Medizinische Hochschule Hannover

Institut für Transfusionsmedizin und Transplantat Engineering

Genetically-engineered T cells for adoptive T-cell therapy: From CARs to TRUCKs and beyond

INAUGURALDISSERTATION

zur Erlangung des Grades einer Doktorin oder eines Doktors der Naturwissenschaften

- Doctor rerum naturalium -

(Dr. rer. nat.)

vorgelegt von

Anna Christina Dragon

aus Hannover

Hannover 2021

Angenommen durch den Senat: 30.07.2021

Präsident:	Prof. Dr. med. Michael P. Manns
Wissenschaftliche Betreuung:	Prof.‘in Dr. rer. nat. Britta Eiz-Vesper
Wissenschaftliche Zweitbetreuung:	Prof. Dr. med. Thomas Moritz

1. Referent/in:	Prof.’in Dr. rer. nat. Britta Eiz-Vesper
2. Referent/in:	Prof. Dr. med. Thomas Moritz
3. Referent/in:	Prof.’in Dr. rer. nat. Renata Stripecke

Tag der mündlichen Prüfung: 30.07.2021

Prüfungsausschuss

Vorsitz:	PD Dr. rer. nat. Matthias Lochner
1. Prüfer/in:	Prof.’in Dr. rer. nat. Britta Eiz-Vesper
2. Prüfer/in:	Prof. Dr. med. Thomas Moritz
3. Prüfer/in:	Prof.’in Dr. rer. nat. Renata Stripecke

Table of Contents

Abstract **1**

Zusammenfassung **3**

1 Introduction **5**

1.1 T lymphocytes are part of the adaptive immune system 5

1.2 Adoptive therapy with genetic engineered T cells 6

1.2.1 Chimeric Antigen Receptor (CAR)-engineered T cells 6

1.2.2 TRUCKs, the next generation of CAR-T cells 10

1.3 The hurdle to choose an ideal target for CAR-T-cell therapy 12

1.3.1 A virus-derived peptide as target for Post-Transplant Lymphoproliferative Disorder (PTLD) 13

1.3.1.1 Development of PTLD 13

1.3.1.2 The T-cell-receptor-like monoclonal antibody TÜ165 15

1.3.2 The disialoganglioside G_{D2} as tumor-associated antigen 16

2 Project Aims **18**

3 Research Articles **20**

3.1 CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation 20

3.2 Design and Characterization of an “All-in-One” Lentiviral Vector System Combining Constitutive Anti-G_{D2} CAR Expression and Inducible Cytokines 38

3.3 GMP-compliant manufacturing of TRUCKS: CAR T cells targeting GD2 and releasing inducible IL-18 62

4 Results and Discussion **106**

4.1 A virus peptide as CAR target provides high specificity and coverage 106

4.2 Peptide-specific TRUCKs exhibit highly improved functionality 110

4.3 Functionality of G_{D2}-targeting TRUCKs for the treatment of solid tumors 112

4.4 Feasibility of GMP-compliant manufacturing of TRUCKs 115

5 Conclusion and Outlook 119

6 References 121

7 Appendix 131

7.1 List of abbreviations 131

7.2 Curriculum vitae 133

7.3 Danksagung 137

7.4 Erklärung 138

Abstract

Anna Christina Dragon

Genetic engineered T cells for adoptive T-cell therapy: From CARs to TRUCKs and behind

A therapeutic approach that gained importance in the last years is the adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells achieving impressive anti-tumor effectivity in patients with hematological malignancies. One hurdle in CAR-T-cell design is the decision for an appropriate tumor target. Post-transplant lymphoproliferative disorder (PTLD) is a severe complication after solid organ and stem cell transplantations and most frequently results from the uncontrolled expansion of Epstein-Barr virus (EBV)-infected B cells in the immunocompromised patients. Since conventional CAR-T cells can only recognize extracellular antigens, the specificity of the T-cell-receptor-(TCR)-like monoclonal antibody TÜ165 targeting an Epstein-Barr nuclear antigen (EBNA)-3C-derived peptide of the EBV latency type III was used to direct CAR-T cells against an intracellular, PTLD-associated target. Such TÜ165 CAR-T cells exhibited specific effector functions against EBV peptide$^+$ target cells.

Most CAR-T-cell products generated to treat other tumor entities than hematological malignancies failed to achieve maintained responses due to lacking CAR-T-cell persistence and the formation of an immunosuppressive tumor microenvironment (TME) by solid tumors. To approach this, T cells redirected for universal cytokine-mediated killing (TRUCKs) were developed, which contain a CAR that is constitutively expressed and a nuclear factor of the activated T cells (NFAT)-sensitive, inducible cassette encoding for a soluble mediator such as the cytokines interleukin (IL)-12 or IL-18. Thereby, CAR activation upon target recognition at the tumor site induces the release of the mediator in a locally-restricted manner enabling re-modulation of the TME, recruitment and activation of other immune cells as well as improvement of the CAR-T-cell function and phenotype. Combining the developed TÜ165 CAR with inducible IL-12 expression to generate TRUCKs enabled robust target specificity for EBV-infected cells, whereas healthy cells were spared, and mediated highly improved functionality regarding improved activation marker upregulation, pro-inflammatory cytokine and cytolytic mediator expression, as well as cytotoxicity towards EBV peptide$^+$ target cells. The target-specific release of IL-12 further mediated recruitment and activation of other immune cells. Thereby, TÜ165 TRUCKs with inducible IL-12

may serve as an innovative therapeutic approach enabling the specific elimination of malignant cells, while healthy immune cells survive and are attracted to the tumor.

