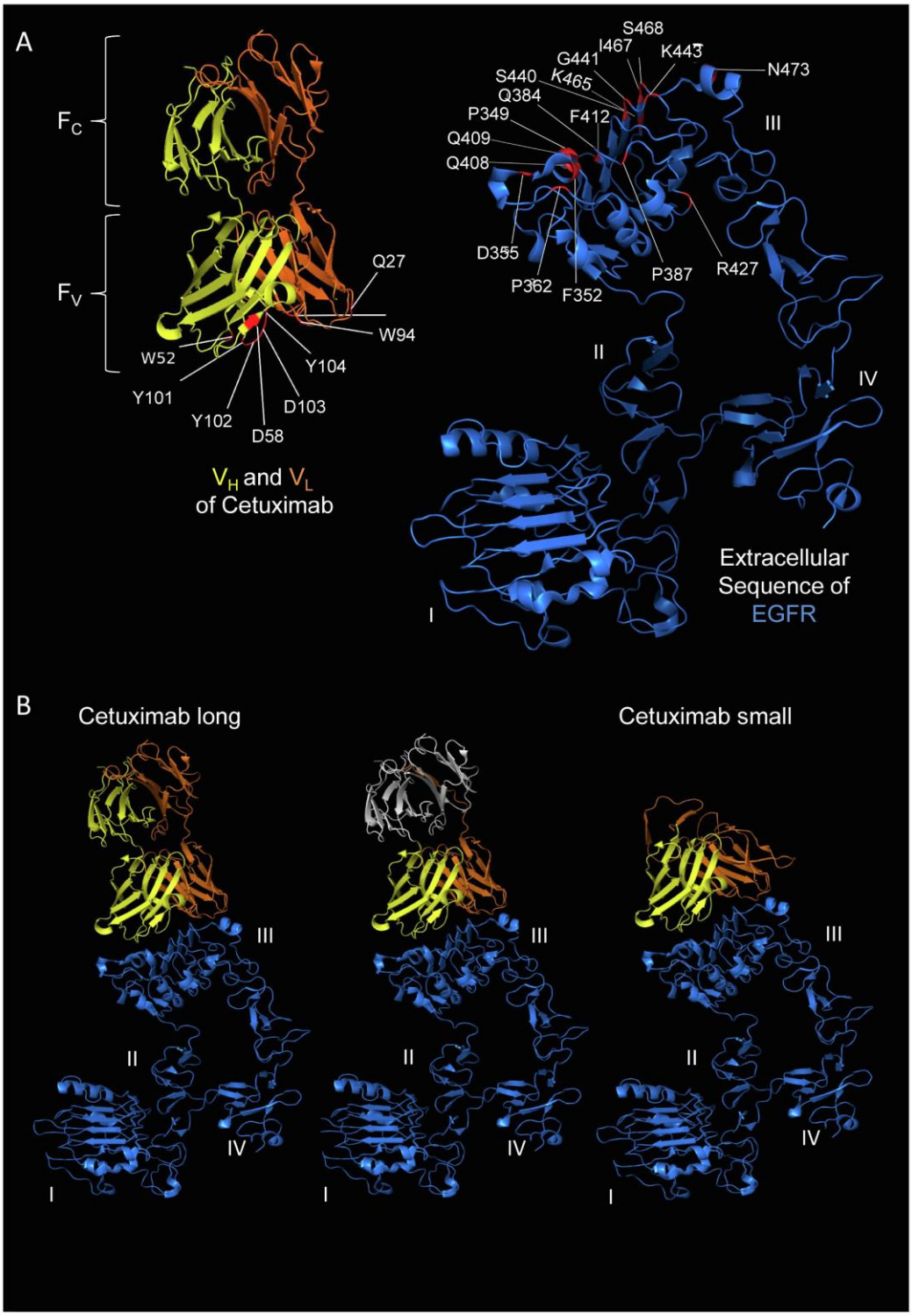

Fig. 1. Interaction of EGFR and cetuximab (Cartoon view). A) Amino acids mutated in cetuximab-resistant tumors or critical for cetuximab binding are shown in red [10,32–34] within the extracellular domains I-IV of EGFR pictured in blue (PDB 1NQL) [62]. Amino acids of the heavy (yellow) and light (orange) 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.)

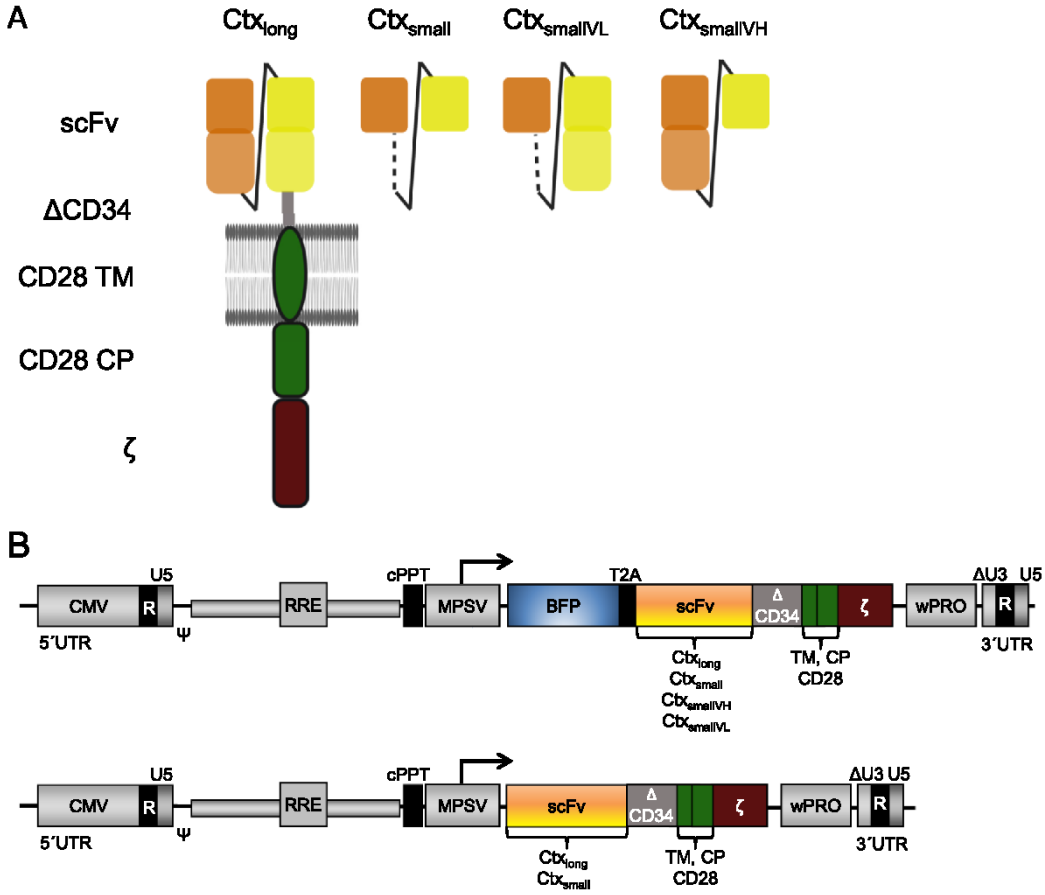

Fig. 2. Design of different cetuximab-based CARs. A) Schematic structure of the different CARs. The constructs contained the four variants of the cetuximab scFv followed by a ΔCD34 hinge domain, a CD28 transmembrane and intracellular domain and the CD3ζ cytosolic domain. Ctx_{long} (original VH and VL), 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

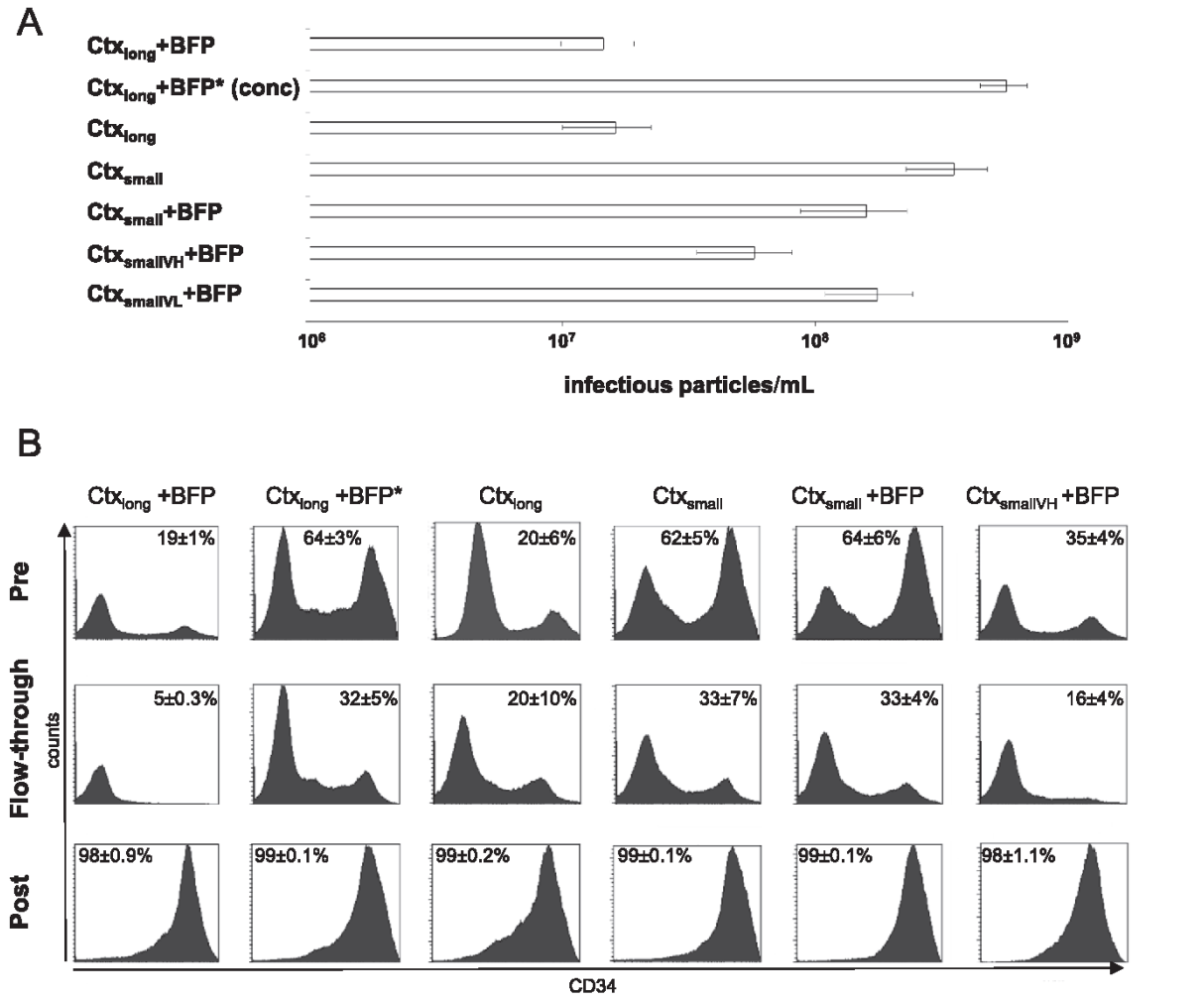

Fig. 3. Shortening of the cetuximab-derived scFv improves lentiviral and titer and transduction efficiency. A) HT1080 cells were lentivirally transduced with serially diluted vectors encoding cetuximab CAR constructs with or without BFP (Ctx$_{long}$, transduction with 15-fold virus supernatant (*(conc)), 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

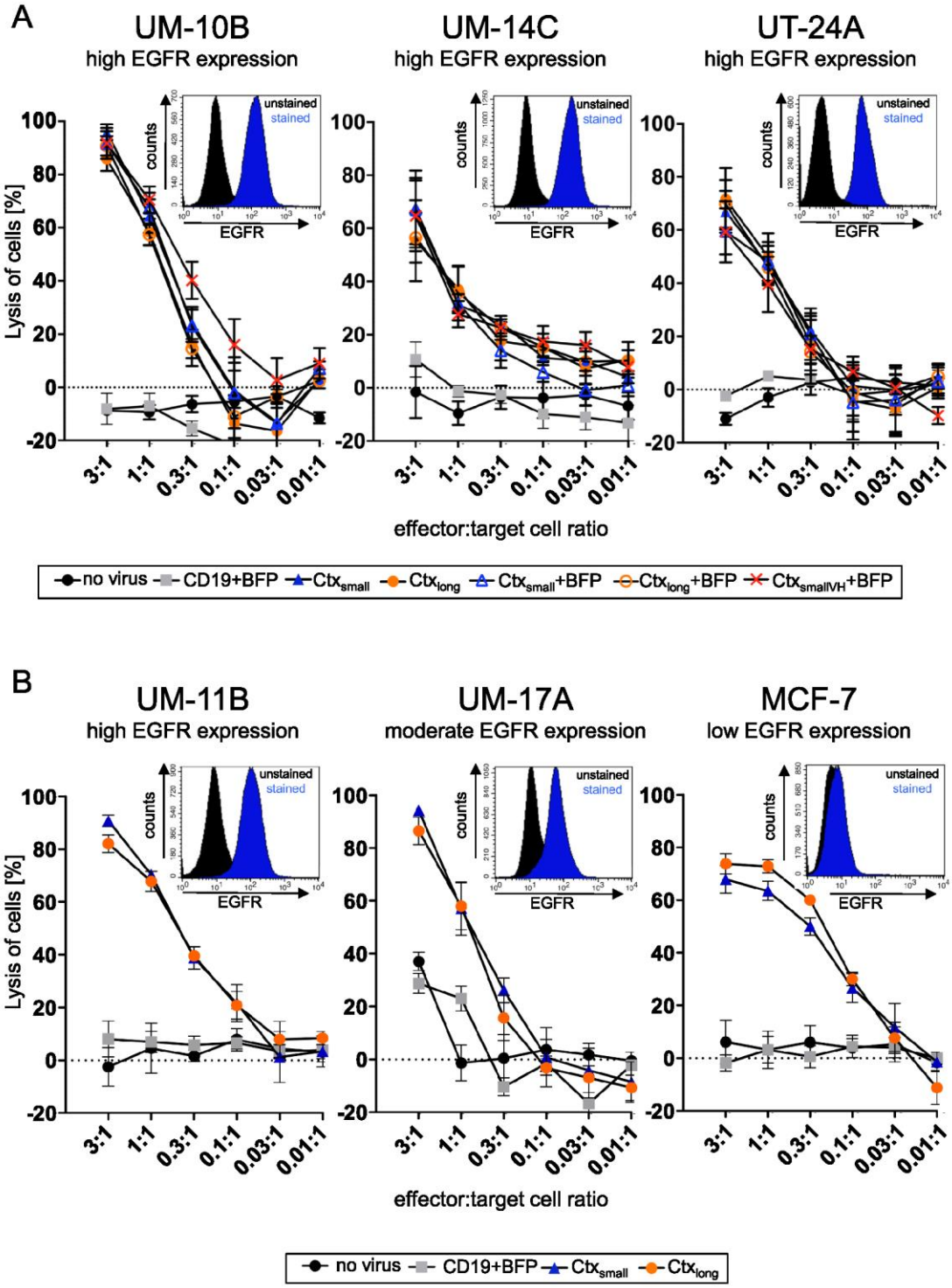

Fig. 4. Ctx-derived CARs eradicated HNSCC cells with high efficacy. EGFR expression was determined by staining with an EGFR-PE antibody. Antigen expression (x-axis) was plotted against the absolute cell count (y-axis) for unstained control cells (black) and stained cells (blue). Primary human T-cells 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.

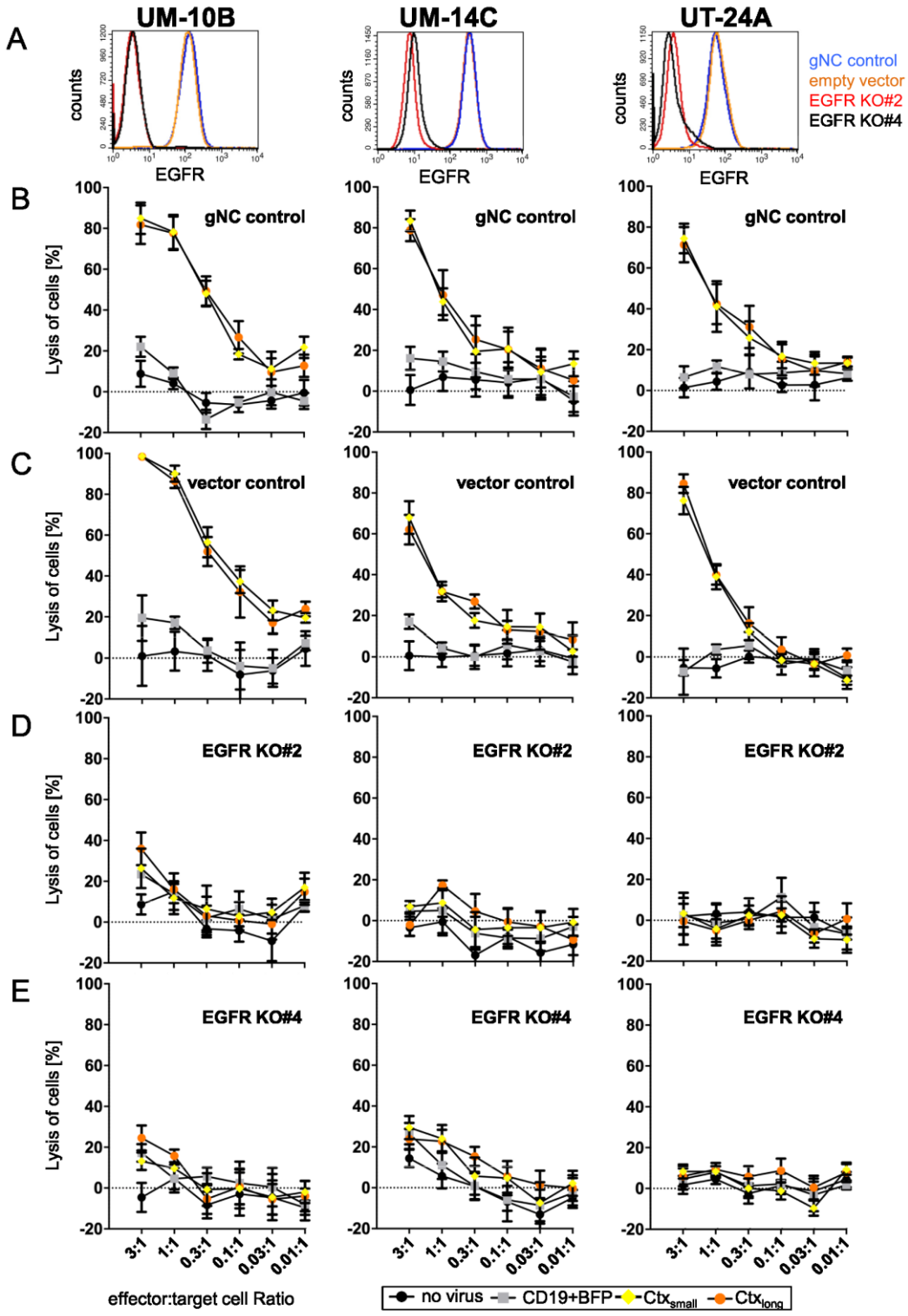

Fig. 5. Ctx$_{long}$ and Ctx$_{small}$ CAR T-cells eradicated HNSCC cells with high efficacy. A) EGFR expression of UM-10B, UM-14C and UT-24A cells transduced with a CRISPR/Cas9 negative control vector (blue), the empty vector (orange) or the two CRISPR/Cas9 gRNA vectors (KO#2 red, KO#4 black). EGFR 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.

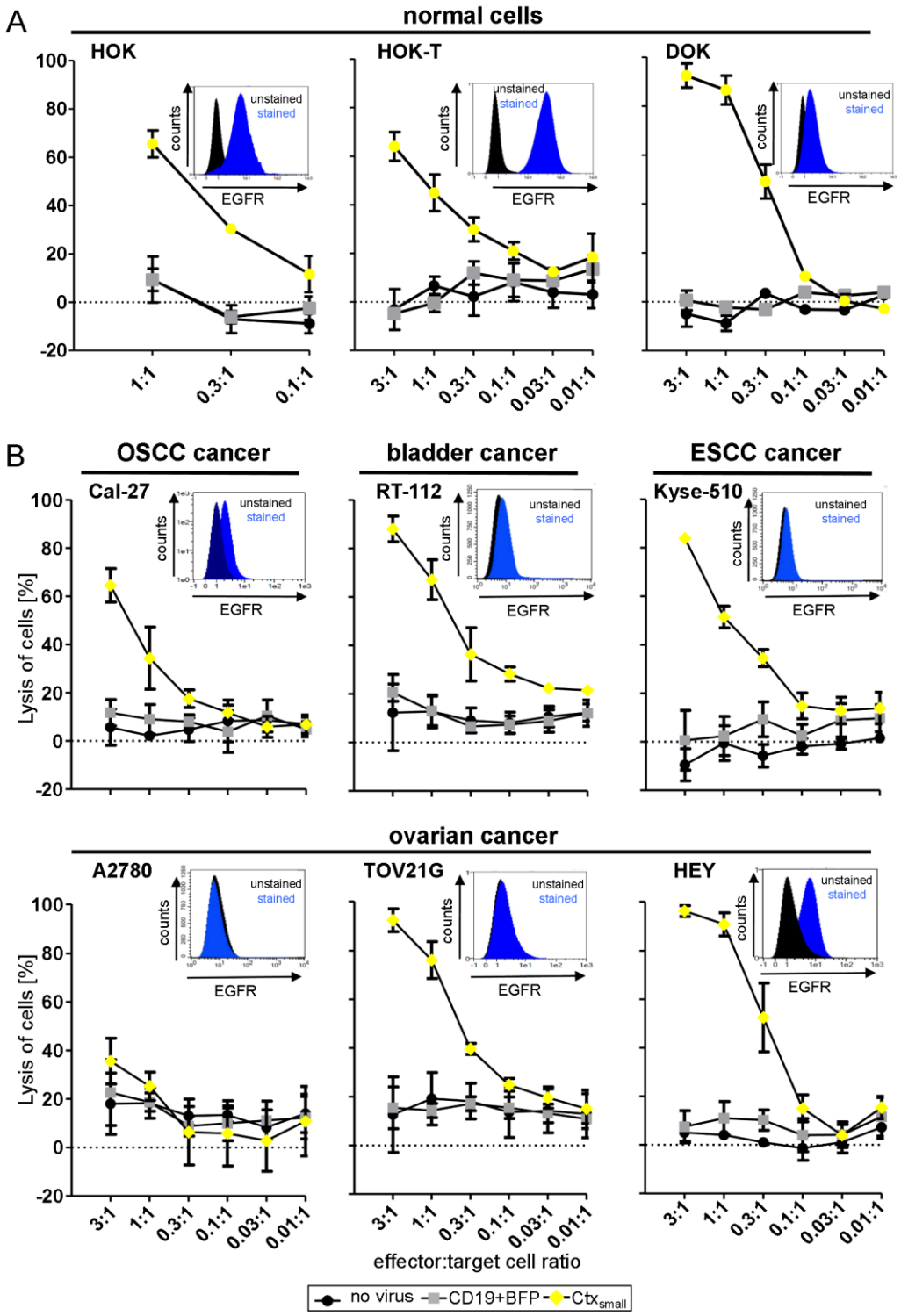

Fig. 6. Ctx_{small} CAR T-cells specifically eradicated EGFR-positive cells of different origin. EGFR expression was assessed by staining with EGFR-PE antibody. Antigen expression (x-axis) was plotted against the absolute cell count (y-axis) for unstained control cells (black) and stained cells (blue). 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.

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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.

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Appendix A. Supplementary material

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2.2.3 Combination therapy with epigenetic modulators

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

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Since cancer cells are often epigenetically dysregulated, this study explored the possibility to combine epigenetic drugs with CAR T-cell therapy. Here, the DNA methyltransferase inhibitor decitabine improved cytotoxicity of the previously described CD44v6 and EGFR CARs against urothelial carcinoma cell lines, but not against healthy control cells. While this effect was not due to changes in antigen or immune checkpoint expression, decitabine altered the balance of pro- and anti-apoptotic gene expression to an apoptosis-sensitive state, which included the upregulation of BID and downregulation of BCL2L1.

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

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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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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).

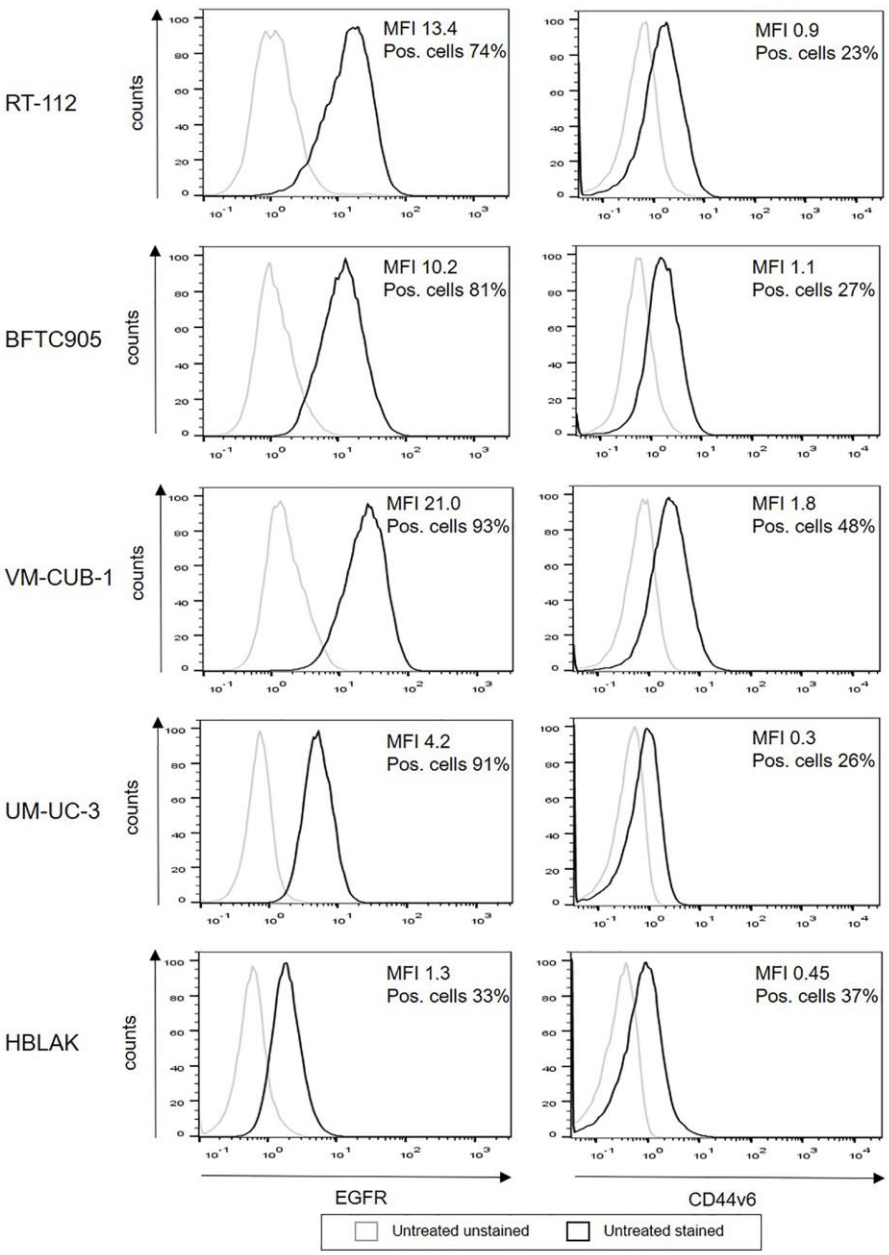

FIGURE 1 | Surface expression of EGFR and CD44v6 on untreated UCC and HBLAK. The UCC RT-112, BFTC905, VM-CUB-1, UM-UC-3, and benign HBLAK were stained with the indicated antibodies and analyzed by flow cytometry. The figure shows representative histograms out of at least three independent experiments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

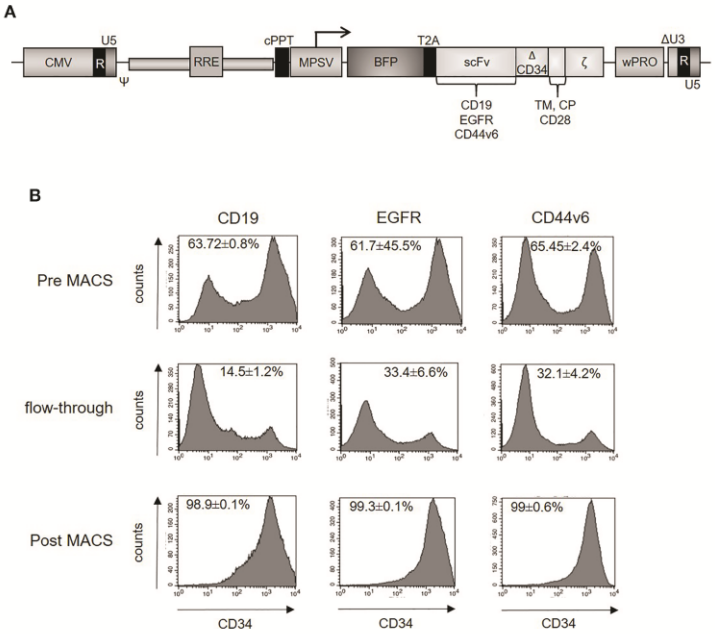

FIGURE 2 | **(A)** Schematic design of the lentiviral vector for expression of the second-generation CARs. The constructs contain CD19, EGFR or CD44v6 scFv, CD34 hinge domain, CD28 transmembrane and intracellular domain, CD3ζ cytosolic domain and blue fluorescent protein (BFP). **(B)** Enrichment of 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

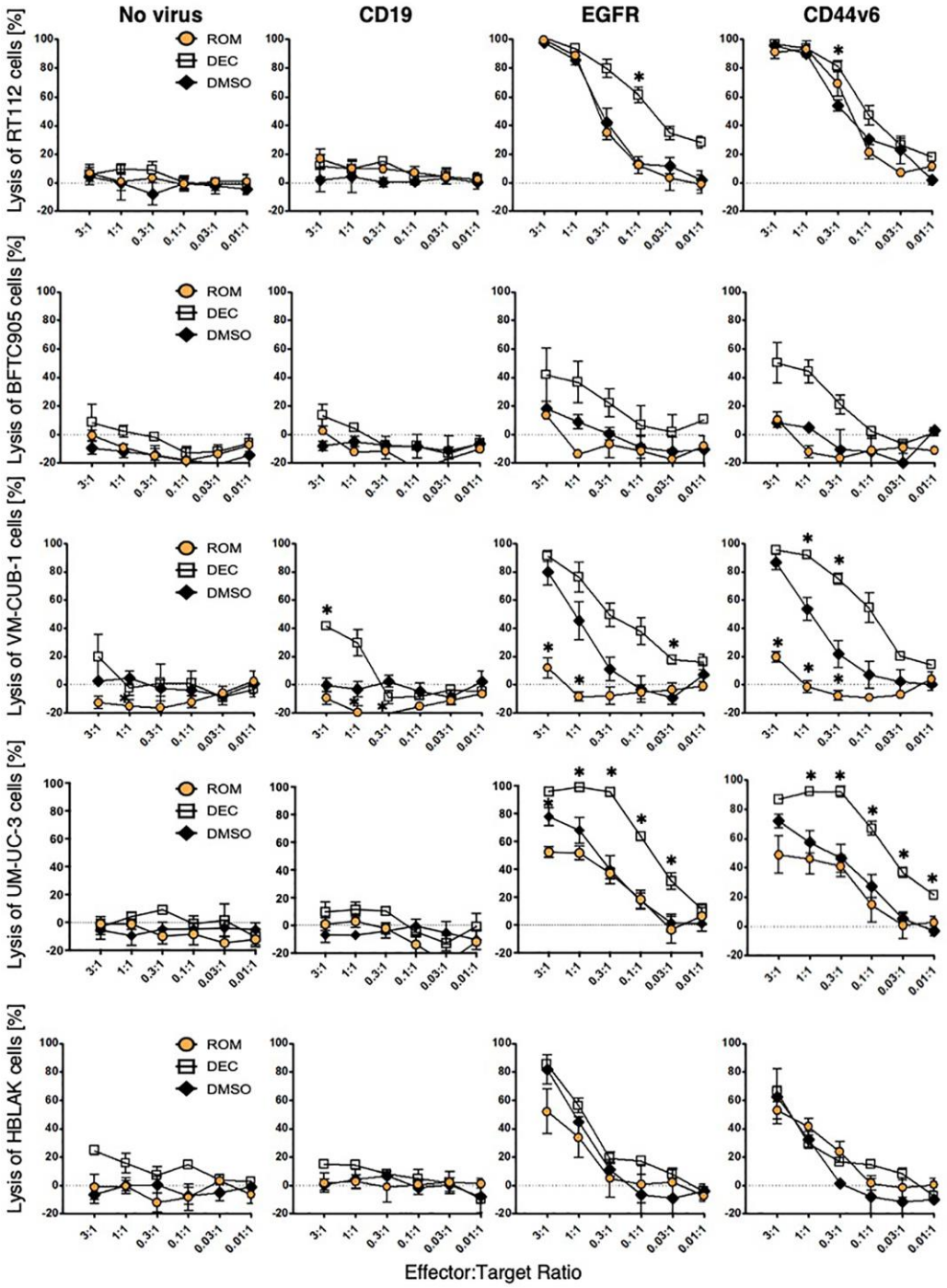

FIGURE 3 | CAR T-cell cytotoxicity can be modulated by epigenetic treatment. UCC and HBLAK cells were pretreated with 100 nM decitabine (white square), 3 nM romidepsin (orange circle), or DMSO (black rhombus) and subsequently co-cultured with CD19-, EGFR-, or CD44v6 CAR T-cells or untransduced (no virus) 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

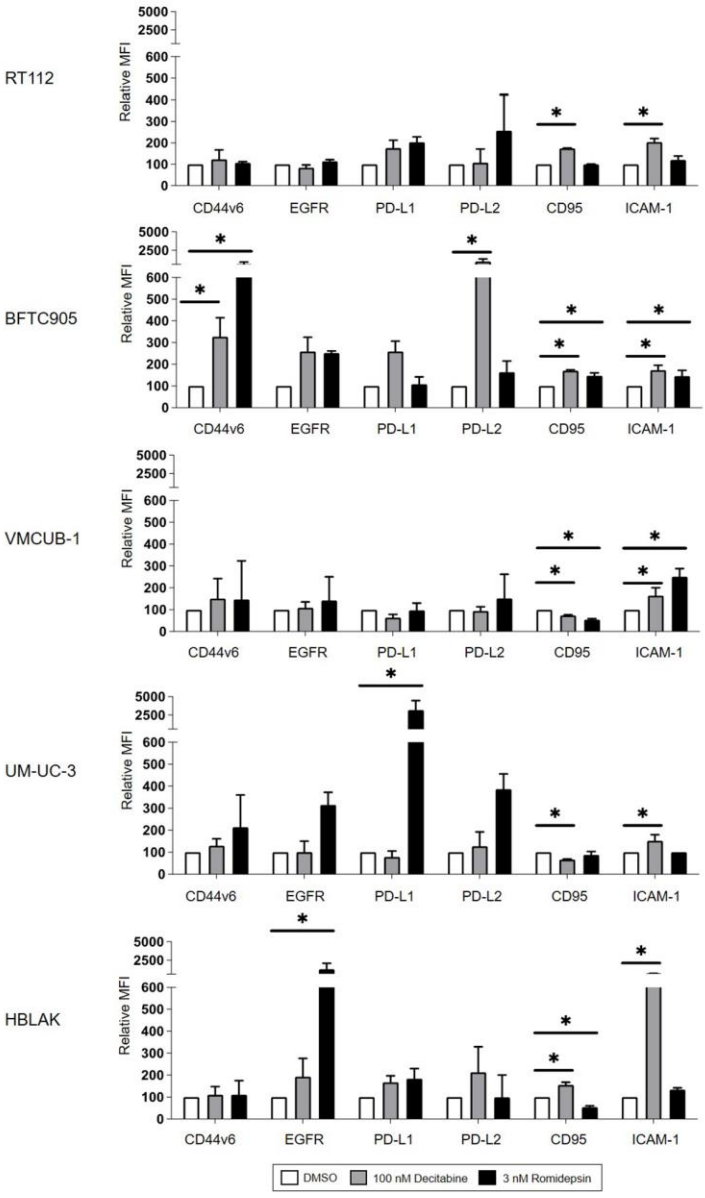

FIGURE 4 | Cell surface protein expression of CD44v6, EGFR, PD-L1, PD-L2, CD95, and ICAM-1 on target cells after DMSO, DEC, or ROM treatment. The graphs represent changes in median fluorescence intensity (MFI) of each marker normalized to DMSO control. White bars represent the values for DMSO-treated 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

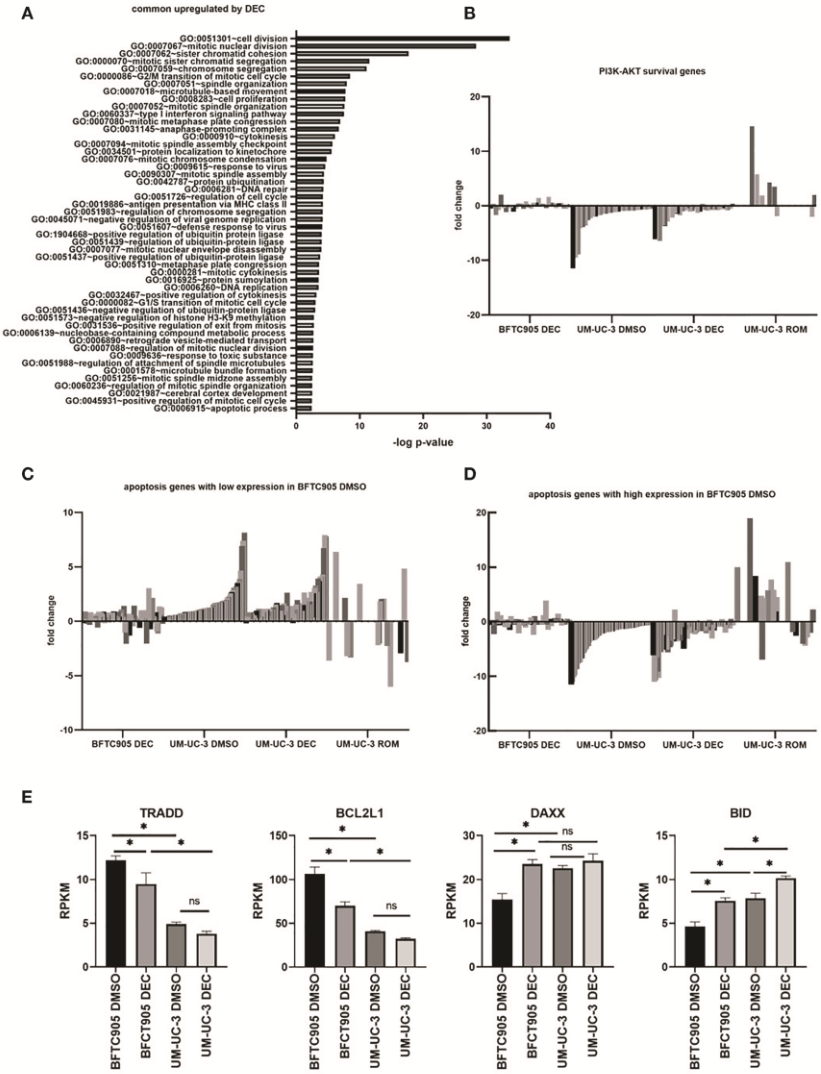

FIGURE 5 | Gene expression changes in UCC BFTC905 and UM-UC-3 after DEC treatment determined by RNA sequencing. **(A)** GO group analysis was performed for 207 genes commonly induced by DEC in both UCC (d7) using the online tool DAVID (34, 41). Only the GOs with p<0.004 are displayed. **(B)** Fold change 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

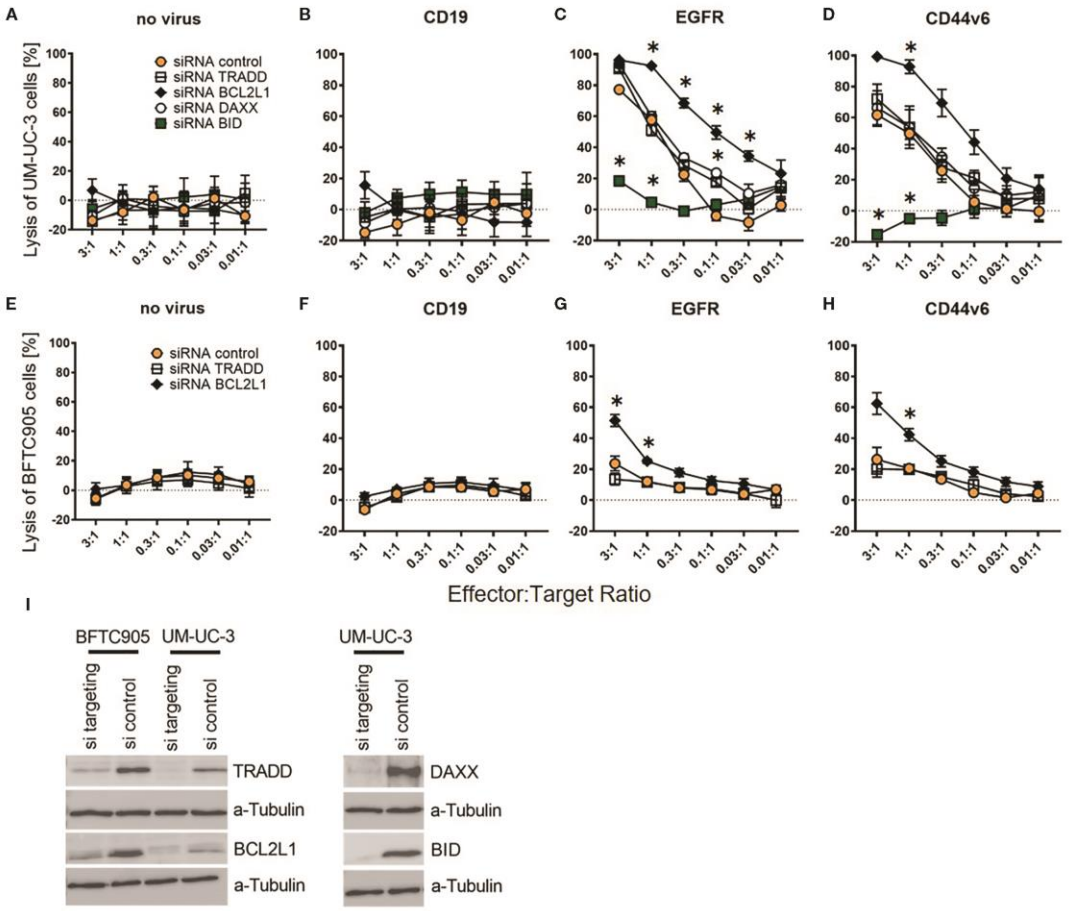

FIGURE 6 | Killing efficiency of EGFR and CD44v6 CAR T-cells against UM-UC-3 and BFTC905 cells after siRNA knockdown of apoptosis regulating genes. **(A)** Cytotoxicity of untransduced (no virus), CD19, EGFR, and CD44v6 CAR T-cells against UM-UC-3 **(A–D)** and BFTC905 **(E–H)** cells transfected with 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.

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SUPPLEMENTARY MATERIAL

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

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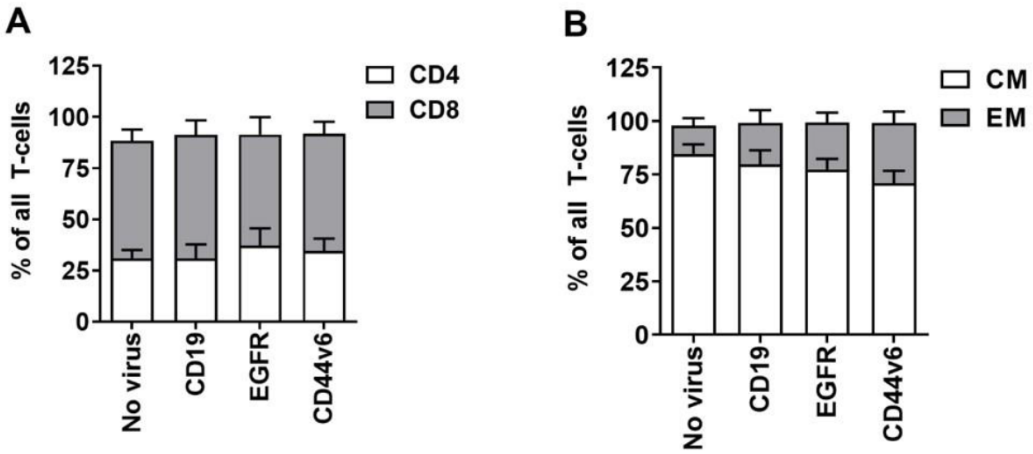
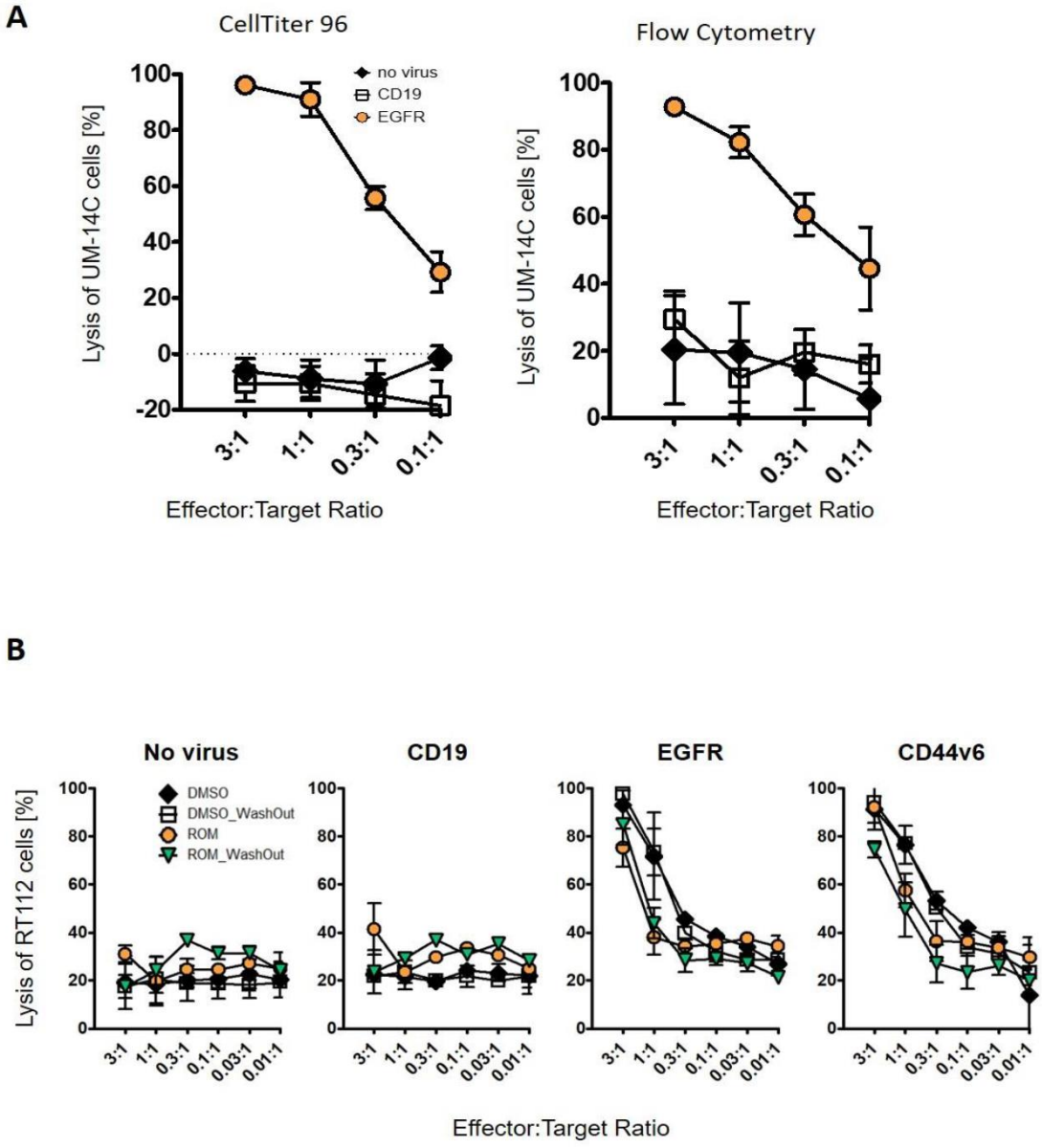
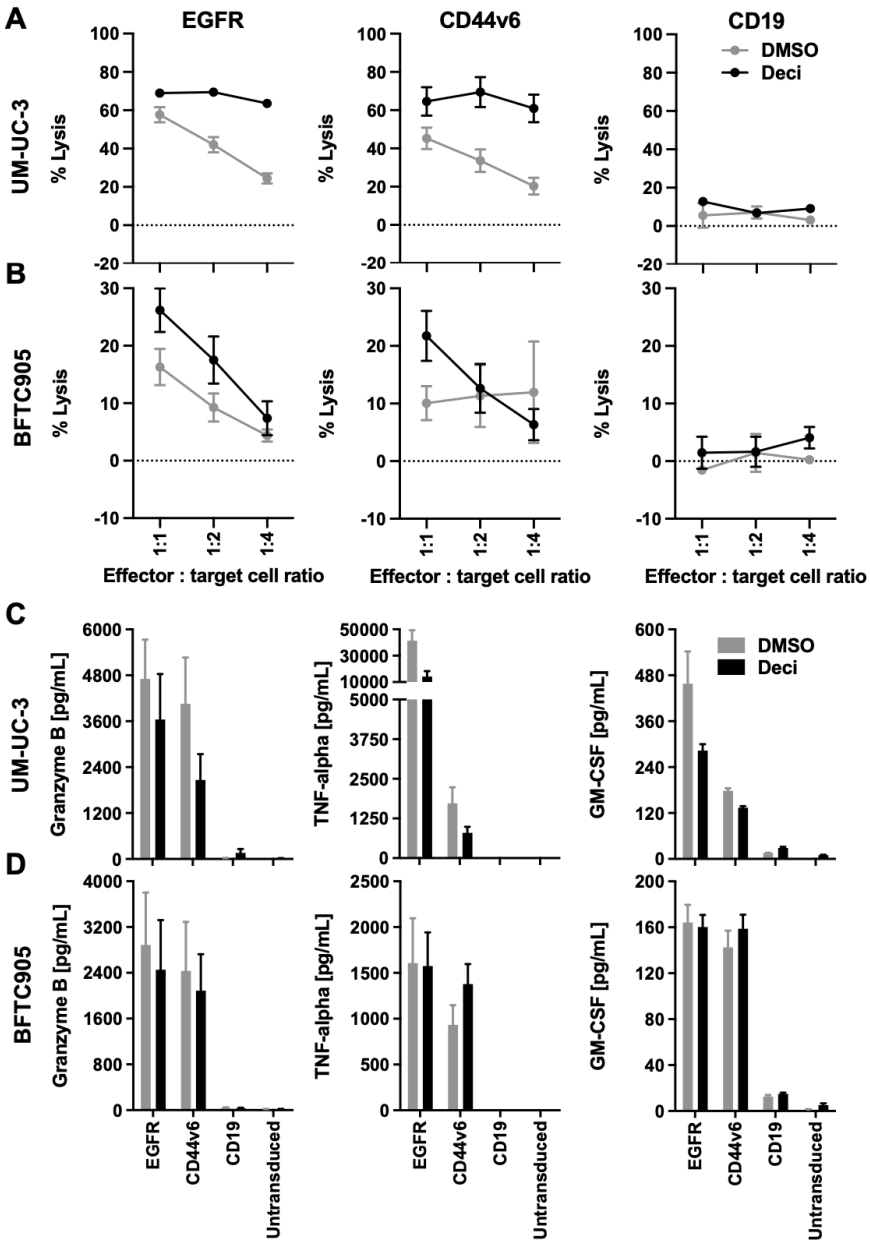
Supplementary Figures



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.

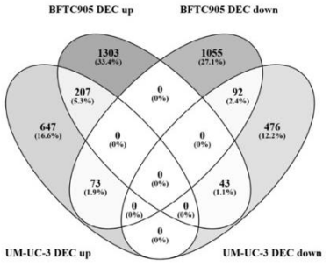


Supplementary Figure S3. CAR T-cell cytotoxicity and cytokine secretion against UM-UC-3 and BFTC905 spheroids. 100 nM DEC (black) and DSMO (grey) pretreated UM-UC-3 and BFTC905 cells were seeded in ultra-low attachment U-bottom 96-well plates. After overnight spheroid formation, CD19-, EGFR- or CD44v6 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.

A

	7 DEC up	7 DEC down
BFTC905	1553	1220
UM-UC-3	927	611

B



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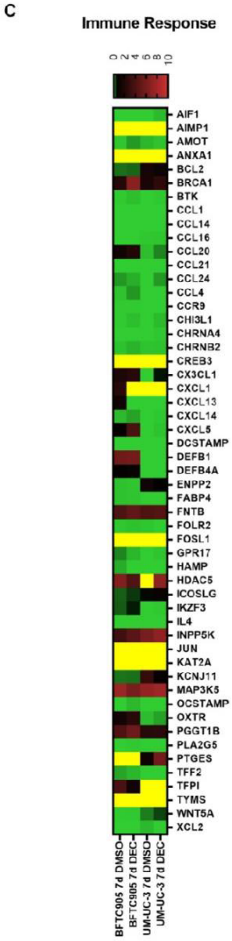
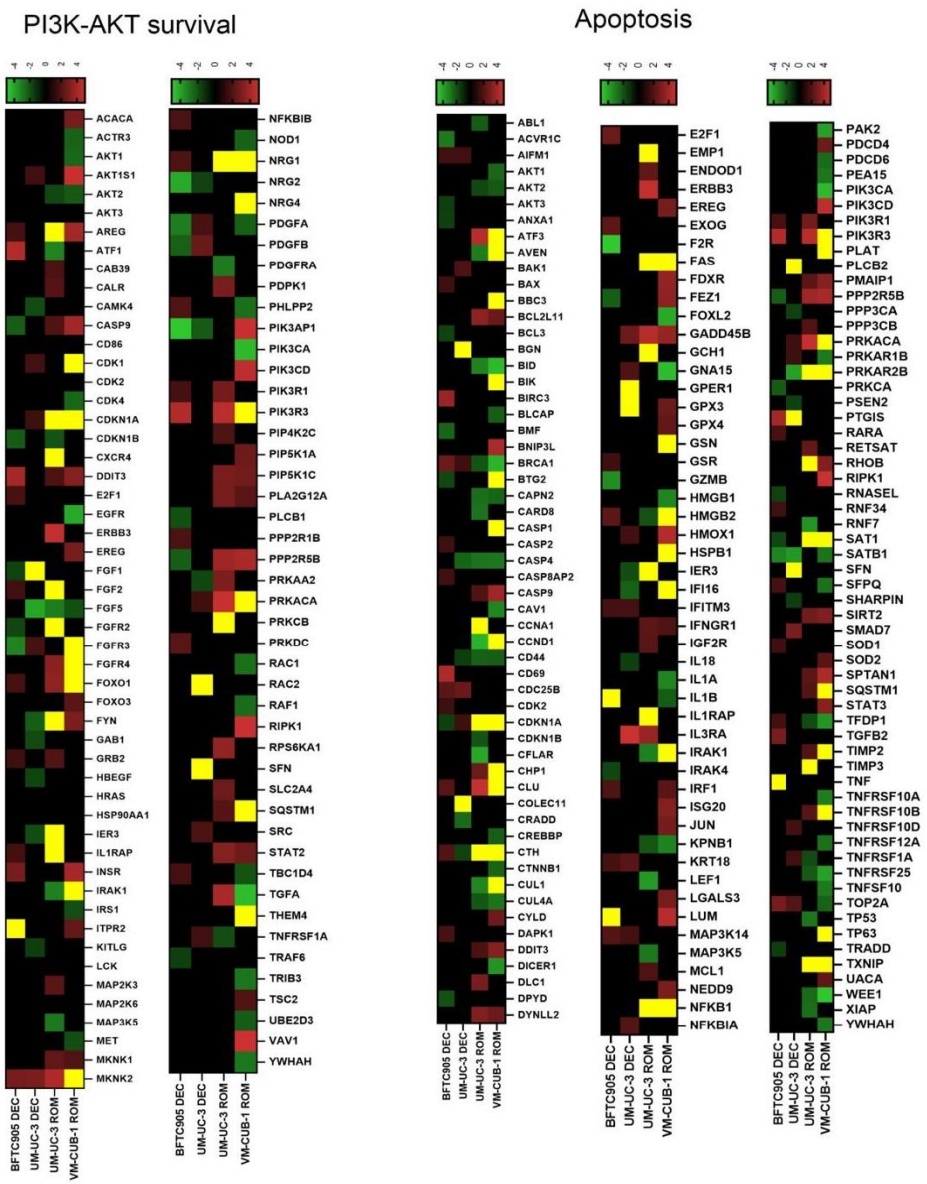


Supplementary Figure S4. DEC induced differential gene expression determined by RNA sequencing. (A) Number of statistical significantly differentially expressed genes seven days after treatment with DEC compared to DMSO controls. As a cut-off we applied ≥ 1.5 fold-change and p ≤0.05 after Bonferroni adjustment. (B) Commonly altered genes between the two cell lines were identified by Venn diagram analysis using the online tool Venny 2.0 [33]. (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.

2.3 Alternative cell types for CAR therapy

2.3.1 Genetic engineering of NK cells for CAR therapy

Title: **Genetic engineering and enrichment of human NK cells for CAR-enhanced immunotherapy of hematological malignancies**

Authors: Maren Soldierer*, **Arthur Bister***, Corinna Haist, Aniththa Thivakaran, Sevgi Can Cengiz, Stephanie Sendker, Nina Bartels, Denise Smorra, Maryam Hejazi, Markus Uhrberg, Kathrin Scheckenbach, Cornelia Monzel, Constanze Wiek, Dirk Reinhardt, Naghmeh Niktoreh, Helmut Hanenberg

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While all previous manuscripts employed T-cells as effector cell, this study focusses on the use of NK cells for CAR therapy. To improve CAR NK cell generation, a protocol for the efficient transduction of NK cells was generated by comparing various lentiviral envelope proteins as well as transduction enhancers and the MPSV promoter was determined as most suited to drive high-level transgene expression in NK cells. Also here, immunomagnetic reagents can be used to produce pure and well defined CAR NK cell populations. Furthermore, to improve the limited persistence of NK cells *in vivo*, NK cells were engineered with IL-15 constructs, which improves their persistence and ultimately also tumor control in an ALL xenotransplantation model.

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Genetic Engineering and Enrichment of Human NK Cells for CAR-Enhanced Immunotherapy of Hematological Malignancies

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The great clinical success of chimeric antigen receptor (CAR) T cells has unlocked new levels of immunotherapy for hematological malignancies. Genetically modifying natural killer (NK) cells as alternative CAR immune effector cells is also highly promising, as NK cells can be transplanted across HLA barriers without causing graft-versus-host disease. Therefore, *off-the-shelf* usage of CAR NK cell products might allow to widely expand the clinical indications and to limit the costs of treatment per patient. However, in contrast to T cells, manufacturing suitable CAR NK cell products is challenging, as standard techniques for genetically engineering NK cells are still being defined. In this study, we have established optimal lentiviral transduction of primary human NK cells by systematically testing different internal promoters for lentiviral CAR vectors and comparing lentiviral pseudotypes and viral entry enhancers. We have additionally modified CAR constructs recognizing standard target antigens for acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) therapy—CD19, CD33, and CD123—to harbor a CD34-derived hinge region that allows efficient detection of transduced NK cells *in vitro* and *in vivo* and also facilitates CD34 microbead-assisted selection of CAR NK cell products to >95% purity for potential clinical usage. Importantly, as most leukemic blasts are *a priori* immunogenic for activated primary human NK cells, we developed an *in vitro* system that blocks the activating receptors NKG2D, DNAM-1, NKp30, NKp44, NKp46, and NKp80 on these cells and therefore allows systematic testing of the specific killing of CAR NK cells against ALL and AML cell lines and primary AML blasts. Finally, we evaluated in an ALL xenotransplantation model in NOD/SCID-gamma (NSG) mice whether human

CD19 CAR NK cells directed against the CD19+ blasts are relying on soluble or membrane-bound IL15 production for NK cell persistence and also *in vivo* leukemia control. Hence, our study provides important insights into the generation of pure and highly active allogeneic CAR NK cells, thereby advancing adoptive cellular immunotherapy with CAR NK cells for human malignancies further.

Keywords: human NK cells, chimeric antigen receptor, genetic engineering, lentiviral vectors (LVS), adoptive cellular immunotherapy, transduction

INTRODUCTION

Cancer immunotherapy, entitled by *Science* as the breakthrough of the year 2013 (1), continues to grow exponentially, and harnessing the power of cellular therapies has contributed significantly to this progress. Although adoptive cellular therapies had been used in the past as rather experimental treatments for patients after stem cell transplantation, with late-stage disease and/or metastatic solid tumors (2–4), the immense potential of cellular immunotherapies became obvious since 2010 through the introduction of second-generation chimeric antigen receptor (CAR) T cells in clinical phase I/II studies (5, 6). Since then, CAR T cells have overcome many limitations of autologous adoptive immunotherapies, which previously were using tumor-infiltrating lymphocytes (TILs) isolated from malignant tissues, expanded *in vitro*, and reinfused into the patients (7). In contrast, CAR T cells do not require extended sampling of tumor tissues, as the patients' autologous circulating T cells are engineered *ex vivo* to recognize tumor-associated antigens, thus making CAR T cell therapy principally applicable for both solid tumors and hematological malignancies (8).

The great clinical success of CAR T cells in early trials for relapsed or refractory hematological malignancies of the B cell lineage has already resulted in the approval of five CAR T cell products, targeting CD19-positive leukemia and lymphoma (Kymriah®, Yescarta®, Tecartus®, and Breyanzi®) or B cell maturation antigen (BCMA)-positive multiple myelomas (Abecma®) (6, 8–10). These second-generation CAR constructs typically consist of extracellular antibody-derived sequences that determine the specificity and two intracellular T cell signaling units, usually the zeta chain of the CD3 complex and either CD28 or 4-1BB/CD137 as co-stimulatory domains. Thus, CARs on competent immune effector cells can recognize surface target antigens independent of any human leukocyte antigen (HLA) constellation and then kill the target cells (6). However, to avoid allogeneic graft-versus-host disease (GvHD), the CAR T cell products need to be generated in autologous settings, which results in very expensive and long manufacturing pipelines. In addition, a high number of clinical trials have reported severe adverse events following autologous CAR T cell treatment, such as life-threatening cytokine release syndrome (CRS) or neurotoxicity (6).

Natural killer (NK) cells are professional immune effector cells of the innate immune system that can recognize and lyse their target cells in a non-antigen-specific manner, thereby enabling them to effectively detect and eliminate malignant cells that have escaped the T cell immune surveillance (11, 12). Most importantly, as NK cells are not HLA-restricted and when transplanted do not cause acute or chronic GvHD, they can readily be administered to HLA-mismatched patients and have, when obtained from healthy donors, significantly shorter manufacturing periods (13). In addition, since large numbers of immune effector cells are required for successful therapeutic transplantation, and leukemia patients often have limited numbers of leukocytes due to their heavy pretreatment regimens, the potential to use allogeneic CAR NK cells of healthy donors for therapeutic infusions would be a major advantage over autologous CAR T cells and allows *off-the-shelf* usage of pre-manufactured products (11). Notably, NK cells can readily be genetically modified with lentiviral vectors (14) and the classical second-generation CARs with either CD28 or 4-1BB signaling domains function well in NK cells and confer additional antitumor effects (11). In preclinical xenograft murine models, the activity of CAR NK cells against malignant cells was similar to that of CAR T cells, albeit with less cytokine release and better overall survival rates, at least for ovarian cancer (15) and acute lymphoblastic leukemia (ALL) (16). Importantly, the first clinical phase I/II trial with CD19 CAR NK cells for the treatment of relapsed or refractory CD19-positive leukemias (NCT03056339) reported high response rates and no treatment-associated occurrence of CRS, neurotoxicity, or GvHD (17). Hence, CAR NK cells for specific target antigens appear to be safe and could potentially be used as *off-the-shelf products*, thus drastically shortening the production time and lowering the costs of CAR-based cellular cancer therapeutics (18).

Compared to a large number of clinical studies with CAR T cells, CAR NK cell therapy development is clearly lagging behind (9). Most of the delay can be attributed to two major problems, the comparably low transduction rates and the challenges in the large-scale genetic engineering of primary human NK cells from healthy donors (19). In contrast to human T cells, which can readily be transduced with lentiviral vectors using the VSV-G glycoprotein as envelope pseudotype (20, 21), NK cells do not express sufficient amounts of the cellular surface molecules of the LDL receptor family that serve as entry receptors for VSV-G (21). Therefore, different envelope pseudotypes are necessary for the efficient entry of the lentiviral particles (22). In their seminal publications, Girard-Gagnepain et al. demonstrated that two constructs derived from the envelope protein of the baboon endogenous virus, BaEV-Rless, and BaEV-TR, are optimally suited for genetically modifying resting hematopoietic cells (14) and also human primary NK cells with lentiviral vectors (21, 23).

The most efficient and reliable large-scale expansion of primary human NK cells up to date can be performed by using genetically engineered cells of the AML cell line K562 as irradiated feeder cells that express the 4-1BB ligand, membrane-bound IL15, and/or IL21 (17, 19, 24, 25). However, using leukemic K562 cells for stimulation of cellular products that will be injected into patients is not ideal.

In this study, we intended to establish the transduction of primary human NK cells derived from peripheral blood in a feeder-cell-free and CliniMACS Prodigy™-compatible system (26) using the NK MACS medium from Miltenyi Biotec and also addressed several issues in genetic engineering of NK cells, including optimized transgene expression, alternative lentiviral pseudotypes, and transduction enhancers. We also established a methodology for the enrichment of human CAR NK cells and developed an assay system to functionally test the CAR-specific cytotoxic activity against leukemic cell lines and primary AML blasts *in vitro*. Finally, we addressed conditions for the prolonged survival of CAR NK cells *in vivo* in immunodeficient NOD/SCID-gamma (NSG) mice with B-cell precursor ALL.

MATERIAL AND METHODS

Cell Culture

The AML cell lines MOLM-14, NOMO-1, CMK, and THP-1 and the ALL cell lines REH and BV-173 were obtained from the DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 GlutaMAX™ medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S; all from Thermo Fisher Scientific, Waltham, MA, USA), referred to as complete RPMI-1640. Human embryonic kidney cells (HEK293T, DSMZ) were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ supplemented with 10% FBS and 1% P/S, referred to as complete DMEM.

Isolation and Culture of Human NK Cells

Primary human NK cells were isolated from peripheral blood of healthy donors, for which they gave written informed consent according to the protocol (#2019-623) approved by the local ethics committee (Heinrich Heine University, Düsseldorf, Germany). Ficoll density gradient centrifugation (Ficoll-Paque Plus; Cytiva Europe, Freiburg, Germany) was performed to collect peripheral blood mononuclear cells (PBMCs). NK cells were then enriched by immunomagnetic negative selection using MojoSort™ Human NK Cell Isolation Kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. NK cell purity and phenotype were determined by flow cytometry (MACSQuant® Analyzer 10, Miltenyi Biotec, Bergisch Gladbach, Germany) on days 0, 7, and 14, using fluorochrome-conjugated antibodies against CD3, CD33, CD56, and CD94 (all REAfinity™ clones from Miltenyi Biotec). NK cells were activated and expanded in NK MACS® Medium (Miltenyi Biotec) supplemented with 5% heat-inactivated human AB serum (Sigma-Aldrich, Darmstadt, Germany), 1% P/S, 500 IU/ml of IL2 (Proleukin, Novartis, Basel, Switzerland), and 10 ng/ml of IL15 (Miltenyi Biotec) at a concentration of 1–2 million cells/ml.

Lentiviral Expression Constructs

All CAR constructs were optimized for human codon usage and synthesized by GeneArt (Thermo Fisher Scientific) or BioCat (Heidelberg, Germany). The CARs were co-expressed *via* a T2A site with the monomeric tag blue fluorescent protein (BFP from Evrogen, Moscow, Russia) under the control of the myeloproliferative sarcoma virus (MPSV) promoter (27) and equipped with our CD34-derived hinge C6 (28), CD28 transmembrane, and co-stimulatory domains as well as the CD3 zeta-chain unit (27). The CD19, CD33, CD123, and epidermal growth factor receptor (EGFR) (clone cetuximab/C225, from now on referred to as Cetux) CARs were previously described (20, 27–29). In the ALL xenograft model, CD19 CARs were co-expressed with codon-optimized soluble human IL15 or human IL15 tethered to the IL15 receptor α-chain (IL15-IL15R). Both IL15 cDNAs used a codon-optimized CD8 leader peptide. The codon-optimized firefly luciferase/enhanced green fluorescent protein (EGFP) fusion (LucEG) (20) and the EGFP/neomycin resistance fusion (EGN) (27) lentiviral expression vectors were previously published. The MPSV, hPGK (human phosphoglycerate kinase 1), modified spleen focus-forming virus (SFFV), and short EF1α (elongation factor 1-α) promoter constructs were also previously published (27). The wild-type SFFV was newly introduced in the pCL6EGNwo vector (27). The optimized EF1α promoter was generated from the long EF1α construct with the wild-type SD and acceptor sites (27) by deleting ~600 bp of the natural intron and mutating an open reading frame (sequences are available upon request).

Production of Lentiviral Particles

The production of lentiviral particles in HEK293T cells was performed on 10-cm dishes as previously described (20, 30). For pseudotyping, the following envelopes were used: the vesicular stomatitis virus glycoprotein (VSV-G, 6 µg per transfection), the codon-optimized baboon endogenous virus lacking the R-peptide (BaEV-Rless, 1 µg per transfection), the codon-optimized BaEV surface and transmembrane units fused to the cytoplasmic units of the amphotropic murine leukemia virus (aMLV; BaEV-TR, 1 µg per transfection), the codon-optimized gibbon ape leukemia virus (GALV) surface unit fused to the transmembrane and cytoplasmic units of aMLV (GALV-TM, 1 µg per transfection), or the previously published (31) feline endogenous virus envelope fused to the cytoplasmic units of aMLV (RD114-TR, 6 µg per transfection). Twenty-four hours after transfection, the culture medium was replaced with 10 ml of IMDM (Sigma-Aldrich) supplemented with 10% FBS, 1% P/S, and 1% L-glutamine (Thermo Fisher Scientific). Cell supernatants containing viral particles were harvested 48 h after transfection, filtered through a 0.45-µm filter, and either used directly or after concentration (5-fold) at 10,000 × *g* for 2 h at 4°C. Lentiviral supernatants were used fresh or stored at −80°C until usage.

Transduction of Human NK Cells

After 7 to 10 days of expansion in a complete NK MACS medium, primary human NK cells were transduced with lentiviral particle-containing supernatants. For the envelope, promoter, and entry enhancer testing, 1 to 1.5 × 10^5 NK cells per well were transduced in flat-bottom 96-well plates in working volumes of 100 µl with serially diluted lentiviral particles encoding EGN. When RetroNectin™ (TaKaRa Bio Inc., Otsu, Japan; from now on referred to as Retronectin) was used as transduction enhancer, non-treated 96-well plates were coated either overnight at 4°C or for 2 h at 37°C prior to use (32, 33). In contrast, Vectofusin®-1 (Miltenyi Biotec, from now on referred to as Vectofusin) was added at a final concentration of 10 µg/ml to tissue culture-treated 96-well plates. For transductions of higher numbers of NK cells, 1 to 1.5 × 10^6 NK cells were transduced on Retronectin-coated 12-well plates (1 ml final volume) or 3.5 to 4 × 10^6 NK cells on Retronectin-coated 6-well plates (2.5 ml final volume) with 500 or 1,250 µl concentrated lentiviral particles, respectively, encoding CD19, CD33, CD123, or Cetux CARs. After 24 h, cells were supplemented with 2 ml (12-well) or 5 ml (6-well) fresh complete NK cell medium. Transduction efficiency was determined 3 to 4 days after transduction by flow cytometry, measuring BFP and/or CD34 (CAR) expression with phycoerythrin (PE)-conjugated QBend-10 CD34 antibody (Thermo Fisher Scientific).

CAR NK Cell Enrichment

Three to four days after transduction, CAR NK cells were enriched on an OctoMACS™ Separator using MS columns or on a QuadroMACS™ Separator using LS columns after staining with CD34 microbeads (clone QBend-10) according to the manufacturer's instructions (Miltenyi Biotec) and similarly as described previously for CAR T cells (28). In order to investigate the MACS enrichment efficiency, the CAR NK cell purity and yield, and the expression of the different CAR constructs on the transduced NK cells, the three cell fractions—before MACS, flowthrough, and after MACS—were collected and analyzed by flow cytometry for BFP expression and/or CD34 positivity. Before any functional analyses were performed, the MACS-enriched CAR NK cells were further cultured for at least an additional 2 days in a complete NK MACS medium.

Blocking and Cytotoxicity Assay

To block the activating receptors, non-transduced and CAR NK cells were incubated with monoclonal REAfinity™ antibodies against NKG2D, DNAM-1, NKp30, NKp44, NKp46, and NKp80 (Miltenyi Biotec) at concentrations of 10 µg/ml in complete RPMI-1640 at 4°C. These REAfinity™ antibodies harbor mutations in their Fc receptors, which prevents their binding to human Fc receptors. Control cells were incubated in parallel with REAfinity™ isotype controls (Miltenyi Biotec). After the 2 h of incubation, the NK cells were added at 3:1, 1:1, and 0.3:1 ratios (without washing) to MOLM-14, NOMO-1, CMK, THP-1, REH and BV-173 cells seeded at 2 × 10^4 cells/well in a 96-well U-bottom plate in complete RPMI-1640 medium. After 16 h, the cultures were harvested, and leukemic cell lysis was analyzed by flow cytometry, using propidium iodide (Sigma-Aldrich) for live/dead cell discrimination. The leukemic cells in the culture were specifically recognized by either EGFP expression (REH, MOLM-14) or after staining with CD15-FITC or CD33-FITC REAfinity™ antibodies. The samples were analyzed on a MACSQuant® Analyzer 10 (Miltenyi Biotec). The specific lysis in % was calculated as [1 – number of viable target cells (sample)/number of viable target cells (control)] × 100%.

NSG Xenotransplantation Mouse Model

All animal experiments were approved by the state animal research committee (LANUV, NRW, Germany). The use of leftover primary blasts from the initial AML diagnosis of children or adolescents treated within the AML-BFM study group was authorized by the ethics committee of the medical faculty at the University of Duisburg-Essen (application number 16-7069-BIO). Each AML-BFM trial was previously approved by their local institutional ethics committees: the Hannover Medical School for AML-BFM 2012 trial and registry (application number 13.03.12/La) and the University of Duisburg-Essen's medical faculty for AML-BFM 2017 registry (application number 17-7462-BO). The primary blasts were injected into 8- to 16-week-old NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (NSG) mice (Charles River Laboratories, Sulzfeld, Germany) and then sacrificed upon the clinical manifestation of the AML (Füchtjohann, Hanenberg, *in preparation*, 2022).

For analyzing the *in vivo* persistence of NK cells, 8- to 10-week-old NSG mice were treated by i.p. injection of 25 mg/kg of busulfan (Busilvex®, Busulfan, Medac, Darmstadt, Germany) and the next day intravenously transplanted with 10 × 10^6 NK cells containing 50% transduced and 50% untransduced NK cells. Transduced NK cells co-expressed either soluble human IL15 and EGFP or human IL15 bound/tethered to the IL15 receptor α-chain (IL15-IL15R) and EGFP. Control mice were transplanted with 10 × 10^6 untransduced NK cells. Each group consisted of 3 animals. Peripheral blood was analyzed at days 5, 10, 20, and 27 after lysis of erythrocytes with BD Pharm Lyse (BD Biosciences, San Jose, CA, USA) on a MACSQuant™ Analyzer 10 for murine CD18, human CD45, CD56, and EGFP-expressing cells (all antibodies were REA clones from Miltenyi Biotec) as described previously (28).

For the engraftment of REH cells expressing a firefly luciferase/EGFP fusion protein (REH^{LucEG}) (28), 7- to 10-week-old NSG mice were treated by i.p. injection of 25 mg/kg of busulfan and the next day intravenously transplanted with 3.5 × 10^6 REH^{LucEG} cells *via* tail vein injections. Two days later, the animals were randomized into different treatment groups and injected with 3.5 × 10^6 NK cells containing 50% positive CD19 CAR NK cells co-expressing BFP, IL15, or IL15-IL15R and 50% untransduced NK cells (6 mice per group, 7 mice as untreated control). Luminescence analysis was performed on days 7, 15, 22, and 27 as previously described (20) to assess the progression/proliferation of the ALL blasts. Peripheral blood was obtained at days 8, 15, and 22 *via* the tail vein and, after lysis of erythrocytes with BD Pharm Lyse, analyzed on a MACSQuant™ Analyzer 10 for EGFP positive REH^{LucEG} cells and BFP, CD34, CD56, and CD94 NK cells (CD34-PE from Thermo Fisher Scientific, all other antibodies from Miltenyi Biotec).

Fluorescence Microscopy

MACS-enriched CAR NK cells co-expressing BFP were stained with the QBend-10 CD34-PE antibody (as described above) and then co-cultured with EGFP-expressing REH cells in complete DMEM at an NK cell to target cell ratio of approximately 0.3:1. For these experiments, gelatin-treated 8-well slides with glass bottom (ibidi GmbH, Gräfelfing, Germany) were used. Bright-field and epifluorescence images were acquired at 37°C for 1–4 h after the seeding using an IX83 Inverted Microscope (Olympus, Hamburg, Germany), a 100× oil objective (NA 1.49, UAPON100XOTIRF, Olympus, Hamburg, Germany), and the cellSens Dimension software (Olympus, Hamburg, Germany). Images were processed using the open-source Fiji software (https://imagej.net/software/fiji/).

Statistical Analysis

Statistical analyses were performed using GraphPad PRISM 9.0 using the log-rank test, one-way ANOVA with Tukey adjustment for multiple comparisons, and unpaired Student's t-test for single comparison. p-Values ≤ 0.05 were considered significant and indicated with an asterisk.

RESULTS

To define optimal conditions required for the generation of CAR-expressing primary human NK cells and their expansion under Good Manufacturing Practice (GMP)-compliant conditions recently published in cooperation with Miltenyi Biotec (26), we systematically tested a number of variables that are known to influence the cytotoxicity of genetically modified NK cells.

Lentiviral Pseudotypes for Efficient Transduction of Human NK Cells

Recently, two constructs derived from the envelope protein of the baboon endogenous virus, BaEV-Rless and BaEV-TR (14) have been published that are optimally suited for genetically modifying NK cells (21, 23). We additionally wanted to assess the transduction efficiency achieved in human NK cells with an alternative envelope pseudotype, GALV-TM, a chimera generated from the gibbon ape leukemia virus and the aMLV envelope proteins (34), that we previously used for the transduction of human CD34+ stem cells (35). For efficient virus production, our lentiviral vector plasmid pCL6EGNwo, expressing a fusion protein of EGFP and the neomycin resistance gene under the control of a modified SFFV promoter (27), was pseudotyped with the three envelopes. As additional controls, we also pseudotyped the lentiviral vector with the feline endogenous virus chimeric construct RD114-TR (31) and VSV-G (20).

Primary human NK cells were isolated and expanded as previously described (26) and then transduced with serially diluted (1:10 to 1:10,000) non-concentrated supernatants of freshly produced lentiviral vector particles on Retronectin-coated 96-well plates. Three to four days after transduction, NK cells were analyzed by flow cytometry for EGFP expression. As shown in **Figure 1A**, BaEV-Rless was the most efficient pseudotype, as 28% of NK cells were EGFP positive using 1:10-diluted viral supernatant; even a 1:1,000 dilution of this pseudotype was associated with a higher transduction efficiency than VSV-G at a 1:10 dilution. The lentiviral vectors pseudotyped with BaEV-TR or GALV-TM transduced approximately 9% (at 1:10) to 0.3% (at 1:1,000) of the NK cells. While being more efficient than VSV-G, the RD114-TR pseudotype was still rather inefficient with gene transfer rates ranging from 2.2% to 0.1%.

Retronectin *vs.* Vectofusin for Enhancing the Transduction of Human NK Cells

To promote the binding of lentiviral particles on the surface of target cells and thereby increase the likelihood of viral entry, cationic culture additives such as polybrene or protamine sulfate have been used during the transduction procedure and neutralize the electrostatic repulsion between the opposing negatively charged membrane bilayers (36, 37). Vectofusin is such a histidine-rich cationic amphipathic peptide that was developed to promote efficient lentiviral transduction of primary human cells (38), including T and NK cells (39). Alternatively, a recombinant chimeric fragment derived from human fibronectin, CH-296 or Retronectin, is used since 1996 in research settings and in clinical trials to promote colocalization of viral particles and mammalian target cells, thereby increasing the efficiency of the genetic modification for both adherent and non-adherent cells (32, 33). Although the effects of Vectofusin and Retronectin on the transduction of different non-adherent cell types including human CD34+ hematopoietic stem cells (38) and also primary human NK cells (23, 39) have been reported, this type of comparison has not been performed for the lentiviral BaEV pseudotype that mediates the most efficient gene transfer into primary human NK cells (**Figure 1A**).

Therefore, we transduced primary human NK cells with the pCL6EGNwo vector (27) pseudotyped with BaEV-Rless, BaEV-TR, or GALV-TM using serial dilutions, ranging from 1:2 to 1:1,000. Transduction was performed in flat-bottom 96-well plates, either coated with Retronectin or after supplementing Vectofusin to the culture medium. Control NK cells were transduced in 96-well cell culture plates without any additional transduction enhancer. The gene transfer efficiencies into the human NK cells were analyzed 3 to 4 days later by flow cytometry, and the values were normalized by assigning 100% to the highest transduction efficiency achieved within the cell series of each donor. Remarkably, the BaEV-Rless pseudotype on Retronectin mediated the highest gene transfer in each of the four donors and was therefore set at 100%, while the other two pseudotypes BaEV-TR and GALV-TM did not even achieve 50% of the BaEV-Rless transduction efficiency (**Figure 1B**). Using Vectofusin with the BaEV-Rless-pseudotyped lentiviral particles resulted in roughly 80% of the Retronectin-assisted gene delivery and was clearly better than the gene transfer efficiencies observed with the other two pseudotypes BaEV-TR and GALV-TM. The gene transfer without any transduction enhancer was very low regardless of the glycoprotein used. Therefore, for the remaining experiments in this study, we chose to perform the lentiviral transductions of primary human NK cells with the BaEV-Rless pseudotype on Retronectin-coated plates.

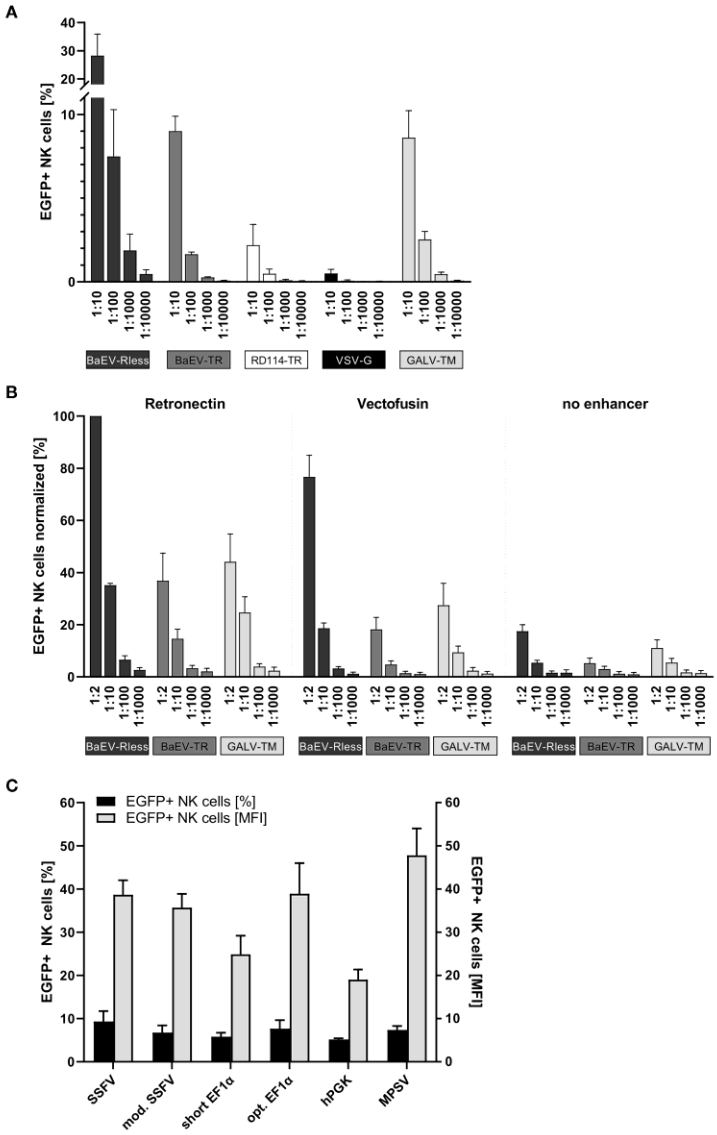

FIGURE 1 | Systematic improvement of primary human NK cell transduction. Primary human NK cells were isolated and cultured as described in the body of the manuscript. **(A)** After 7 to 10 days of expansion, NK cells were transduced with serial dilutions of EGFP-expressing lentiviral particles pseudotyped with BaEV-Rless, BaEV-TR, RD114-TR, VSV-G, or GALV-TM. Three to four days after transduction, EGFP expression was analyzed by flow cytometry. Data are represented as mean ± SEM of four biological replicates. **(B)** Primary human NK cells were transduced with serial dilutions of EGFP-expressing lentiviral particles pseudotyped with BaEV-Rless, BaEV-TR, or GALV-TM, using Retronectin, Vectofusin, or no transduction enhancer, respectively. Three to four days after transduction, EGFP expression was analyzed by flow cytometry. Data are represented as mean ± SEM of five biological replicates. **(C)** Primary human NK cells were transduced with serial dilutions of lentiviral particles pseudotyped with BaEV-Rless, expressing EGFP under the control of the wild-type or modified SFFV, the wild-type or optimized EF1α, the hPGK, or the MPSV promoter. Three to four days after transduction, the expression of EGFP was analyzed. From the serial dilutions, samples with gene transfer rates of between 5% and 10% were analyzed for their EGFP expression intensity [mean fluorescence intensities (MFIs)]. Data are represented as mean ± SEM of four biological replicates.

A Promoter for High-Level Transgene Expression in Human NK Cells

We have previously shown that the U3 region of the MPSV as an internal promoter in our self-inactivating (SIN) lentiviral vectors is the best choice for achieving high-level gene expression in primary human T cells (27). Here, in order to identify an optimal internal promoter for stable high-level CAR expression in primary human NK cells, we tested the MPSV and two

additional viral promoters as internal promoters in our lentiviral pCL6EGNwo vector (27): the U3 regions from SFFV (40) with one (mod. SFFV) or with two enhancer regions (SFFV). We also included two promoters of human cellular housekeeping genes, namely, the human phosphoglycerate kinase-1 (hPGK) (27) and the human elongation factor 1α (EF1α) promoter with (opt. EF1α) or without (short EF1α) an optimized splicing unit as internal promoters.

Primary human NK cells were transduced with serially diluted BaEV-Rless-pseudotyped lentiviral vectors expressing the EGFP-neomycin fusion as marker gene under the control of the different promoters. Three to four days after transduction, NK cells were analyzed for EGFP expression by flow cytometry. We compared the mean fluorescence intensity (MFI) of the different promoters in samples with a gene transfer of approximately 5%–10%, as cells with this gene transfer rate most likely carry only one copy of the vector integrated into their genome (27), thereby eliminating the bias of multiple integrations on the transgene expression levels. As shown in **Figure 1C**, the hPGK and short EF1α promoters with MFIs of 19.0 and 24.9, respectively, were associated with the lowest transgene expression levels. Optimizing the splice unit of EF1α by shortening the intron and correcting an open reading frame in the intron improved the transgene expression levels of the optimized EF1α promoter to those of the SFFV promoters with MFIs ranging from 35.7 to 38.9. However, the MPSV U3 promoter with an MFI of 47.8 still provided the highest level of transgene expression, and thus it was chosen for the expression of CAR constructs in primary human NK cells for our subsequent experiments.

Transduction and Enrichment of Human CAR NK Cells

After the establishment of an optimized transduction protocol, primary human NK cells with low CD33 expression were transduced with 5-fold concentrated BaEV-Rless-pseudotyped lentiviral particles encoding BFP *in cis* with CD19, CD33, CD123, or EGFR CARs, harboring our CD34-derived hinge (20, 29), on Retronectin (**Figures 2A, B**). Three to four days after transduction, the expression of the CARs was analyzed after staining of the C6 hinge region with the QBend-10 antibody and detecting BFP expression by flow cytometry. As shown in **Figure 2C**, CD34/CAR expression strongly correlated with BFP expression for all four CAR constructs. To purify the CAR NK cells, we then performed MACS enrichment on an OctoMACS™ separator using MS columns and CD34 microbeads binding to the C6 hinge. As an indicator of the content of transduced NK cells, BFP expression was analyzed in three fractions: before MACS, the flowthrough, and after MACS (representative samples in **Figure 2D**). Before MACS enrichment, we obtained on average of between 38.8% ± 3.4% (Cetux) and 47.0% ± 3.7% (CD123) CAR-positive NK cells. Enrichment of CAR NK cells on MACS columns led to purification rates of between 96.2% ± 1.2% (CD123) and 97.7% ± 0.8% (CD19). However, the flowthrough contained relatively high percentages of CAR NK cells (between 24.4% ± 2.2% for CD123 and 29.0% ± 4.2% for Cetux), albeit with much lower MFIs than the CAR NK cells before or after MACS (**Figure 2E**).

Reduction of Background Killing of Leukemic Cell Lines by Activated Human NK Cells

For more than 40 years, it is well established that after stimulation, primary human NK cells can exhibit high cytotoxic activities against human leukemic cells (41). This cytotoxicity is often mediated by the expression of antigens on the leukemic blasts that are ligands for activating receptors on NK cells (42). As *in vitro* expanded NK cells are highly activated (26), they often recognize and thus kill leukemia cells independently of CAR antigen binding (42). In order to eliminate this "background" killing for follow-up assays, we planned to systematically block activating receptors of the NK cells in cytotoxic experimental settings. To this end, we co-cultured cells of four AML cell lines and two B-cell precursor ALL cell lines overnight with three ratios of PB-derived 7- to 14-day-old NK cells with the effector-to-target cell ratios of 3:1, 1:1, and 0.3:1. In parallel, overnight co-cultures were set up with the same NK cells, albeit preincubated with monoclonal antibodies that recognized and block either three (NKG2D, NKp30, and NKp46) or six (NKG2D, DNAM-1, NKp30, NKp44, NKp46, and NKp80) activating receptors on NK cells (11, 43). Although these recombinant antibodies were all mutated in their Fc domains and therefore should not bind to human Fc receptors, we still included a co-culture condition with NK cells that were preincubated with the appropriate isotype controls.

As demonstrated in **Figure 3**, the degree of target cell lysis by activated NK cells seemed to be cell line-specific. On the other hand, three AML cell lines [MOLM-14 (AML-M5), THP-1 (AML-M4), and CMK (AML-M7)] and two B-cell precursor leukemic lines [REH and BV-173] were killed efficaciously by the activated NK cells in the absence of blocking antibodies and also in the presence of isotype controls; NOMO-1 (AML-M5) cells were resistant to NK cell killing. Importantly, preincubation with all six antibodies ameliorated the cytotoxicity of the NK cells against the five susceptible cell lines quite efficiently; however, using the three antibodies against NKG2D, NKp30, and NKp46 only partially reduced the NK cell-mediated killing for two AML cell lines, MOLM-14 and CMK (**Figure 3**).

CAR NK Cells Effectively Kill AML and ALL Cell Lines as Well as Primary AML Blasts *In Vitro*

After successfully establishing a blocking protocol, we specifically tested the functionality of CAR NK cells against one ALL and two AML cell lines. To this end, MACS-enriched CAR NK cells of at least five healthy donors with low CD33 expression (data not shown) were blocked with all six monoclonal antibodies described above and then co-cultured overnight with NOMO-1, MOLM-14 (both CD19− CD33+ CD123+), and REH (CD19+ CD33− CD123-) cells at various effector-to-target cell ratios. Flow cytometric analysis revealed the expression patterns of

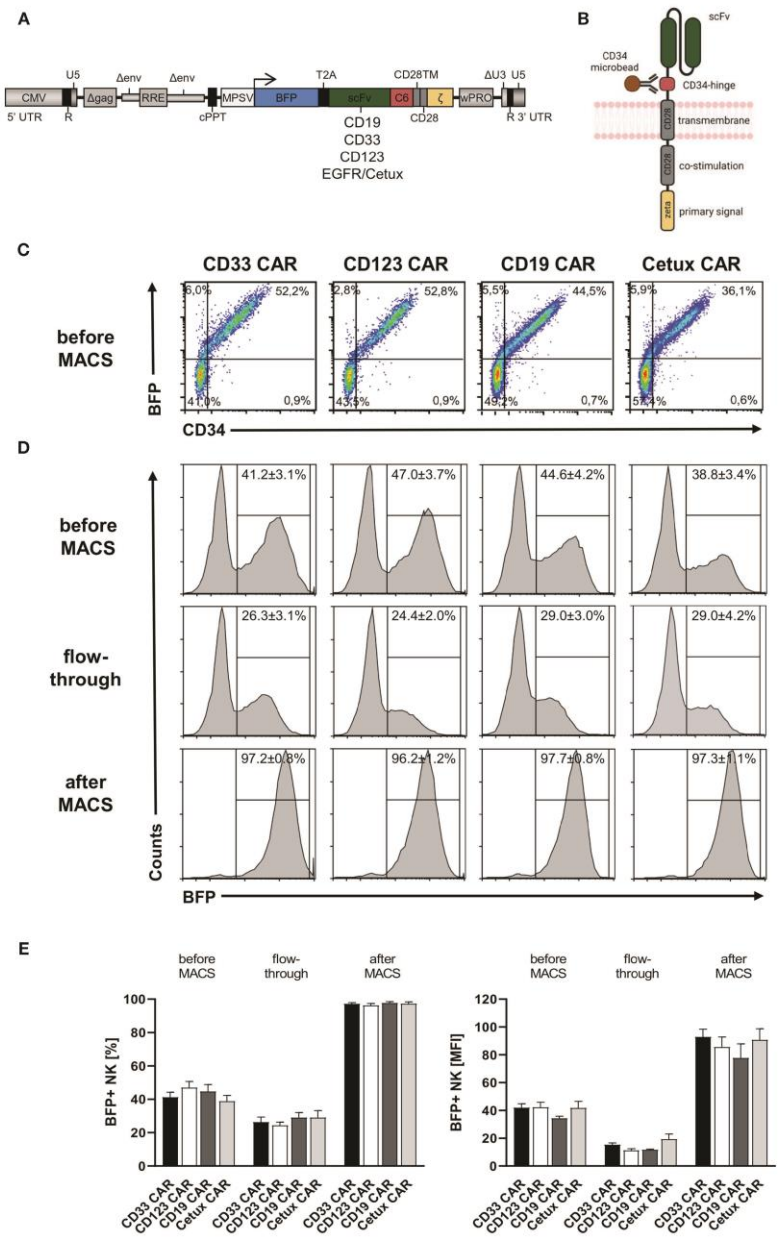

FIGURE 2 | CD34-hinged CAR expression and enrichment in primary human NK cells. Primary human NK cells were transduced with the lentiviral vectors delineated in panels **(A, B)** encoding BFP *in cis* with CD34-hinged CD33, CD123, CD19, or Cetux CARs. **(B)** Schematic representation of CAR construct including CD34-derived C6 hinge for microbead interactions. **(C)** Three to four days after lentiviral CAR transduction, NK cells were analyzed for BFP and CAR (via CD34-PE staining) expression by flow cytometry. Representative dot plots are shown. **(D, E)** CD34-hinged CAR NK cells were enriched *via* magnetic cell sorting using CD34 microbeads and three fractions (before MACS, flowthrough, and after MACS) were analyzed for the content of NK cells expressing BFP by flow cytometry. The histogram blots show representative data. Data are represented as mean ± SEM of at least four biological replicates.

CD19, CD33, and CD123 for each of the target cell lines (**Figure 4A**). After 16 h of co-culture, CD33 and CD123 CAR NK cells efficaciously killed NOMO-1 and MOLM-14 cells with approximately 90% specific lysis at an effector-to-target cell ratio of 1:1 (**Figure 4B**). Interestingly, although CD123 is expressed much lower on NOMO-1 cells than CD33 (**Figure 4A**), CD123 CAR NK cells performed as well as CD33 CAR NK cells in the NOMO-1 co-cultures. REH cells were highly efficaciously (>95%

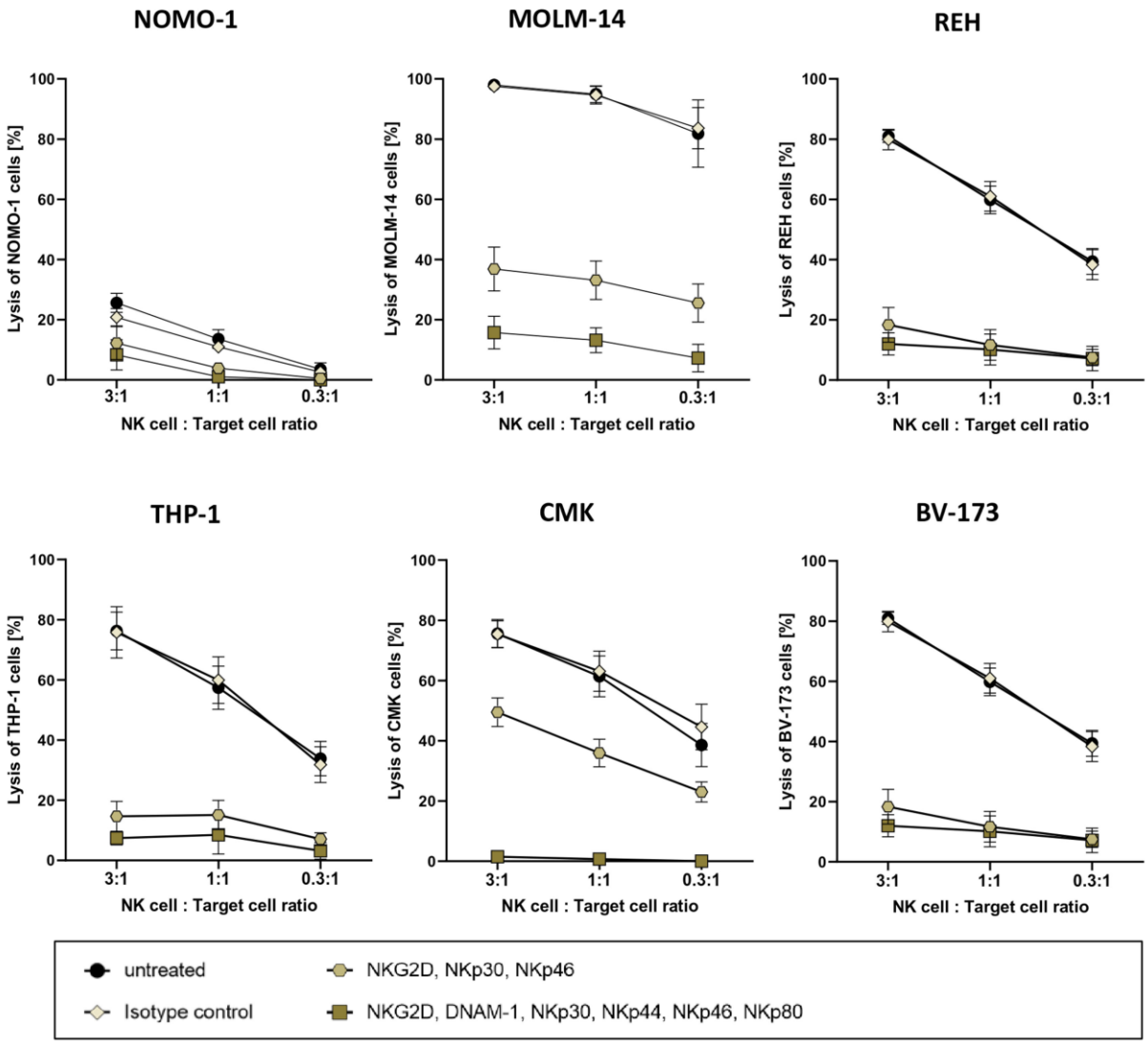

FIGURE 3 | Cytotoxic potency of activated primary human NK cells against acute leukemia. Killing of various leukemic cell lines by activated primary human NK cells was analyzed by co-culture of NK cells and target cells at several ratios. Activated primary human NK cells were incubated with blocking antibodies against activation receptors (as stated) and co-cultured with NOMO-1, MOLM-14, REH, THP-1, CMK, and BV-173 cells at various ratios to determine cytotoxic activity of NK cells. After 16 h of co-incubation at 37°C, the cytotoxicity of the treated and untreated NK cells was analyzed *via* flow cytometry, and the degree of the cell lysis was analyzed as described in the *Material and Methods*. The graphs represent mean ± SEM of four biological replicates.

at 1:1) eradicated by CD19 CAR NK cells, but not by the other CAR constructs (**Figure 4B**). Importantly, the lysis was strongly dependent on the specific antigen expression, and we did not observe off-target toxicities in these experiments.

Next, we hypothesized that the CAR NK cells would also specifically kill primary AML blasts obtained at diagnosis and expanded in NSG mice (Füchtjohann, Hanenberg, *manuscript in preparation 2022*). As AML blasts can express low levels of CD19, we used the EGFR-recognizing Cetux CAR NK cells as negative controls. Flow cytometric analysis revealed the expression patterns of EGFR, CD33, and CD123 for the primary AML blasts used (**Figure 4C**). The results in **Figure 4D** demonstrated that the CD33+ CD123+ primary AML-M4 blasts were specifically killed (up to 90% at 1:1), while only 60% of CD33+ CD123+ primary AML-M5 blasts were eliminated by both CD33 and CD123 CAR NK cells at 3:1 and 1:1 ratios, respectively.

Finally, we took advantage of the fact that our CARs contained the CD34-derived C6 hinge to visualize the specific interaction of CD19 CAR NK cells with the CD19-positive REH

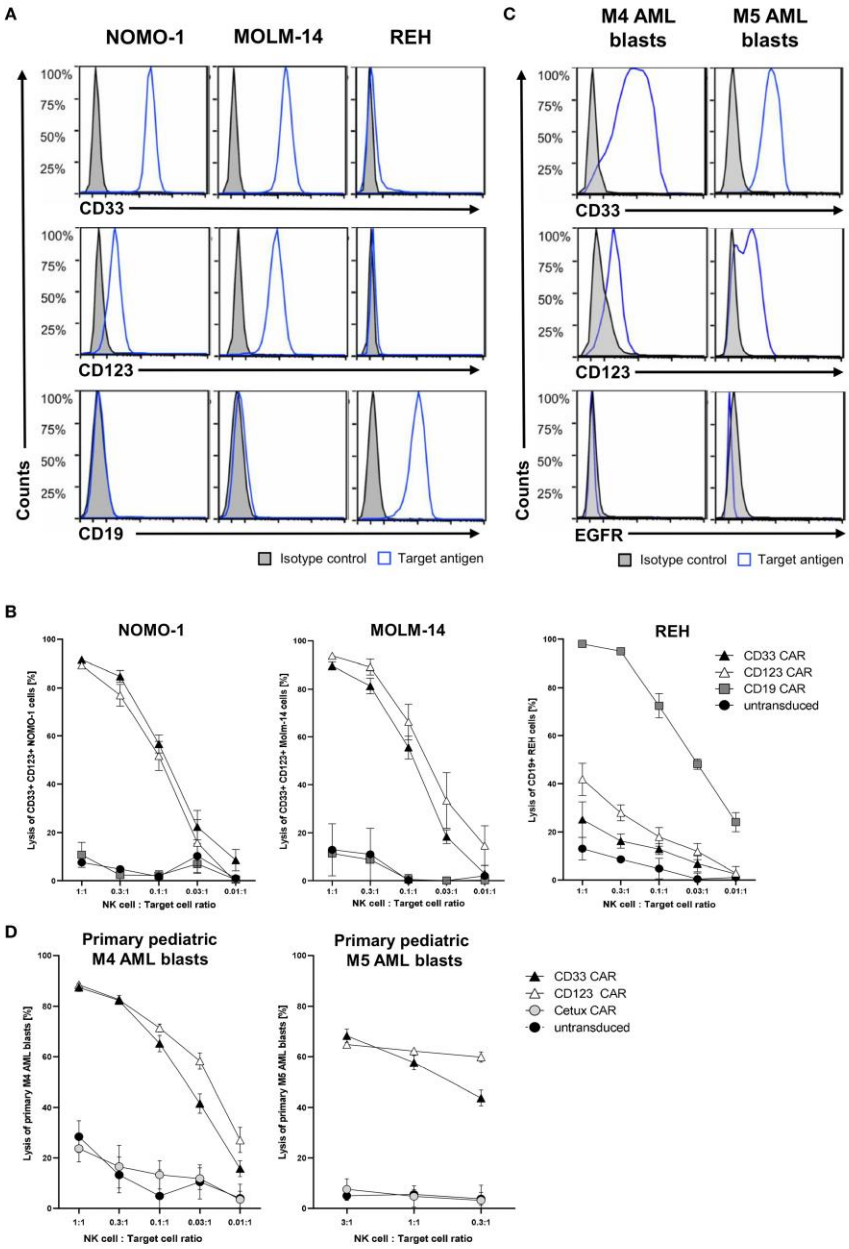

FIGURE 4 | Cytotoxic activity of CAR NK cells against leukemic cells. **(A)** AML cells NOMO-1 and MOLM-14, and ALL cells REH were analyzed for their surface expression of CD19, CD33, and CD123. **(C)** Primary pediatric M4 and M5 AML blasts were analyzed for their surface expression of CD33, CD123, and EGFR using conjugated antibodies by flow cytometry. CAR-transduced and microbead-enriched primary human NK cells were blocked as described previously and co-cultured with NOMO-1, MOLM-14, and REH cells **(B)** or primary pediatric M4 and M5 AML blasts **(D)** at various effector-to-target cell ratios to determine the specific cytotoxic activity of CAR NK cells. The graphs represent mean ± SEM of at least five (cell lines) or three (AML blasts) biological replicates.

cells in the presence of blocking antibodies. For the fluorescence microscopy analysis, the PE-conjugated QBend-10 antibody was employed to label the hinge region of CD19 or CD33 CAR NK cells, which both co-expressed BFP, while the target REH cells expressed EGFP. As indicated by the white arrow in **Figure 5A**, the CD19 CAR constructs on the transduced NK cells accumulated in immunological synapses formed between 1 and 4 h between NK and REH cells. In contrast, the CD33 CAR

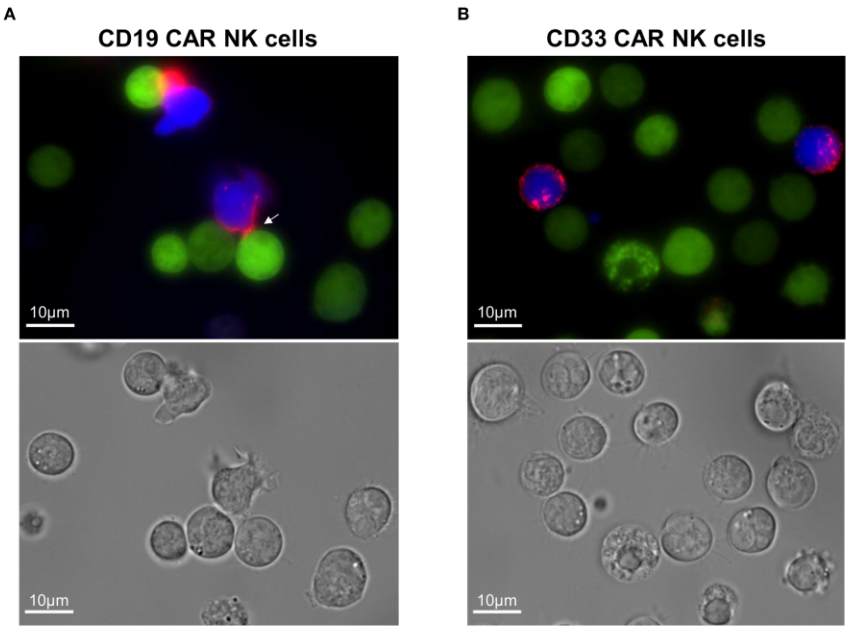

FIGURE 5 | Visualization of the immunological synapses formed between CD19 CAR NK cells and CD19+ REH cells. **(A)** The immunological synapse between CD19 CAR NK cells and CD19+ REH cells was analyzed by fluorescence microscopy, visualizing the CAR expression (CD34-PE-conjugated antibody, red) on MACS-enriched CAR NK cells (BFP expression, blue) in relation to REH cells (EGFP expression, green). **(B)** MACS-enriched CD33 CAR NK cells served as negative controls. Contrast settings are uniform for both images for the channels: brightfield, 377/447 and 470/525 nm. The contrast in channel 545/620 nm was adjusted to maximize the visibility of the cell-to-cell contacts.

constructs were equally dispersed on the cell surfaces of CD33 CAR NK cells (**Figure 5B**), suggesting that no specific interaction with the CD33-negative REH cells occurred.

CD19 CAR NK Cells Effectively Kill CD19+ ALL Cells *In Vivo*

For *in vivo* application of activated NK cells in humans, it is paramount that NK cells do not possess autocrine stimulation loops that would ensure their survival beyond a few days in patients (44, 45). Therefore, allogeneic NK cell therapies are usually accompanied by the subcutaneous application of IL2 or intravenous infusions of lentiviral IL15; both treatments are actually associated with severe side effects in patients (44, 45). In order to evaluate the impact of IL15 signaling on the persistence of NK cells *in vivo*, we generated two lentiviral IL15 vectors (**Figure 6A**): the first expressed soluble human IL15 and EGFP, and the second human IL15 tethered to the IL15 receptor α-chain (IL15-IL15R) and EGFP. Importantly, both IL15 constructs were able to mediate the expansion of primary NK cells in *in vitro* experiments in the absence of any other growth factors (data not shown). Next, NSG mice were injected with 5×10^6 EGFP+ NK cells expressing either soluble IL15 or IL15-IL15R and 5×10^6 untransduced NK cells. We used this 1:1 ratio *in vivo* to investigate potential survival advantages of IL15 expressing NK cells over untransduced NK cells in each mouse and also to understand the impact of IL15-tethering for both populations. The persistence of human NK cells was assessed for 27 days (**Figure 6B**), using the gating strategy shown in **Figure 6C** for the peripheral blood analysis on day 10. **Figure 6D** reveals that the NK cell survival was highly dependent on IL15 signaling, as the percentages of NK cells in the blood of the mice transplanted with untransduced NK cells progressively decreased over time. In contrast, both strategies to express IL15 *in vivo* drastically improved the initial NK cell engraftment and also the persistence of NK cells at all sampling time points. Remarkably, the ratio of EGFP positive to negative cells stayed rather constant at 1:1 for the soluble IL15, while the IL15-IL15R expressing NK cells appeared to slowly outgrow the untransduced cells over time: after 27 days, approximately 70% of all NK cells in the blood of the animals were EGFP positive.

Finally, we used an ALL xenograft model in NSG mice to evaluate the impact of IL15 signaling for the survival and leukemia control of CAR NK cells *in vivo*. For this experiment, three CD19 CAR vectors were generated (**Figure 7A**) co-expressing *via* a T2A site either BFP, soluble human IL15 (17), or human IL15 tethered to the IL15 receptor α-chain (IL15-IL15R) (46). For the *in vivo* experiments, NSG mice were transplanted intravenously with 3.5×10^6 REH cells expressing a firefly luciferase/EGFP fusion protein (REH^{LucEG}). Two days later, 3.5×10^6 total primary human NK cells with 50% non-transduced and 50% transduced cells were injected *via* the tail

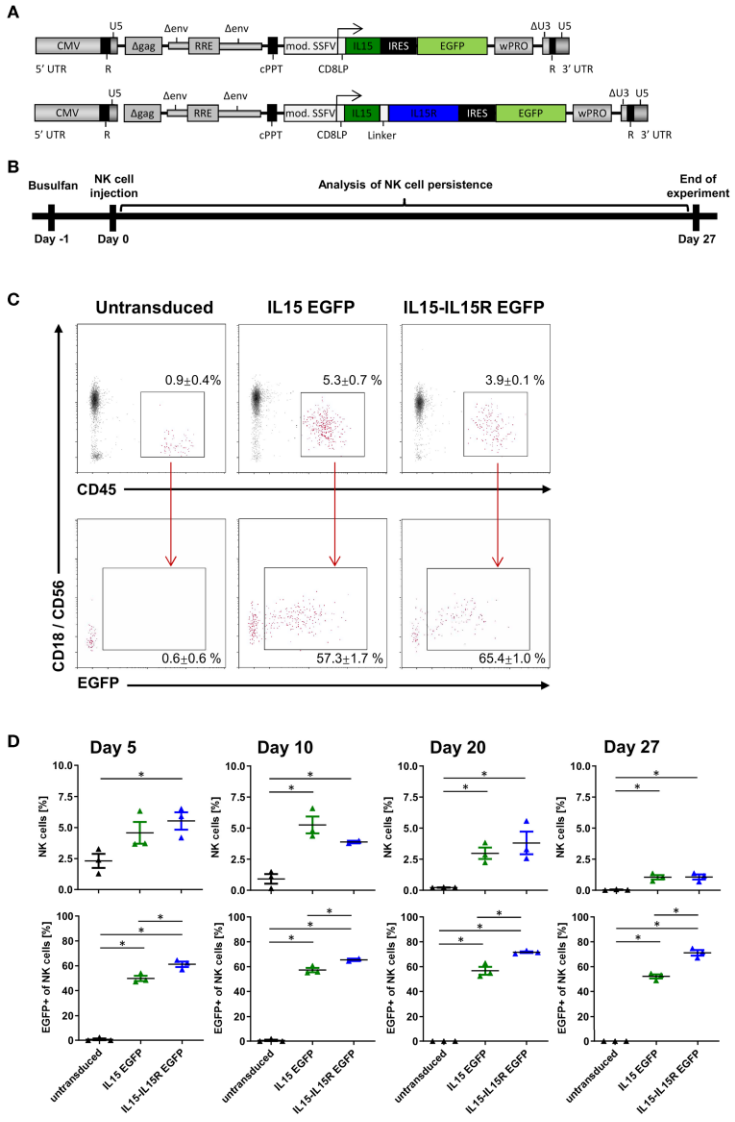

FIGURE 6 | NK cells rely on endogenous IL15 production for *in vivo* persistence in NSG mice. **(A)** Schematic representation of the vector maps of the IL15 constructs used for NK cell transduction. **(B)** Primary human NK cell preparations consisting of 50% EGFP/IL15 or EGFP/IL15-IL15R and 50% untransduced NK cells (or 100% untransduced cells as control group) were injected into busulfan-preconditioned mice (3 animals per group). On days 5, 10, 20, and 27, NK cell persistence in the bloodstream was analyzed by mouse CD18, human CD45, and CD56 staining as well as EGFP expression. **(C)** Example of gating strategy showing representative data from day 10. Values indicate mean ± SEM of all three animals. **(D)** NK cell persistence in the blood including ratios of transduced to untransduced NK cells. Data are represented as mean ± SEM. Significances of $p<0.05$ are indicated by an asterisk (*).

vein (**Figure 7B**). Untreated NSG mice showed rapid leukemia progression and had to be sacrificed between days 17 and 19 (**Figures 7C, D**). Surprisingly, treatment of the mice by infusion of BFP/CD19 CAR NK cells did not improve the survival at all (**Figures 7C, D**). For these two groups, the REH cells made up between 0.3% and 0.4% of peripheral blood cells on day 7 and between 3.3% and 7% on day 15 (**Figure 7E**). Interestingly, the BFP/CD19 CAR NK cells were still detectable at day 8 (2.3% of peripheral blood cells) but did not persist in the blood beyond this time point (**Figure 7F**). When the CD19 CAR NK cells additionally expressed IL15 or IL15-IL15R instead of BFP, the leukemia progression was markedly reduced as seen by luminescence imaging (**Figure 7C**) and peripheral blood analysis (**Figure 7E**). We attributed the improved survival of the animals to the robust increase in the persistence of CAR NK cells, as these immune effector cells were detected at high levels

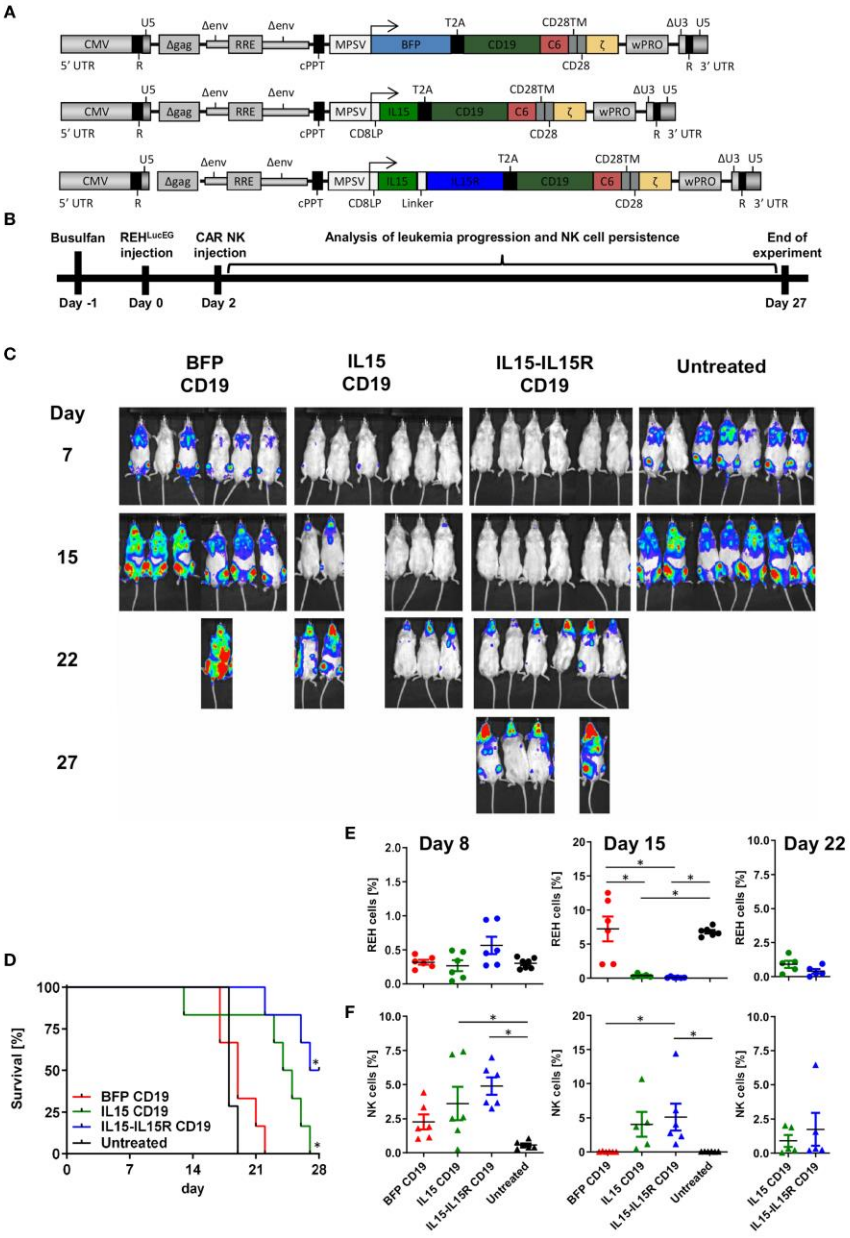

FIGURE 7 | CAR NK cells rely on endogenous IL15 production for leukemia control in an ALL xenograft model. **(A)** Schematic representation of vector maps of CAR constructs used for NK cell transduction. **(B)** Primary human NK cell preparations consisting of 50% BFP/CD19 CAR, IL15/CD19 CAR, or IL15-IL15R/CD19 CAR and 50% non-transduced NK cells were injected 2 days after xenotransplantation of REH^{LucEG} into busulfan-preconditioned NSG mice (6 animals per group, 7 animals as untreated control). **(C)** On days 7, 15, 22, and 27, leukemia progression was analyzed by luminescence imaging in a Caliper device. **(D)** Kaplan–Meier survival curves for the three treatment groups and the untreated control animals. **(E)** REH^{LucEG} and **(F)** NK cell persistence in the bloodstream was analyzed by CD19, CD34, CD45, and CD56 staining as well as EGFP expression by flow cytometry. Data are represented as mean ± SEM. Significances of $p<0.05$ are indicated by an asterisk (*).

on day 15 (4% for IL15/CD19 and 5.1% for IL15-IL15R/CD19 CAR NK cells) and were even present at lower levels on day 22 in the peripheral blood of the animals (**Figure 7F**). Importantly, however, the animals in the IL15 and IL15-IL15R groups all showed ALL persistence in central nervous system (CNS) lesions (**Figure 7C**). These lesions partly led to neurological deficits/abnormalities, and the majority of these animals had to be sacrificed between days 22 and 27. At the termination of the

experiment, three NSG mice of the IL15-IL15R/CD19 CAR NK cell group were still alive, albeit all affected by CNS leukemia.

DISCUSSION

The application of risk-adapted treatment protocols has greatly improved the survival of children and adolescents with ALL and acute myeloid leukemia (AML) during the past four decades (47). However, a significant number of patients, at least 10% of pediatric ALL and 25% of pediatric AML, still suffer from relapse and/or refractory disease and despite all efforts ultimately succumb to their leukemia (48–51). For these patients, phase I/II clinical trials with adoptive cellular therapies generally were only beneficial in post-transplantation settings, where lymphocyte infusions of the stem cell donors are often sufficient to treat minimal residual disease and even full-blown relapses (52). Infusions of allogeneic NK cells have also been tested in clinical trials and were tolerated well without any GvHD; however, the therapeutic benefits were rather limited, and the concomitant treatment with IL2 or IL15 was associated with profound acute toxicities (11). Recently, antibody-based immunotherapies with bispecific T cell engagers or CAR T cells have achieved remarkable initial success, predominantly for B cell-associated malignancies (53, 54). However, for patients with relapsed/refractory leukemias, the generation of autologous CAR T cells often takes too much time, is technically quite difficult in small children or heavily pretreated patients, and rather impossible for AML patients due to the lack of suitable target antigens for the long-term persistent CAR T cells (53, 54). Here, generating allogeneic CAR NK cells for *off-the-shelf* usage and with limited persistence *in vivo* in patients might be ideal for inclusion in salvage treatment protocols for relapsed/refractory patients, e.g., as a blast-reductive treatment/bridge to allogeneic stem cell transplantation. Taking advantage of the new possibilities to generate allogeneic CAR NK cells on the CliniMACS Prodigy® platform (Miltenyi Biotec) with chemically defined media, we systematically tested variables affecting the transduction and cytotoxic efficacy of allogeneic NK cells with these tools.

The efficacy of CAR immune effector cells to eliminate their target cells is influenced by several factors, including the affinity of the scFv present in the CAR construct, the expression levels of the CAR itself on the effector cells, and availability of the targeted antigen/epitope on the target cell (8, 29). Thus, for CARs with a low affinity or when the target antigen is expressed at low levels, high and stable CAR expression is crucial to ensure excellent antitumor cytotoxicity. To this end, the internal promoter in the SIN lentiviral vector and the CAR/transgene sequence(s), e.g., after the codon optimization, are the main tools to improve CAR expression levels. As we already employed cDNAs optimized for human codon usage, the systematic testing of various promoters was our final step to optimize CAR expression in primary human NK cells. To our knowledge, only Allan et al. tested multiple internal promoters in lentiviral vectors for the transduction of human primary NK cells and finally favored the short human EF1α promoter as the best out of eight candidates for their bicistronic CAR expression cassette (55). In our study, we observed the same relationships when comparing the transgene expression levels of the short EF1α, the EF1α with optimized splicing cassette, and the hPGK promoters, but all eight promoters from Allan et al. still appear to be much weaker than the MPSV promoter that we originally established for robust lentiviral CAR expression in human T cells (27). The original EF1α (56), the human CMV (14), or the SFFV promoter (23, 39) was also used in other studies to express CAR constructs in primary human NK cells. However, since NK cell culture and transduction protocols differ greatly between groups, we analyzed the activity of these commonly used promoters in NK cells expanded in the NK MACS medium from Miltenyi and realized that the MPSV promoter outperformed the hPGK, both SSFV and both EF1α promoters, which is in line with our CAR T cell study (27).

A major advantage of HIV-1-derived lentiviral vectors is their promiscuity in accepting multiple heterologous viral envelopes as pseudotypes, thus allowing a wide tropism of target cells (57). While T cells can readily be genetically modified with the VSV-G pseudotype, neither quiescent nor activated NK cells express sufficient levels of the low-density lipoprotein receptors that are employed by VSV-G pseudotypes to enter target cells (23, 56). Changing the lentiviral pseudotype to BaEV-TR (23, 56) or RD114-TR (39) has resulted in better transduction efficiencies, as the cellular glycoproteins that serve as viral receptors, ASCT-1 and/or ASCT-2, are higher expressed on activated but not on naïve/resting NK cells (23, 56). Noteworthy here is that IL2 priming of human NK cells especially leads to upregulation of the glutamine transporter ASCT2 (14, 58). We also showed that GALV-TM-pseudotyped lentiviral vectors facilitate robust transduction of primary human NK cells, probably as the viral receptor PIT1 is sufficiently expressed on activated human NK cells (59, 60). The three envelopes BaEV-TR (14), RD114-TR (31), and GALV-TM (34) are actually chimeric/fusion proteins between the surface units of the three viral envelopes and the cytoplasmic domains of the aMLV; this is necessary as the lentiviral protease from HIV-1 cannot cleave the R-peptide of the three wild-type glycoproteins but can process the one of aMLV during the extracellular maturation process of the budding lentiviral particles (14, 31, 34). In the BaEV-Rless glycoprotein, the R-peptide is already removed from the wild-type envelope, which resulted in a drastically enhanced fusogenic capacity of this pseudotype already during virus production in the HEK293T cells, as described by Girard-Gagnepain et al. and others (14, 61). Nevertheless, despite the huge syncytia formation, the release of physical particles seems to be almost similar for both pseudotypes, BaEV-Rless and BaEV-TR (14). In agreement with this work, our human codon-optimized BaEV-Rless version also here outperformed all other envelopes and provided by far the most efficient gene transfer into primary human NK cells.

In our studies, the use of the transduction enhancer Retronectin was associated with an 8-fold increase in gene transfer efficiency over the control wells without any enhancer

and thus superior to Vectofusin, which mediated a 7-fold increase. For research purposes, these values are quite comparable; however, for clinical applications, Miltenyi has adapted the use of their Vectofusin reagent for the automated processes in the closed tube system on the CliniMACS Prodigy® (26). Thus, using Vectofusin will thus be the easiest way to achieve high transduction efficiencies for clinical CAR NK cell products.

We previously established a novel element for CARs, the CD34-derived C6 hinge, that facilitates detection and enrichment of CAR T cells in clinical processes and does not negatively influence the functional characteristics of genetically transduced and enriched T cells *in vitro* and *in vivo* in mice (28). In the present study, we demonstrated that C6 as a hinge in CAR constructs expressed on human NK cells also allows to easily detect transduced NK cells *in vitro* or the peripheral blood of mice using the QBend-10 antibody by flow cytometry. Additionally, the C6 hinge also facilitates rapid enrichment of genetically modified CAR NK cells to purities of >95% using CD34 microbeads on MACS columns. However, compared to CAR T cells (28), enriching CAR NK cells with the same CD19 CAR construct with the C6 hinge resulted in a higher loss of CAR NK cells in the flowthrough, 29.0% for CAR NK cells (**Figure 2**) compared to only 11.7% for CAR T cells (28). We believe that these differences in the MACS enrichment efficiency are due to the lower expression levels (MFIs) of the CAR constructs on NK cells (data not shown). Even when the CAR T and NK cells originate from the same donors and both cell types showed similar gene transfer rates, more CAR NK cells are lost in the flowthrough (data not shown). One possible explanation is that the MPSV promoter, although still the best promoter tested for NK cells, might not express transgenes in NK cells as strongly as it does in T cells. However, in clinical settings, the loss of transduced NK cells in the flowthrough might not be as relevant as for autologous CAR T cell therapy, as the NK cell numbers can easily be adapted in allogeneic settings and one CAR NK cell product from apheresis might still be sufficient for several recipients (26). The key point here is that allogeneic NK cells with high CAR expression levels can readily be purified on a system that is compatible with GMP conditions and where the CD34 microbeads might not need to be removed (62, 63).

In order to verify the specific functionality and cytotoxic activity of our enriched CAR NK cells, we overcame the impediment of profound NK cell killing of leukemic cells by introducing blocking antibodies against six activating NK cell receptors in the co-cultures. Previously, a decrease in the NK cell cytotoxicity was reported when singularly blocking DNAM-1 (64) or NKG2D (65) on NK cells prior to incubation with AML cells. Additionally, Boermann and colleagues tested the effects of blocking NKG2D, DNAM-1, NKp30, NKp44, and NKp46 on primary human NK cells prior to co-culture with rhabdomyosarcoma cell lines (66). Others just overexpressed the target antigen for their CAR NK cells on cells that were not attacked per se by activated NK cells (26). Currently, we are in the process of systematically analyzing the effect(s) of each ligand on leukemic blasts for NKG2D, DNAM-1, NKp30, NKp44, NKp46, and/or NKp80 on *in vitro* activated and expanded NK cells (data not shown). So far, the success of blocking individual receptors seems to be target cell line-dependent. Paramount is that despite the blocking of their activating receptors, the MACS-enriched NK cells expressing CD19, CD33, or CD123 CARs still exhibit highly specific cytotoxic activity against ALL and AML cells *in vitro*. Fluorescence microscopy of CD19 CAR NK cells attacking CD19-positive REH target cells revealed that the C6 hinge is also an ideal tool to visualize the immunological synapses where the direct interaction between the CAR NK cells and the target cell occurs. We additionally demonstrated that primary AML blasts from pediatric patients expanded in NSG mice can be used as important target cells to determine the specific cytotoxicity of allogeneic CAR NK cells against these primary blasts.

Although CD19 CAR NK cells efficaciously killed the two CD19-positive B-cell precursor ALL cell lines REH and BV-173 in overnight cultures *in vitro*, our *in vivo* experiments clearly demonstrated that support of the CD19 CAR NK cells by IL15 co-expression is an absolute prerequisite for the control of the ALL blasts *in vivo*. While the co-expression of soluble IL15 was required to increase survival of the animals, as also reported by others (17, 67), the group of animals that received CAR NK cells, where IL15 is tethered to the IL15 receptor α-chain, lived slightly longer. These results seemed to confirm previous observations by Imamura et al. (68), where the tethered IL15 sustained NK cell survival and expansion in an autocrine stimulation and might mediate survival and growth advantages over NK cells co-expressing non-membrane-bound IL15. However, the initial accumulation of REH cells in the CNS already at day 15 and then strongly at day 22 suggested that migration and/or defect for NK cells with soluble IL15 expression might exist for the CNS, while human CAR NK cells with IL15 tethered to the IL15R might be better suited to survive and function in the CNS of mice. Thus, the survival of the REH leukemic blasts in the CNS of the animals might simply reflect the reduced capabilities of NK cells to cross the blood–brain barrier of the animals. Noteworthy here is that we used the same REH ALL mouse model in our previous publication (28) where we showed that the C6 hinge in a CD19 CAR is as efficacious as a commonly used CD8-derived hinge to control B-cell leukemia *in vivo* by CAR T cells. In this work, all mice in the CD19 CAR T cell groups survived, and no REH leukemia cells were detected in the CNS of the animals at any time point (28). Whether human CAR NK cells have defects in the homing to the CNS only in NSG mice or whether diminished migration of human CAR NK cells might also be a problem in humans needs to be addressed in future studies.

In summary, we established an efficient protocol for expansion and transduction of primary human NK cells with BaEV-pseudotyped CAR lentiviral vectors, which allow enrichment of the CAR NK cells to high purities. We also developed a simple method for blocking activation receptor-dependent killing of target cells by NK cells, thereby paving the way for successful evaluation of CAR NK cells targeting a variety of antigens on leukemic blasts and also on other malignant cells

in future studies. Since the MACS enrichment protocol can easily be established under GMP conditions, our study seems to be highly informative for the *off-the-shelf* manufacturing of CAR NK cells for clinical use in cellular immunotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The use of peripheral blood from healthy donors was approved by the local ethics committee (Heinrich Heine University, Düsseldorf, protocol #2019-623).

AUTHOR CONTRIBUTIONS

MS, AB, CH, NB, MU, CW, DR, NN, and HH planned the experiments. MS, AB, CH, AThi, SCC, NB, SS, ATho, DS, MH, and NN conducted the experiments. MS, AB, CH, AThi, SCC, SS, NB, ATho, DS, MH, MU, KS, CM, CW, DR, NN, and HH critically analyzed data. MS, AB, CH, NN, and HH wrote the manuscript with the help of the other authors. All authors approved the final manuscript.

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Conflict of Interest: HH and CW are inventors on a patent describing the CD34 hinge for CAR immune effector cells.

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

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3 Discussion: Challenges of CAR therapy

Cancer therapy has made significant progress within the last two decades. With a profound switch from traditional and non-tumor specific systemic therapies towards targeted and personalized approaches, these novel strategies have achieved dramatically increased response and survival rates and especially long-term therapy outcomes improving for poor-prognosis cancer patients. Immunotherapies with antibodies or CARs, declared as the scientific breakthrough of the year 2013 by the Journal *SCIENCE*, are on the forefront of this paradigm shift [166]. Especially cellular therapies, which employ genetically-modified immune cells for the treatment of cancer, are a promising approach to induce tumor clearance and stable disease remission. Since their first description in 1989 as chimeric antigen receptors [18], CAR cellular products have come a long way and it took more than 20 years for their breakthrough in clinical studies and almost 30 years for their market approval. Currently, more than 500 clinical trials are utilizing CAR T-cells for the treatment for various malignancies and, in addition to the six already approved ones, more CAR T-cell products are expected on the market in the upcoming years [23]. Nonetheless, while the field rapidly advanced within the last decade, CAR designs still need to be optimized and the broad applicability of this type of therapy for cancer is still a great challenge to overcome. In this thesis, I describe CAR design improvements, which tackle different aspects of CAR therapy. These were:

1) The establishment of novel hinge domains derived from human CD34 and NGFR/CD271 enables the detection and enrichment of CAR T- as well as NK cells, which will be a key factor to drive allogeneic therapies forward. In various systems, these two hinges proved to be as safe and as effective as widely used and clinically approved hinge domains.

2) CAR T-cell therapy for solid cancers is still lagging behind mainly due to the lack of safe antigens, heterogeneity of solid tumors, impeded T-cell trafficking as well as the hostile TME. To improve the therapy for head and neck as well as bladder cancer, CD44v6 was established as an important target antigen, an EGFR-targeting CAR was engineered to improve lentiviral production and T-cell transduction, and the combination therapy with the epigenetic drug decitabine enabled to prime tumor cells towards apoptosis to increase CAR T-cell efficacy.

3) NK cells could be superior to T-cells as effector cells for broader cellular immunotherapy for several reasons, including inherent anti-tumor responses, risks for fewer adverse events and the potential for allogeneic *off-the-shelf* therapies. As part of this dissertation, a workflow for the generation of (pure) allogeneic CAR NK cell products was established.

3.1 Engineering the optimal CAR construct

When constructing a CAR, each domain has distinct effects on the function and thus the cellular consequences for the CAR expressing cell. Obviously, the **antigen-binding domain** dictates to which target antigen the CAR binds and thus which cells are being eliminated. The most successful CAR target by a good margin has been CD19, which is almost exclusively expressed on cells of the B-cell lineage and also uniformly present on B-cell leukemias and lymphomas. Especially the high-affinity clone FMC63, on which all clinically approved CD19 CAR T-cell products are based upon, proved to be highly effective in controlling tumor growth and induce remission in patients [88, 89, 107]. Also in this dissertation, the FMC63 scFv was used to efficaciously eradicate various CD19+ leukemia and lymphoma cell lines or as a widely accepted negative control for solid cancer cells. Similarly to scFv-engineering as a parameter to tune CAR affinity and consequently the required antigen threshold for activation signaling in the CAR cell [46], the linker between the heavy and the light chain can influence dimerization and signaling of the CAR construct, thereby ultimately affecting efficacy and therapy outcome in cellular studies [167]. Here, we employed scFv-engineering to shorten the Cetuximab-derived scFV for an EGFR-CAR. While these changes did not confer improved effector function to the T-cells, the lentiviral vector production as well as transduction efficiency of primary human T-cells was dramatically improved. With the lentiviral vector and subsequent CAR T-cell manufacture being a main cost factor in CAR T-cell therapy, such an improvement will be key to reduce costs for a potential clinical application. Besides scFvs, CAR constructs can be also equipped with ligands [41], cytokines [42], extracellular parts of receptors [43] or nanobodies [168] to recognize their target antigens on malignant cells. Recently, CARs with antigen-binding domains derived from TCRs have been established as an intriguing option to target intracellular proteins [169], which nevertheless require presentation on MHC molecules and thus are prone to MHC loss by the tumor cells.

Throughout this dissertation, the combination CD28-CD3ζ was used as signaling domains in 2nd generation CARs for targeting hematological as well as solid cancer cells. However, it is still a matter of discussion, which **co-stimulatory domain** will provide the most utility to CARs. Although CD28 and 4-1BB are currently the most employed options [170], it is widely accepted that CD28 leads to a more rapid and strong T-cell proliferation as well as anti-tumor response [15] and requires a lower antigen threshold for signaling [171]. Due to this stronger response and the induction of IL-2, which counteracts TGF-β-mediated immunosuppression in the TME [36], CD28 is thought to be the better candidate for solid tumors. This hypothesis has been confirmed in preclinical solid tumor models, where CD28-co-stimulated CARs showed better tumor infiltration, expansion and tumor clearance [172]. Also for hematological malignancies, CD28-equipped CD19 CARs showed superior response and survival rates in the treatment of DLBCL patients when compared to 4-1BB-equipped counterparts [173]. These studies therefore indicate,

that CD28 indeed seemed to be the correct choice for the applications within this thesis.

Nonetheless, further engineering of the CAR constructs by adapting the co-stimulatory domain could significantly change the CAR signaling. Importantly, CD28 signaling in CARs is more prone to cause T-cell exhaustion and activation-induced cell death [25, 26]. To counteract CD28-mediated exhaustion, the CD28 endodomain [174] or ITAMs within CD3ζ [175] can be mutated to reduce the signaling strength of the CAR without losing CAR efficacy. This might also help to reduce neurotoxicities, as these side-effects are attributed to strong CAR T-cell activation and are more frequent in CD28-based CARs compared to 4-1BB-based constructs [176]. Moreover, CD28 also induces IL-10 production and secretion, which can inhibit immune cells. Here, the coupling of CD28 with OX40 signaling, which represses IL-10 secretion, is an option to prevent IL10-mediated immunosuppression [29].

NK cells are an attractive effector cell type for CAR therapy, since these cells are not MHC-restricted in their capacity to kill malignant cells and can be used independent of any HLA constellations for allogeneic therapies [155]. In contrast to T-cells, however, NK cells do not physiologically express CD28. Nonetheless, CD28-based CARs are completely functional when expressed in NK cells, although their signaling does not induce autocrine expression of proliferative cytokines such as IL-2 [177, 178, 179]. Thus, CAR NK cells heavily rely on further stimulation on top of the CAR signaling to improve *in vivo* persistence, as was shown in the ALL mouse model, when CD19 CAR NK cells with a CD28-CD3ζ configuration could not control the progression of the CD19+ ALL blasts (see section 2.3). Only co-expression of the CD19 CAR with either soluble or membrane-tethered IL-15 improved the persistence of NK cells *in vivo* and impeded the ALL progression. Similar results were obtained in comparable preclinical studies [177, 178] and also in a clinical trial [180], where IL15-armored NK cells achieved complete remission in 7 out of 11 patients without observable toxicities. Interestingly, while NK cells do not carry TCRs, they still do express CD3ζ, which complexes with the Fc receptor CD16 to mediate ADCC of pathogens or cancerous cells [152]. Thus, when incorporated in CARs and expressed in NK cells, the ITAMs within the CD3ζ shift the net activation signal to induce elimination of the targeted cell. Although CD3ζ remains the gold standard, the intracellular domains of NK cell-associated activation domains such as DNAX-activation protein 10 or 12 (DAP10, DAP12) or NKG2D have also been used as primary activation signal for CAR engagement in NK cells [15, 179].

The **hinge domain** plays a crucial role in CAR design as it gives the CAR the flexibility and length to reach its target and thus indirectly mediates CAR signaling. Currently, CD8- and CD28-derived hinges are the go-to-candidates for clinical applications, but as for the co-stimulating domains, the best choice is still a matter of discussion and might rely on the specific situation. In 2nd generation

CAR constructs with a CD28-CD3ζ configuration expressed in human T-cells, CD28-hinged CD19 CAR constructs were reported to produce higher levels of cytokines, especially IL-2, exhaust more quickly and are more prone to activation-induced cell death (AICD) when compared to their CD8-hinged counterparts [181]. While both CD8- and CD28-derived hinges are relatively short, appr. 40 aa (amino acids), and are already well established in clinical CAR products, both antigens are naturally present on T-cells and partially also on NK cells. In contrast, the immunoglobulin-derived CH_2CH_3 hinges are much longer, approximately 230 aa, and if non-mutated associated with off-target toxicities [132]. As longer hinges are needed to reliably target membrane-proximal and sterically-hindered epitopes, our CD34- and NGFR-derived hinges with lengths of 99 aa (C6), 120 aa (N3) and 162 aa (N4) are certainly attractive options to target antigens, for which the CD8- and CD28-derived hinges are not long enough. From these three, each has its merits and thus also here it is a matter of functional testing, which one is the best candidate for a clinical application. Obviously, N4 should mediate the best binding for membrane-proximal epitopes, while C6 and N3 on the other hand might be better suited for an one-size-fits-all approach.

Indeed, when incorporated into the respective CAR, the CD34-derived C6-hinge mediated not only eradication of CD19, CD5, ROR1, CD5, CD33 or CD123 leukemia and lymphoma cells (see section 2.1.1), but also of EGFR- or CD44v6-positive head and neck as well as urothelial carcinoma cells (see section 2.2). In addition, CD34 selection with immunomagnetic reagents is in clinical use for more than 15 years for hematopoietic stem cell transplantation and CD34-fusion proteins have been used to select transgenic T-cells in clinical trials [182]. Thus, using CD34 as a selection marker with subsequent magnetic selection is highly feasible for a clinical application within CAR therapy.

The safety of cytoplasmatically truncated NGFR as a selection marker has been controversially discussed in the past. An early study reported leukemia induction after transduction of murine hematopoietic stem/progenitor cells with oncoretroviral vectors expressing truncated NGFR; this malignant transformation was presumably caused by the specific insertion locus and by dimerization and signaling of other growth factor receptors with the truncated NFGR [183]. Thereafter, however, another study demonstrated safe transduction of truncated NGFR into more than 9×10^9 bone marrow cells and primary T-cells of various species [184] and truncated NGFR has been used as selection marker in various clinical trials including a recently started trial with ΔNGFR-hinged CD44v6 CAR T-cells (NCT04097301; [74]). Regarding the NGFR hinges, N3 could potentially be the safer option compared to N4, as it lacks the 2nd CRD, which is apparently needed for binding of NGF and other neurotrophins [185]. Generally speaking, when designing new CAR hinges, potential unknown binding partners should be kept in mind, as these could cause specific *off-target* toxicities, as we saw with the longer CD34

hinge, C7, which caused unspecific toxicity against a not yet identified antigen on various AML cell lines. Although the only described ligands for CD34 are selectins [186], we could exclude these since their binding to CD34 relies on glycosylation of CD34 [187, 188] and the unspecific toxicity even occurred when the C7-hinge was mutated to prevent glycosylation (see section 2.1). Similarly, CD80 has been described as an unexpected binding partner for NGFR [189], although the physiological role of this interaction is still unclear.

Marking of transgenic cells with a cell surface marker is a desirable feature for cellular therapy, as it allows to track and eventually also eliminate the transgenic cells. In line with this, the lentiviral vector of Breyanzi™, the CD19 CAR T-cell therapy by Juno Therapeutics/Bristol Meyer Squibb, contains a truncated EGFR as such a marker. Consequently, CAR T-cells can be easily detected in the bloodstream of the patient by staining with EGFR antibodies and also be eliminated in case of adverse reaction by the administration of the clinically used EGFR antibody cetuximab [190, 191]. As the persistence of CAR T-cells is a key indicator for therapy success, tracking of CAR T-cells in the bloodstream can be used to predict therapy outcome. The direct incorporation of such a marker into the CAR backbone carries additional benefits, as this reduces the vector size compared to a vector expressing the CAR and a second cell surface marker; it also opens up the space for expression of a 2nd transgene in the vector. The reduction of the vector size is an option to increase virus titers and to improve the transduction efficiency [192], which then reduces the costs of CAR T-cell manufacture and thus CAR therapy itself. As second transgene, especially suicide genes such as the inducible Caspase-9 [193] are an intriguing option to eliminate the CAR T-cells *in vivo* when needed. In contrast to the elimination by an antibody, e.g. cetuximab, the suicide gene specifically eliminates only transgenic cells and thus causes less side effects, as systemically administered antibodies would also damage healthy tissues carrying the marker. Artificial sequences to detect and eliminate CAR T-cells have also been incorporated into the CAR backbone [194, 195], but due to their artificial nature, they are more likely to provoke immunogenic reactions.

If the hinge within the CAR construct not only allows detection, but also enrichment with e.g. immunomagnetic reagents under GMP conditions, which is readily possible with our C6, N3 and N4 hinges, inclusion of a suicide gene would facilitate to generate a pure transgenic cell product that is completely controllable by the suicide gene. This approach is the prerequisite for the generation of allogeneic CAR therapies (if the TCR is not knocked-out), as a potential graft-versus-host-disease can be stopped via suicide gene activation. Thus, our newly developed hinges present an essential first step towards the establishment of allogeneic CAR T-cell therapies.

3.2 Alternative CAR designs to improve therapy efficacy

While CAR T-cell therapy demonstrated unpreceded response rates for r/r malignancies with historically bad prognoses and outcomes, resistance and relapse mechanisms that prevent straight-forward CAR T-cell application are emerging in the clinical trials [15]. The most common relapse mechanism, that has been reported so far, is the loss of the targeted antigen leading to the outgrowth of an antigen-negative tumor cell population. Between 10 and 20 % of ALL patients treated with CD19 CAR T-cells relapse with CD19- disease [196]. In a recent study, even as many as 12 out of 17 ALL patient relapsed due to CD19 loss [197]. On a genomic level, each of these patients had a unique mutation which truncated CD19 before or in the transmembrane domain and thus abolished cell surface expression. Similarly, also the down-regulation of the target antigen, albeit not complete loss, as observed after CD33 CAR T-cell therapy, can induce relapse [198]. Here, it also has been reported that CARs can pull out their target antigen from the tumor cell membrane and internalize it in a process called trogocytosis, which results in tumor cell populations with decreased antigen expression [199]. Besides simply "losing" antigen expression, other mechanisms could also result in evasion from the CAR T-cells. Here, especially mutations in the epitope region of the antigen mediate CAR unresponsiveness and thus loss of CAR efficacy [200]. Through alternative splicing the recognized epitopes can be spliced out of the protein, which has been reported for CD19 CAR T-cell therapy [200] and treatment of AML with gemtuzumab-ozogamicin, a CD33-directed antibody-drug-conjugate. Another rare event, that can cause relapse, is the unfortunate transduction of leukemic blasts present in the apheresis product with CARs leading to the masking of the target epitope. Here, the transduction of a single leukemic blast has been reported to be enough to cause relapse after CD19 CAR T-cell therapy [201].

All of these mechanisms, which can be summarized under "**antigen escape**", enable the malignant cells to hide from and evade the CAR T-cells. Underlying is a selective pressure, as antigen-positive tumor cells are eradicated by the CAR T-cells, thus giving a growth advantage to tumor cells with lost, mutated or masked epitopes. To prevent or at least reduce the risk of antigen-negative relapse in the first place, the simultaneous treatment with two or more 2nd or 3rd CARs has been under both preclinical as well as clinical evaluation (**Figure 5**). When using these **dual CARs**, it is feasible to co-target antigens from the same lineage, which ensures to restrict the *on-target off-tumor* toxicities to this lineage; for B-cell malignancies, CD19 has been co-targeted with CD20 [202] or CD22 CARs [203]. When using two CAR constructs in one T-cell, it has been reported that the transmembrane as well as hinge domains of the CARs enable dimerization with innate receptors on T-cell such as CD8 and CD28 [81]. Thus, to prevent dimerization of the two CARs with one another or with these molecules, which is a driver of tonic signaling and unspecific CAR activation, each CAR should be equipped with a unique hinge and transmembrane domain. Here, the C6 and N3

or N4 hinge are prime candidates for hinges to be used in such dual-CAR systems, since neither CD34 nor NGFR are expressed on T- or NK cells, which means that they cannot form heterodimers with native CD34 or NGFR on T- or NK cells. CD34 and NGFR are also not known to interact with each other, thus reducing the risk that the CARs themselves could form heterodimers via the interaction of their two hinges. Importantly, when one CAR is equipped with C6 and the other one with an NGFR-derived hinge, T- or NK cells can be enriched for cells expressing both CARs to produce a homogenous dual CAR T- or NK cell product.

In general, the transfer of two complete CARs represents a higher genetic load compared to a bi-cistronic CAR. However, there are multiple ways to transduce the CARs into the T-cells: The two receptors can either be expressed in mono-cistronic cassettes using two vectors or in a single bi-cistronic gene expression cassette using a single vector. Here, bi-cistronic cassettes are preferable as they require the production of only one (lentiviral) vector, which reduces therapy costs and eases the running/application of clinical trials and market approval. Regarding one-vector systems, two-promoter and IRES systems do not necessarily result in adequate expression of both transgenes [204]. In contrast, a 2A site, as was already employed on several occasions in this dissertation and is used in Breyanzi™, the CD19 CAR T-cell therapy by Juno Therapeutics/Bristol Meyer Squibb, drives robust and proportional co-expression of two transgenes [204] and thus might be the best option to co-express two CARs.

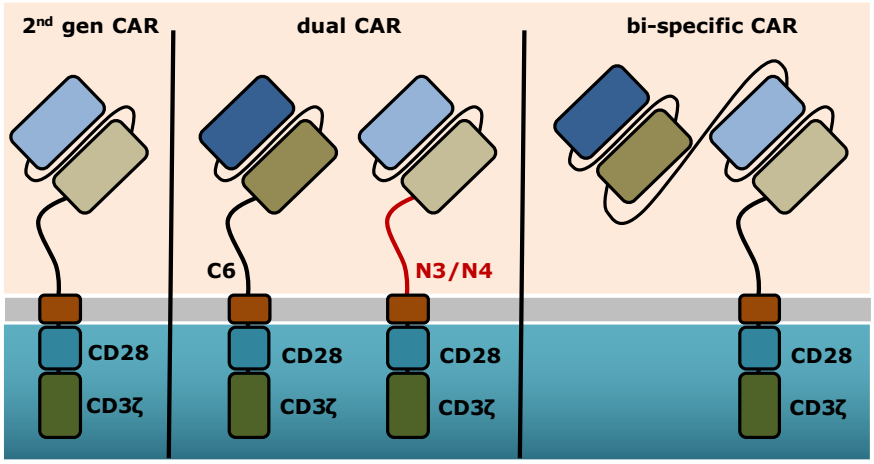

Figure 5: Alternative CAR designs to improve CAR therapy efficacy. In contrast to approaches with standard 2nd generation CARs with one scFv targeting moiety, dual and bi-specific CARs allow to target two antigens by expressing two CARs with distinct antigen specificity or adding a 2nd scFv to the CAR, respectively.

Alternatively, CARs can be equipped with two separate scFvs to enable the effector cell to respond to two different antigens (**Figure 5**). **Bi-specific CARs** require the expression of one only construct and thus do not need bi-cistronic gene expression cassettes or more than one vector; however, one has to pay close attention to the design of the construct regarding the two scFvs. Depending on

where the scFvs bind the corresponding antigen, as in proximally to or distally from the membrane, the orientation of the scFvs is crucial to ensure binding of both domains to its antigens [205]. Consequently, in a CD19/CD20 bi-specific CAR, the CD20 scFv, which binds a membrane-proximal epitope and thus requires a long hinge for antigen binding in a classical mono-specific construct, had to be located at the N-terminus of the bi-specific CAR [205]. Here, the downstream located CD19-scFv presumably functioned as an additional spacer to bridge the CD20-scFv to its epitope. Importantly, in bi-specific antibody preparations, mispairing of the heavy and light chains is a serious problem, which could generate new scFvs with unknown specificity [206]. Although this has not been reported for bi-specific CARs so far, it remains at least a theoretical risk associated with bi-specific CARs. Interestingly, a CD19/CD22 bi-specific CAR required a looping configuration, where the two chains of the CD19-scFv surround the CD22-scFv chains resulting in a $CD19V_H$-$CD22V_H$-$CD22V_L$-$CD19V_L$ sequence [125]. In a recent trial, six out of six patients completely responded to a CD19/CD22 bi-specific CAR, although one patient finally relapsed due to lost CD19 and reduced CD22 expression [207].

3.3 Alternative CAR designs to improve therapy selectivity

On-target off-tumor toxicities are still one of the main concerns and limitations of CAR T-cell therapy. While the eradication of the normal B-cells regularly occurs in the CD19 CAR therapies for B-cell malignancies, regular immunoglobulin infusions can be used for these patients to cope with the B-cell aplasia. Beyond CD19 and other B-cell associated antigens, however, only few cell surface antigens can be targeted without risking or causing severe toxicity [15]. Hence, CAR T-cell therapy for other hematologic malignancies, which are not B-cell derived, is lagging far behind.

AML is derived from progenitor/precursor cells in the myeloid lineage and has a relatively dire prognosis in adult patients. Therefore, new treatment modalities are direly needed for r/r both pediatric and adult patients. However, the two most intriguing CAR targets on AML, CD33 and CD123, are expressed across the myeloid hematopoietic system including very early hematopoietic precursor cells, thus posing a risk of lasting myeloid immunosuppression, especially anemia, agranulocytosis and thrombocytopenia, or even myeloablation [208]. Nonetheless, clinical studies to assess CAR therapy for AML are ongoing. The first patient treated with CD33 CAR T-cells showed severe adverse events during therapy including cytopenias [198]. Initially, CD33 CAR T-cells markedly reduced AML burden, but CD33+ AML blasts reoccurred in the presence of CD33 CAR T-cells and the patient succumbed to his disease. Another recent phase I study investigated the feasibility and safety of CD33-targeted CAR T-cells for heavily pretreated r/r AML patients [209]. To limit toxicity, a low dose of just 3×10^5 cells per kg of body weight was used. While the therapy was well tolerated, success was quite limited, as none of

the patients responded and all died within one month following CAR T infusion due to disease progression. Similarly, first results with transiently expressed CD123 CARs showed only temporary adverse events, but also no antileukemic effect, as the disease quickly progressed in all treated patients [210]. On the contrary, lentivirally transduced CD123 CAR T-cells induced complete responses and remissions in some patients without overt toxicity and treatment-related cytopenia [211]. Importantly, all studies experienced problems regarding the manufacture of CAR T-cell products in time, as r/r AML is an aggressive disease with rapid progression and patients commonly died before being able to receive CAR T-cells. Moreover, the manufacture process itself proved to be problematic, as the patients' immune systems were frequently damaged due to previous treatments and AML burden, leading to the production of insufficient CAR products.

There are several systems to potentially limit the toxicity of CAR T-cell against healthy tissues. In ***Split-CARs,*** the signaling domains of a 2nd or 3rd generation CAR are split into two distinct CARs, each targeting a different antigen (**Figure 6**). Thus a 1st generation CAR with the CD3ζ domain targets the first antigen, while a co-stimulatory receptor carrying one or more co-stimulatory domains targets the second antigen. While engagement of only one receptor diminishes or even abolishes T-cell activation, only the simultaneous binding of both receptors induces full T-cell activation and efficient target cell lysis. Consequently, the *Split-CAR* system creates, in contrast to the bi-specific CARs, an "AND-gate", where both targets need to be bound simultaneously by two first generation CARs. Therefore, this approach allows to increase the specificity from a single-targeting to a dual-targeting strategy. In preclinical research, this system has already been successfully used to co-target ErbB2 and MUC1 for breast cancer [212], mesothelin and a-folate receptor for ovarian cancer [213], CEA and mesothelin for pancreas carcinoma [214], PSMA and PSCA for prostate carcinoma [215] and CD13 and TIM3 for AML [216]. While the *Split-CAR* is an attractive approach to tune CAR T-cell specificity, the translation could prove to be difficult as the system requires a delicate balance between the two CARs and stable expression of two antigens. This might also be the reason that in the almost 10 years since first being described, the *Split-CAR* was only tested a handful of times in preclinical studies and the concept has not been introduced in clinical trials.

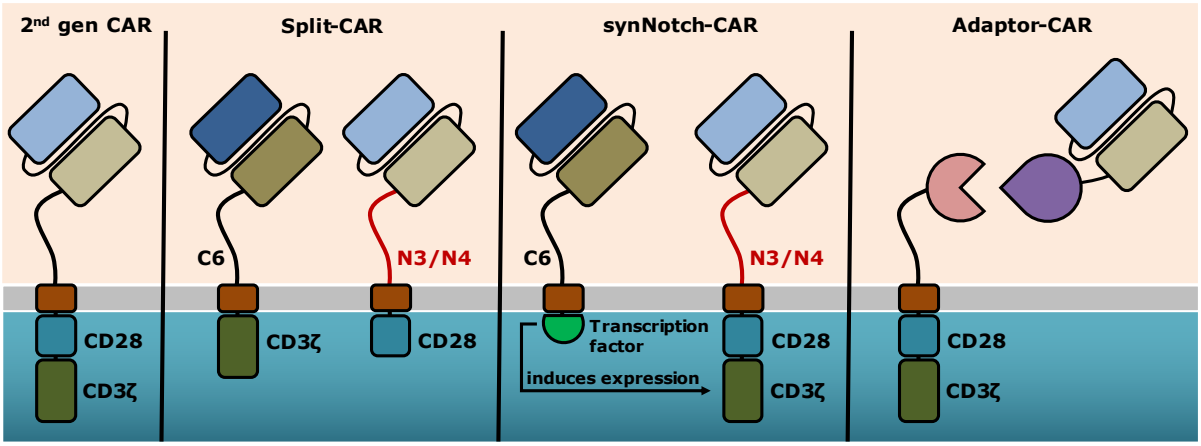

Figure 6: Alternative CAR designs to improve CAR therapy selectivity. In *Split-CARs*, the signaling domains of a 2nd generation CAR are separated into two distinct CARs, each targeting a different antigen. synNotch receptors induce the expression of a second fully functional CAR when activated. Adaptor CARs rely on the supplementation of an adaptor molecule, that mediates the interaction between the target and the CAR T-cells, and thereby dictates the antigen specificity.

Synthetic Notch (synNotch) receptors (**Figure 6**) [217] are also able to increase CAR T-cell specificity, however, they do not require such a delicate design and are more straightforward to establish. Here, the first CAR carries intracellularly a transcription factor that is cleaved off the receptor upon ligand binding. The transcription factor then induces expression of a second functional 2nd or 3rd generation CAR targeting a different antigen. Preclinically, this system has been successfully used in mouse models against hematologic [218] as well as solid tumor cell lines [219]. However, in contrast to the *Split-CAR*, the synNotch system employs a boolean "IF-THEN"-gate rather than an "AND"-gate. Thus, since a fully functional 2nd or 3rd generation CAR is expressed, the risk of *on-target off-tumor* adverse events remains, especially if the two antigens are co-expressed on co-localized or locally proximal tissues [219]. Consequently, the system cannot be used for every target antigen combination and the antigens need to be carefully chosen with regards to expression in the body.

A more sophisticated approach to limit *off-tumor* toxicities is the use of **adaptor-based CAR designs** (**Figure 6**), which are currently in preclinical development. Here, the T-cells are transduced with a CAR construct that lacks a specific antigen-binding domain. Thus, the activation of the CAR T-cells and the killing of the target cells rely on the addition of an adaptor molecule that links the CAR to a target antigen [220]. These adaptor systems usually use CARs which binding a distinct tag recognizing the tagged antibodies or scFvs that bind the TAA of choice. Here, FITC [221], small peptides [222, 223, 224], avidin [225], α-biotin linkers [226] or leucine-zipper motifs [227] have been used as tags that mediate CAR binding and activation. Theoretically, the therapy can be adapted to the needs by increasing or decreasing the adaptor affinity or dose to reduce off-tumor toxicities or increase anti-tumor efficacy [227]. Adaptor molecules can have a serum half-life ranging from just a few

minutes or hours to several days [220]. Thus, CAR T-cell activity relies on repeated/continuous administration of the adaptor fragments, offering another possibility to control the therapy. As the adaptor system not only allows to increase the affinity but also to switch the target itself, this approach is also suited to target the heterogeneity of solid tumors or other malignancies that developed antigen escape mechanisms during immunotherapy. Here, adaptors against different antigens can either be administered simultaneously or subsequentially, depending on the degree of T-cell activity one wants to achieve. Moreover, to increase specificity, adaptor systems can be used for a combinatorial antigen targeting, where the threshold for T-cell activation can only be achieved via the binding of multiple adaptors and thus antigens [226]. However, the biggest hurdle of this approach will be to deliver both the T-cells as well as adaptor molecules to the sites, where the malignant cells reside.

3.4 Improving CAR therapy for solid malignancies

As part of this dissertation, CARs against CD44v6 and EGFR were developed for the treatment of head and neck as well as urothelial carcinomas. As previously mentioned, CAR T-cell therapy for solid tumors is lagging behind due to the lack of safe antigens, the high tumor heterogeneity, the poor infiltration of immune cells into the tumors and the hostile tumor microenvironment (TME). Ongoing research tries to address these issues and will be discussed in the following chapter (**Figure 7**).

Choosing a suitable antigen

The **ideal CAR target** should be abundantly expressed on tumor tissues but not on healthy tissue. However, this has been a pipedream in the field of immunotherapy for the longest time. While the antigen expression for CD19 is highly stable and homogenous on malignant B-cells, this is rarely the case for solid tumors, where antigen expression patterns not only differs between different tumor locations but also within the same tumor nodule, thus limiting CAR T-cell efficacy [228]. To counteract this heterogeneity, multiple antigens have been targeted with CAR T-cells. Here, a bi-specific CAR against ErbB2 and IL13Rα2 not only proved to be more efficacious than the single-specificity CARs, but also mitigated antigen escape in a preclinical model of glioblastoma [229]. Similarly, a scFv recognizing EGFR as well as the mutant EGFR splice variant EGFRvIII was, when expressed in a CAR construct on a NK cell line, effective against glioblastoma cells even when only one antigen was present [230]. A **ligand-based CAR**, which recognizes all four members of the ErbB family (EGFR, ErbB2, ErbB3 and ErbB4) proved to be effective when targeting heterogeneous mesothelioma [231] and a clinical trial testing with the multivalent ErbB CAR for head and neck cancer is currently ongoing (NCT01818323, [232]).

However, targeting of multiple antigens also increases the risk of considerable adverse events as was seen for an advanced cholangiocarcinoma patient, who was treated with successive administration of EGFR- and CD133 CAR T-cells [233], suggesting that tumor-specific rather than tumor-associated antigens should be targeted. Here, CD44v6, a splice variant of the hyaluronic acid receptor CD44, is an intriguing target, as it is expressed on AML, head & neck, colon and several other solid cancers, but expression in healthy tissues is largely restricted to keratinocytes in the skin and oral mucosa and monocytes. Moreover, CD44v6 expression promotes epithelial-to-mesenchymal transition and thus metastasis as well as invasiveness, making it an intriguing target for immunotherapy [234]. Indeed, as part of this dissertation, T-cells were redirected towards CD44v6+ head and neck as well as urothelial carcinoma cells with high specificity, as CD44v6- cells were not targeted, suggesting that it is feasible to target CD44v6 for a clinical application. Likewise, other groups used CD44v6 CARs for lung and ovarian cancer [235] as well as sarcoma [236] in *in vitro* and *in vivo* models. Currently, three clinical trials employ CD44v6-targeted CAR T-cells for AML and multiple myeloma (NCT04097301), breast cancer (NCT04430595) and various other solid malignancies (NCT04427449), although no results have been reported yet. Here, first reports will also indicate whether treatment with CD44v6 CARs is tolerable and which toxicities occur. Another intriguing target for solid malignancies is EGFRvIII, since this genomic deletion of exons 2 to 8 appears to be specific for malignant cells [237]. In contrast to CD44v6, EGFRvIII has been intensively investigated as CAR target and several preclinical studies evaluated efficacy for brain [238], lung [239] and liver cancer [240]. Clinically, EGFRvIII is targeted especially for recurrent brain tumors, mostly gliomas, glioblastomas and gliosarcomas. Here, EGFRvIII-directed CAR T-cells demonstrated tumor control and prolonged survival beyond the expected outcome of recurrent disease [241]. However, similar to other malignancies and CAR targets, EGFRvIII loss or mutation has been reported as evasion mechanism [242] and thus also here combinations with other high-affinity CARs will be key to ensure successful therapy outcome.

One of the biggest drawbacks of conventional CARs is that they are restricted to targeting cell surface antigens, as the scFv can only "see" what is expressed on the cell but not what is present inside. However, being able to target also intracellular proteins would open the door to a whole new world of antigens to target, as only about 1 % of the human proteome is expressed on the cell surface [243]. As a surveillance mechanism and to detect infected or transformed cells, the complete proteasome of a cell is constantly being processed via a complex machinery involving proteases, chaperones and transporters, thus generating peptides, which are presented on MHC molecules on the cell surface. T-cells screen such peptides via their endogenous TCR; in case of a match between **peptide-loaded MHC** and a specific TCR, the T-cell becomes activated and lyses the target cell [244]. Thus, to redirect T-cells towards intracellular proteins, T-cells

have been transduced with exogenous TCRs, which recognize distinct MHC:peptide complexes. Especially neoantigens that arise due to mutations of intracellular proteins are an ideal candidate to target, as these are highly tumor-specific and thus should not cause *on-target off-tumor* toxicities [245]. Here, well-known tumor-specific mutations of e.g. in KRAS were already targeted with exogenous TCRs (NCT03745326, NCT03190941). To drive the identification of other suitable neoantigens forward, screening platforms based on exome sequencing [246] or screening libraries [247, 248] have been utilized. Such platforms might be employed in the future to detect patient- or tumor-specific neoantigens, identify reactive T-cell clones and their TCRs and thus use highly personalized TCRs for cancer therapy [249]. Another class of intriguing intracellular targets are antigens that are expressed in cancers, but only in immune-privileged tissues in the human body such as the testis or only during embryonic development. MAGE-A4 and NY-ESO1 are so called **cancer testis antigens** due to their abundant expression in various malignancies but restricted expression in the testis in the body [250, 251]. Both antigens have been targeted with TCR-modified T-cells for solid malignancies including esophageal cancer [252] and sarcoma [253], although with limited success. Recently, so-called TCR-mimic CARs were developed to target intracellular targets with CARs rather than TCRs. To do so, the variable domains of TCRs or scFvs that recognize a distinct MHC:peptide complex rather than a cell surface antigen have been used as antigen-binding motifs. After successful preclinical testing of these constructs against WT-1 [169], NY-ESO1 [254], SSX2 [255], MELAN-A and TGFbR2 [256], the first TCR-mimic CARs are currently under clinical investigation [257] .

Improving tumor infiltration

In contrast to hematological malignancies, which are mostly present in the blood and lymphatic system and thus easily accessible for CAR T-cells, solid tumors frequently form dense ECM formations and stroma that represents a **physical barrier** for the CAR T-cells [258]. In order to overcome this barrier, CAR T-cells have been modified to express enzymes to degrade ECM components [259] or to eliminate fibroblasts within the TME; this latter approach improved CAR T-cell infiltration into the tumor and thus helped to clear tumors in preclinical lung cancer [260] and ovarian carcinoma mouse models [261]. Also the overexpression of **chemokine receptors** such as CXCR2 [262, 263, 264] or CCR2b [265], that match the chemokines present in the tumor microenvironment, directs the T-cells towards the tumor site. In preclinical models, these strategies improved CAR T-cell infiltration into the tumor and efficacy against brain [265], pancreas [262] and liver cancer [264]. The **local application** of CAR T-cells directly into the tumor site however proved to be superior compared to intravenous injection [266, 267, 268]. Clinically, this route proved to be more effective in the treatment of glioblastoma [269] as well as head and neck cancer [232]. Importantly, the local administration not only mitigates the need of the CAR T-cells to traffic to and infiltrate into the tumor, but also limits *on-target off-tumor*

toxicities as the CAR T-cells mostly remain in the tumor site and thus do not eliminate antigen-positive normal cells in other tissues.

Armoring T-cells against the TME

The TME not only presents a physical barrier for infiltrating immune cells, but is also a hostile environment, where immune cells are inhibited or driven into anergy or even apoptosis by **inhibitory cytokines** or **checkpoint molecules**. Here, strategies to either make the tumor cells more susceptible to T-cell killing, disrupt T-cell inhibition or armor T-cells for the hostile TME are being researched. As the expression of inhibitory receptors on tumor and tumor-resident cells greatly impedes CAR T-cell function and persistence, blockade of these receptors is a straightforward approach to improve therapy outcome. Importantly, several checkpoint inhibitors against PD-1 (Nivolumab, Pembrolizumab, Cemiplimab), PD-L1 (Avelumab, Atezolizumab) and CTLA-4 (Ipilimumab) are approved for clinical use for patients with melanoma, renal, colorectal and urothelial carcinoma, breast, lung, bladder and head and neck cancer as well as several lymphomas [270], thereby paving the way for a combination therapy. Indeed, in preclinical models blockade of the PD-1/PD-L1 axis improved cytotoxicity, cytokine production as well as proliferation of GD2 [271], CEA [272], ErbB2 [273, 274] and mesothelin CAR T-cells [275]. Clinical data on these combination therapy approaches are still limited, but first clinical trials for patients with malignant pleural mesothelioma [276] and neuroblastoma [277] showed promising outcomes and, most importantly, no combination therapy-associated toxicities. Nonetheless, the extrinsic modification of the T-cell response has inert drawbacks, as therapy success also here depends on sufficient delivery or local application of the checkpoint inhibitors. More sophisticatedly, researchers have established T-cell intrinsic strategies to overcome checkpoint-mediated T-cell inhibition. As such, the CRISPR/Cas9-mediated knockout of PD-1 [278, 279, 280, 281] or CTLA-4 [282] in the CAR T-cells greatly improves T-cell effector functions. Likewise, the truncation of inhibitory receptors, which still bind the ligand but lack signaling [275, 283], or the fusion of the extracellular domain of PD-1 or the TGFβ receptor to an intracellular CD28 or IL-7 chain, which switches the inhibitory signal into a stimulatory one [284, 285], greatly improve anti-tumor effects of these armored CAR T-cells. However, to date there are not enough clinical data of these approaches to give clear-cut answers regarding their effectiveness. Also the use of 4th generation CARs/TRUCKs secreting pro-inflammatory and proliferation-stimulating cytokines such as IL-12 [286] or IL-18 [37] to improve T-cell persistence and modulate the immune response is in pre-clinical development as well as under clinical investigation (NCT03542799, NCT03932565, [287]).

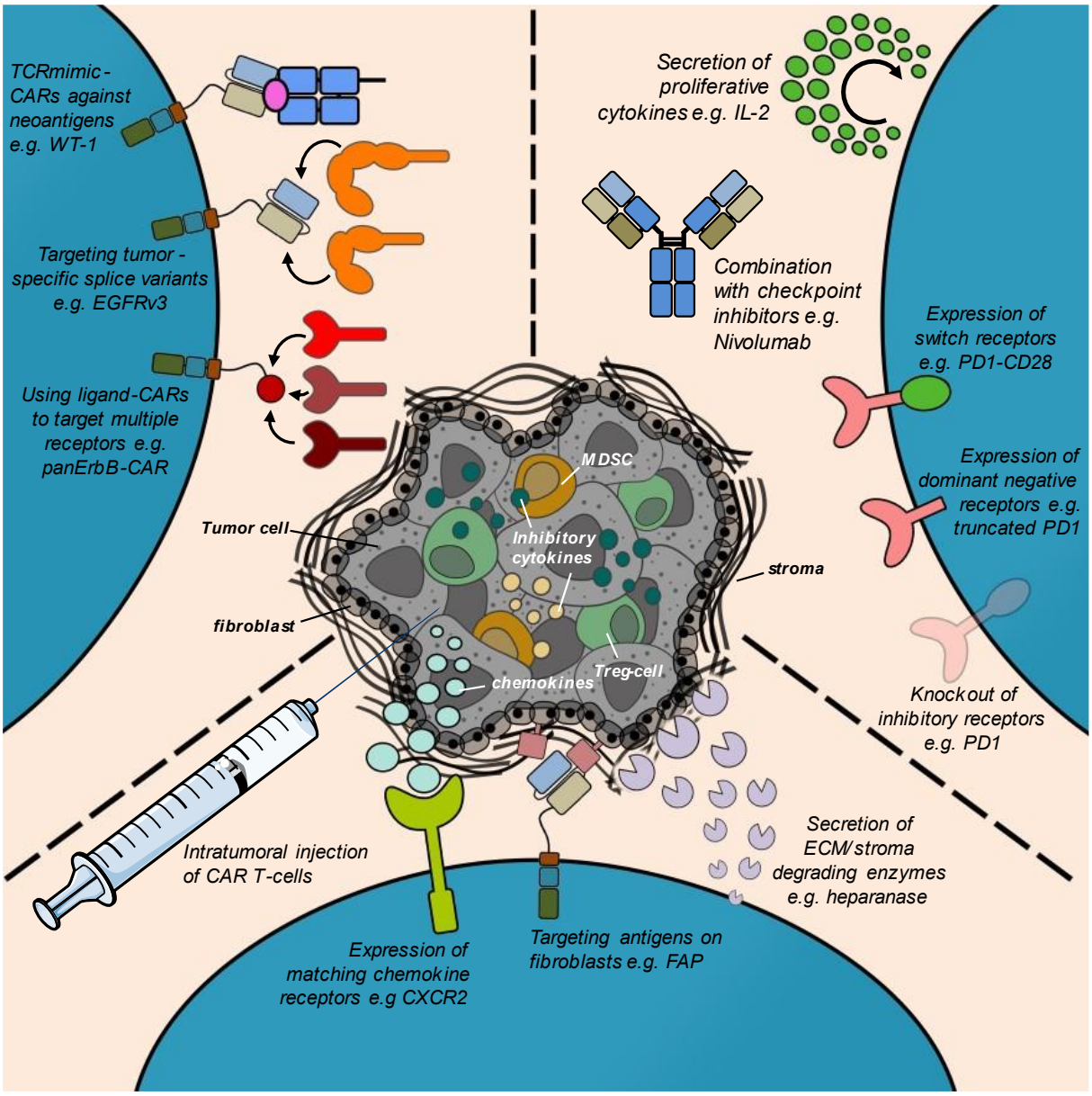

Figure 7: Improving CAR therapy for solid tumors. Since solid tumors present a hostile environment, are difficult to infiltrate and good and safe target antigens are lacking, there is a need address these issues to improve CAR T-cell therapy against solid tumors.

Epigenetic combination therapy

One of the hallmark events that lead to cancer development and progression is the altered epigenetic landscape, leading to transcriptional imbalance and the up-regulation of pro-proliferative and down-regulation of pro-apoptotic genes, thereby causing cell cycle dysregulation [13]. This helps cancer cells, even in the presence of apoptosis-inducing factors, to continue proliferating without entering cell cycle arrest, becoming senescent or undergoing apoptosis. Thus, epigenetic treatment to inhibit DNA methyltransferases (DNMTs) and/or histone deacetylases (HDACs) leads to the activation of epigenetically silenced genes and is already

under pre-clinical as well as clinical investigation for several malignant entities and combination therapy with CAR T-cells [288].

Decitabine blocks DNA methylation by inhibiting DNMTs and is in clinical use for myelodysplastic syndrome and AML [289]. In our hands, decitabine primed urothelial carcinoma cells for more efficacious lysis by CAR T-cells, which was at least partly to the decitabine-induced upregulation of BID and downregulation of BCL2L1, both members of the BCL2 family. In another study, decitabine up-regulated CD19 on lymphoma cells, thus priming the cells for elimination by CD19 CAR T-cells *in vitro* and also *in vivo* in two r/r B-cell lymphoma patients [290]. Currently, two clinical trials prime tumor cells for a combination therapy with CAR T-cells and decitabine in r/r B-cell lymphoma (NCT04850560). Importantly, epigenetic treatment does not only affect the tumor cells, but can also modulate T-cell phenotype and responses. TET2 is a DNA demethylase, which is a key regulator of hematopoiesis. Interestingly, disruption of *TET2* massively improves CAR T-cell proliferation and induced tumor clearance [291]. Similarly, decitabine has been reported to drive favorable T-cell phenotypes [292] and improve tumor rejection in a AML xenograft mouse model [293]. Currently, decitabine-primed CAR T-cells are employed in a clinical trial for patients with B-cell lymphomas (NCT04697940).

Outlook: A non-viral & decentralized allogeneic CAR therapy

Ten years after the first application of CD19-targeted 2nd generation CARs for the treatment of chronic lymphocytic leukemia, a high percentage of patients with B-cell malignancies remain in remission, due to CAR T-cells still circulating in their bloodstream [99]. These findings make hope that CAR T-cell therapy for B-cell malignancies is indeed truly curative rather than a bridge-to-transplant treatment. Nonetheless, autologous CAR T-cell therapy remains a last-line option and, due to its hefty price tag and individualized manufacturing for every patient, not applicable on a broad basis. Thus, one of the next steps for CAR therapy will be to make this immunological therapy broadly available with decreased costs, de-centralized production and shorter manufacturing times. To address these problems, **allogeneic immune effector cells** may be an attractive alternative to autologous T-cells in CAR therapy. Currently, there are three main approaches that are being employed to drive allogeneic CAR therapies forward.

Switching to allogeneic cells for CAR therapy

The first one comprises, like done in this thesis, the use of **NK cells** as effector cells. Unlike T-cells, NK cells do not recognize peptide-loaded MHC molecules and consequently do not cause GvHD when infused into patients. However, NK cell therapy is significantly lagging behind CAR T-cell therapy [23]. In 2020, there were only 17 clinical trial employing CAR NK cells – a fraction compared to the over 500 reported clinical trials with CAR T-cells [23]. One of the

main reasons remains the challenge in the efficient large-scale generation of CAR NK cells, however significant progress has been made here in recent years and the translation of these findings to the clinic is just a matter of time.

The two other strategies employ allogeneic T-cells as immune effector cells. Here, in order to prevent MHC-restricted GvHD, **α/β T-cells** genetically modified to lose expression of the T-cell receptor or γ/δ T-cells, which lack expression of a TCR that recognizes MHC proteins, have been used. The knockout of the TCR of α/β T-cells has been accomplished using CRISPR-Cas9 to specifically target the *TRAC* and *TRBC* loci. The CRISPR-Cas9 system has also been used to knock-in the CAR constructs into one of these loci, which apparently stabilizes CAR expression, reduces tonic signaling and improves ALL rejection by the CAR T-cells [294].

In contrast to α/β T-cells, the TCR of **γ/δ T-cells** does not bind MHC molecules. Rather, the γ/δ TCR binds antigens directly with a particular variable region. These antigens cover a range of potentially immunogenic ligands and include metabolites [295], stress signals [296] and stress receptors [297]. As such, γ/δ T-cells form a bridge between the innate and adaptive immune system and, similar to NK cells, take up a prominent role during infection and tumor immunosurveillance.

All three of these approaches have their strengths, but also merits. Due to their role in tumor immunosurveillance, NK and γ/δ T-cells are destined to be used in adaptive cellular therapies. However, they are only fraction of peripheral blood mononuclear cells (PBMCs), in contrast to α/β T-cells, which make up about 70 % of PBMCs [298, 299]. Consequently, larger starting materials (e.g. apheresis products) or longer *ex vivo* cultivation times would be required to reach the critical cell number for an adoptive therapy. However, these cells do not need additional genetic modifications besides the CAR integration, as there is no need to knockout the TCR to prevent GvHD. This not only prevents potential problems associated with the knockout or additional transduction procedures, but also eases the regulatory framework needed for approval.

Decentralizing CAR T- or NK cell production

As previously mentioned, CAR T-cells are currently produced at centralized locations, e.g. the Fraunhofer Institute for Cell Therapy and Immunology in Leipzig, Germany, from where they are sent to the treatment sites [142]. The whole process is quite time consuming, labor-intense and requires an elaborate infrastructure, which is reflected by the price as well as the production time for the CAR T-cell products. Consequently, to decrease costs and accelerate treatment, there is a need to decentralize the whole process. Here, fully-automated and closed systems, such as the Prodigy™ or Cocoon™ devices developed by Miltenyi Biotec and Lonza, respectively, offer a means to equip a great number of university treatment

centers with stations to manufacture adoptive cellular products themselves. After programming, the machines automatically run the manufacturing of CAR products, including selection of the initial effector cell type, transduction with CAR genes and subsequent expansion. Importantly, both NK [300] as well as T-cells [301], including γ/δ T-cells [302], are compatible with these platforms and as such these can be used for allogeneic CAR therapies employing the three previously mentioned approaches.

Translating non-viral gene transfer methods to CAR therapy

The use of lentiviral vectors for cell and gene is controversially discussed, especially since long-term effects in patients are not well known yet. While lentiviral vectors enable great transductions efficiencies with stable transgene expression, also when transducing T- or NK cells and non- or rarely replicating cells such as stem cells [303] in different pseudotypes [304], these vectors still do integrate randomly into the genome [305]. Theoretically, this random integration poses the risk of activating oncogenes or inactivating tumor suppressor genes, especially when multiple copies of the vector are integrated into the genome. This insertional mutagenesis also translates to the regulatory framework, where the safety of the cellular products has to be demonstrated in surrogate tests e.g. by a maximum of vector copy number integrations into the genome of the transduced cells. Moreover, lentiviral vectors make up a significant amount of the therapy costs, since the lentiviral vectors have to be produced under biosafety level II conditions in an extremely regulated GMP environment. Thus, in order to avoid these issues and reduce therapy costs, non-viral methods might be the go-to approach in the future. Here, the development of transposon-based system as well as CRISPR/Cas9 and its most recent iterations could provide means to do so.

In recent years, the **sleeping beauty (SB) transposition system** has gained momentum for use in cell and gene therapy. The SB transposon, which was originally found in fish genomes, is a synthetic DNA transposon that stably integrates its template into the genome via a cut-and-paste mechanism [306]. As it is more likely to integrate into *safe harbors* and thus less likely to integrate close to cancer-related genes and also simply DNA, it is presumably safer [305] and certainly cheaper to use compared to lentiviral vectors. Very recently, a new study demonstrated feasible and efficient electroporation-mediated introduction of CD19 CARs into TCR-KO T-cells with the SB transposon system [307]. These cells mediated efficient control of leukemia and lymphoma cells *in vitro* as well as *in vivo* and thus prove the feasibility for SB-mediated CAR T-cell generation. Indeed, an ongoing phase I/II clinical trial assesses the use of allogeneic SB-engineered CD19 CAR T-cells for r/r pediatric and adult ALL patients and found promising response rates especially for patients treated with a high-dose of CAR T-cells, as here six out of seven patients showed complete responses and CAR T-cells persisted for up to 10 months [308]. Importantly, the treatment proved to be safe without severe toxicities and with a favorable transgene integration profile. The SB system has also been

used to modify NK cells to express CD19 CARs against ALL; although the generated CAR NK cells were functional with a favorable transgene integration, the gene transfer rates were lower compared to the previously mentioned study employing T-cells [309]. Thus, at this stage the CD34- or NGFR-derived hinges could be used to enrich the CAR NK cell fraction to generate a uniform cellular product.

Novel **CRISPR/Cas9-based technologies** could represent the most exciting alternative to transfer CAR genes into T- or NK cells. Nowadays, these systems even allow integration of genes into the genome without causing DNA double strand breaks or without the need to deliver an exogenous template [310]. Both for T- [311] as well as NK cells [312] electroporation protocols to introduce ribonucleoproteins (RNPs) or mRNAs encoding for Cas9 are readily available. As for the SB transposon system, the production of RNPs or mRNAs costs only a fraction of that of lentiviral vectors, which would help to scale down costs of the therapy. Most importantly, the integration site of the CAR cDNA can be specifically chosen by the guide RNA, which dictates where the Cas9 cuts or nicks the genome. Therefore, CARs can specifically be introduced into the TCR locus [294] and thereby simultaneously achieve a knockout of the TCR *TRAC* and *TRBC* genes. Alternatively, CAR genes can be specifically integrated into safe harbor sites such as *ROSA26* in the genome to prevent the random activation of oncogenes or inactivation of tumor suppressor genes [313]. Targeting to specific genomic loci also limits the number of genomic integration loci of the CAR cDNA to two, since the gene can only be integrated once per allele. This ensures genomic stability of the CAR effector cells and also eases the regulatory framework since cellular products have to demonstrate safe vector copy numbers, before they can be administered to the patient.

In recent years, so-called **nanoblades** have been developed to efficiently transfer genes into primary cells [314]. These are virus-like particles that can be loaded with Cas9 protein and a template to integrate the gene of interest into the target cell. Similar to viral particles, they carry envelope proteins such as VSV-G on their surface, which enables efficient transfer into cells carrying the respective entry receptor and also to alter tropism by adjusting the envelope protein. Indeed, nanoblades have also been pseudotyped with the baboon endogenous virus (BaEV) glycoprotein, which enabled efficient gene editing not only in human T- but also B-cells and hematopoietic stem cells and allowed gene knock-in into hematopoietic stem cells [315]. Although not being tested in NK cells so far, BaEV-pseudotyped nanoblades are an intriguing tool to transfer CAR genes into NK cells, since they are highly efficiently transduced with this glycoprotein (see section 2.3).

Conclusion

We have come a long way since chimeric antigen receptors have been described for the first time in 1989 [19], which ultimately resulted in the approval of

six CAR T-cell therapies just within the last six years. So far CAR therapy remains a last-line therapy approach and cannot be used on a broad basis due to its immense price tag and some safety concerns. However, by transitioning to a donor-based therapy, where CAR T- or NK cells are produced by non-viral means on site in GMP-compatible closed systems (**Figure 8**), therapy costs will come down and the inclusion of suicide genes or switches will allow to stop the therapy in case of serious adverse events. Thus, it remains just a matter of time and coordination, until we will see the next step of the CAR-driven immunotherapy revolution, where CAR therapies can be employed as a safe first-line therapy for hard-to-treat malignancies to a huge amount of patients.

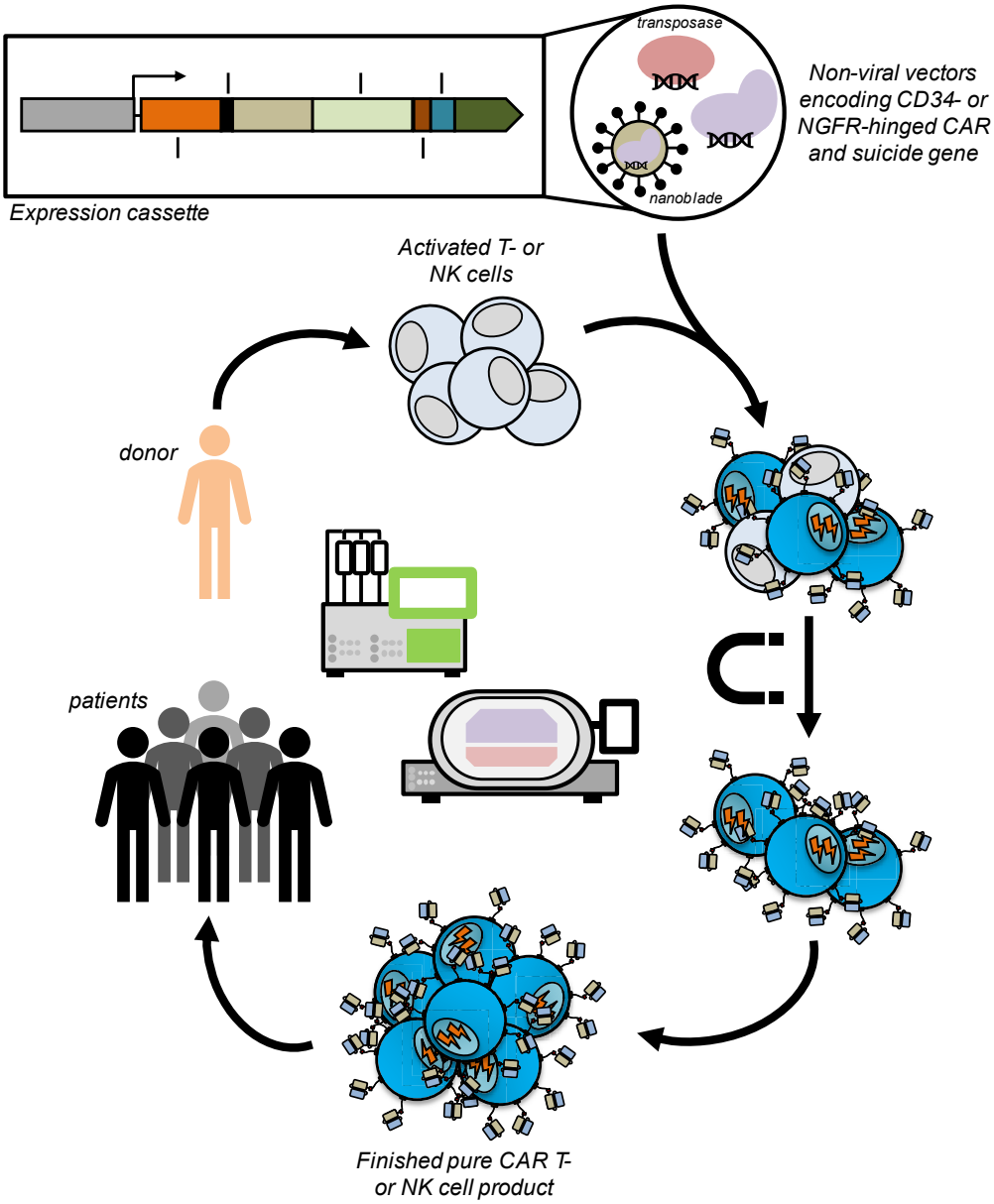

Figure 8: Proposed clinical application of allogeneic CAR T- or NK cells. Contrary to autologous CAR T-cell therapy, T- or NK cells are isolated from a donor and modified with a non-viral system to transfer to express a CAR and a suicide gene. After magnetic enrichment (enabled via CD34- or NGFR-derived hinges), pure CAR T- or NK cells are expanded and administered to multiple patients.

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6 List of abbreviations

A

aa	Amino acid
AICD	Activation-induced cell death
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
appr.	Approximately

B

BaEV	Baboon endogenous virus
BCL2	B-cell lymphoma 2 (antigen)
BCMA	B-cell maturation antigen
BFP	Blue fluorescent protein
BID	BH3 interacting domain death agonist
BiKE	Bispecific killer engager

C

CAR	Chimeric antigen receptor
CD (e.g. CD19)	Cluster of differentiation
CD44v6	CD44 variant 6
CEA	Carcinoembryogenic antigen
CH_2CH_3	Constant heavy 2 constant heavy 3
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

D

DAP10	DNAX-activation protein 10
DAP12	DNAX-activation protein 12
DEC	Decitabine
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMTi	DNA methyltransferase inhibitor

E

ECM	Extracellular matrix
e.g.	Exempli gratia
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
EMA	European Medicines Agency
EpCAM	Epithelial cell adhesion molecule
ErbB2	Erythroblastic oncogene B2

F

FcR	Fc receptor
FDA	Food and Drug Administration

FITC	Fluorescein isothiocyanate

G

GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
GvHD	Graft versus host disease

H

HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma

I

ICOS	Inducible T-cell costimulator
IFN-γ	Interferon gamma
IgG (e.g. IgG1)	Immunoglobulin G
IL (e.g. IL-2)	Interleukin
IRES	Internal ribosomal entry site
ITAM	Immunoreceptor tyrosine activation motif

J

JAK	Janus kinase

M

mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAGE-A4	Melanoma-associated antigen A4
MCL	Mantle cell lymphoma
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompability complex
MM	Multiple myeloma
MPSV	Myeloproliferative sarcoma virus
mRNA	Messenger RNA

N

NFAT	Nuclear factor of T-cell activation
NGFR	Nerve growth factor receptor
NK cell	Natural killer cell
NSG	NOD-SCID-gamma
NY-ESO-1	New York esophageal squamous cell carcinoma-1 (antigen)

P

PBMC	Peripheral blood mononuclear cells
PCMA	Prostate stem cell antigen
PD-L1	Programmed death-ligand 1
PE	Phycoerithrin
PMBCL	Primary mediastinal B-cell lymphoma
PSMA	Prostate-specific membrane antigen

R

RNA	Ribonucleic acid
RNP	Ribonucleoprotein

ROM	Romidepsin
ROR1	Receptor tyrosine kinase-like orphan receptor 1
r/r	refractory/relapsed

S

SB	Sleeping beauty
scFv	Single chain fragment of the variable region
SSX2	Synovial sarcoma, X breakpoint 2 (antigen)
STAT	Signal transducer and activator of transcription
synNotch	Synthetic Notch

T

TAA	Tumor associated antigen
TCR	T-cell receptor
TET2	Tet-methylcytosine-dioxygenase 2
TGF-β	Transforming growth factor beta
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
T_{reg} cell	Regulatory T-cell
TriKE	Trispecific killer engager
TRUCK	T-cells redirected for antigen-unrestricted cytokine-initiated killing

V

V_H	Variable region of the heavy chain
V_L	Variable region of the light chain
VSV-G	Vesicular stomatitis virus glycoprotein

W

WT-1	Wilms tumor protein 1

7 Congress contributions

Posters

Ibach T, Roellecke K, Wiek C, **Bister A**, Haist C, Altvater B, Rossig 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, Hanenberg H & 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, Roellecke 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, Gattermann 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 27th Annual 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 27th Annual Congress, Barcelona, Spain*

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Scheckenbach K, **Bister A**, Schulte E, Hanenberg H, Wiek C, Wagenman 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.*

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Haist C**,** Konig C, **Bister A**, Wiek C,Scheckenbach K, Niegisch G, Hanenberg H, Hoffmann MJ & Grunewald CM (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*

Presentations

Bister A (2018): Development of CAR T-cell therapy for mantle cell lymphoma (MCL) with safety-improved lentiviral vectors. 6. *Retreat of the Düsseldorf School of Oncology, Düsseldorf, Germany*

Bister A (2019): Simultaneous targeting of CD5 and CD19 to increase specificity towards double-positive mantle cell lymphoma cells in CAR T-cell therapy. *7. Retreat of the Düsseldorf School of Oncology, Düsseldorf, Germany*

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

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Eidesstattliche Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbstständig ohne unzulässige fremde Hilfe unter Beachtung der ”Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf” erstellt worden ist. Die Dissertation habe ich in dieser oder in ähnlicher Form noch bei keiner anderen Institution vorgelegt. Ich habe bisher keine erfolglosen oder erfolgreichen Promotionsversuche unternommen.

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