As a different tumor-associated antigen, G_{D2} is highly expressed on various solid tumors including neuroblastoma. However, G_{D2}-specific CAR-T cells failed to sustain remission in neuroblastoma patients in clinical trials due to lack of T-cell persistence indicating incorporation of a cytokine expression in the CAR construct could improve the clinical outcome. For this, modular "all-in-one" vectors were generated that comprise the constitutive expression of a G_{D2}-targeting CAR and the NFAT-inducible expression of IL-12 or IL-18, respectively. Both G_{D2}-specific TRUCKs with inducible IL-12 and IL-18 facilitated high target specificity and functionality in co-cultures with G_{D2}^{+} target cells, and further mediated recruitment of other immune cells by the target-induced release of the respective cytokine. Up-scaling the manufacturing process, the good manufacturing practice (GMP)-compliant generation of TRUCKs for the potential use in future clinical trials, exemplary shown for IL-18 TRUCKs targeting G_{D2}, yielded a large amount of highly functional cells reflecting all effector functions evaluated in the pre-clinical testing. Since the manufacturing of TRUCKs was smooth to implement, this evokes optimism for the feasibility to generate further TRUCK products for the potential clinical use.

Altogether, this brings TRUCK technology as an improvement for current CAR-T-cell approaches and especially for innovative strategies to specifically target malignant cells via intracellular antigen recognition, one step closer to clinical application.

Zusammenfassung

Anna Christina Dragon

Genetic engineered T cells for adoptive T-cell therapy: From CARs to TRUCKs and behind

Ein therapeutischer Ansatz, der in den letzten Jahren zunehmend an Bedeutung gewonnen hat, ist der adoptive Transfer von CAR-modifizierten T Zellen, der in Patienten mit hämatologischen Malignomen eine beeindruckende Antitumor-Effektivität erzielen konnte. Eine Herausforderung im Design von CAR-T-Zellen ist die Auswahl eines geeigneten Tumortargets. PTLD ist eine Komplikation nach Organ- und Stammzelltransplantationen, die meist aus einer unkontrollierten Expansion von EBV-infizierten B-Zellen in den immunsupprimierten Patienten resultiert. Da konventionelle CAR-T Zellen nur extrazelluläre Antigene erkennen können, wurde der T-Zellrezeptor-ähnliche, monoklonale Antikörper TÜ165 gegen ein EBNA-3C-abgeleitetes Peptid der EBV-Latenzphase III benutzt, um die Antigenspezifität von CAR-T-Zellen gegen ein intrazelluläres, PTLD-assoziiertes Target zu richten. Die so generierten TÜ165 CAR-T Zellen entwickelten spezifische Effektorfunktionen gegen EBV-Peptid$^+$ Zielzellen.

CAR-T-Zellprodukte, die für die Behandlung weiterer Tumorentitäten neben hämatologischen Malignomen entwickelt wurden, konnten aufgrund fehlender T-Zellpersistenz und der Ausbildung einer immunsuppressiven Tumormikroumgebung (TME) durch solide Tumoren bislang keine anhaltende Tumorantwort erwirken. Um dem zu begegnen, wurden TRUCKs entwickelt, die einen konstitutiv exprimierten CAR und eine NFAT-sensible, induzierbare Kassette für einen löslichen Mediator, wie beispielsweise das Zytokin IL-12 oder IL-18, enthalten. Dadurch induziert die CAR-Aktivierung nach Zielerkennung am Tumorort die Ausschüttung des jeweiligen Moleküls in einer lokal beschränkten Art und Weise, wodurch eine Remodulation der TME, eine Anlockung und Aktivierung weiterer Immunzellen, und eine Verbesserung der Funktionalität und des Phänotyps der CAR-T-Zellen ermöglicht wird. Die Kombination des entwickelten TÜ165 CARs mit einer induzierbaren IL-12-Expression zur Generierung von TRUCKs ermöglichte eine verlässliche Spezifität für EBV-infizierte Zellen, wohingegen gesunde Zellen nicht erkannt wurden. TÜ165 TRUCKs wiesen außerdem im Hinblick auf eine erhöhte Aktivierungsmarkerexpression, die Ausschüttung von pro-inflammatorischen Zytokinen und zytotoxischen Mediatoren, sowie Zytotoxizität gegenüber EBV-Peptid$^+$ Zielzellen eine stark verbesserte Funktionalität auf. Durch die targetspezifische Freisetzung von IL-12 wurde weiterhin die Rekrutierung und Aktivierung

weiterer Immunzellen vermittelt. Dadurch haben TÜ165 TRUCKs mit induzierbarem IL-12 das Potential als neuer, innovativer Therapieansatz, um die spezifische Eliminierung von malignen Zellen zu ermöglichen, während gesunde Immunzellen verschont und mobilisiert werden.

Als weiteres tumor-assoziiertes Antigen wird G_{D2} auf vielen soliden Tumoren, wie beispielsweise dem Neuroblastom, hoch exprimiert. G_{D2}-spezifische CAR-T-Zellen haben es in klinischen Studien jedoch bislang nicht geschafft, in Neuroblastom-Patienten eine langfristige Remission zu erhalten. Dies liegt wahrscheinlich an einer mangelnden T-Zell-Persistenz und legt die Vermutung nahe, dass das klinische Ergebnis durch das Hinzufügen einer Zytokinexpression in das CAR-Konstrukt verbessert werden könnte. Dazu wurden modulare „all-in-one"-Vektoren generiert, die die konstitutive Expression eines G_{D2}-spezifischen CARs und die NFAT-induzierbare Expression von entweder IL-12 oder IL-18 vereinen. Beide G_{D2}-spezifischen TRUCKs mit induzierbarem IL-12 oder IL-18 wiesen eine hohe Spezifität und Funktionalität in Ko-Kulturen mit G_{D2}^+ Zielzellen auf, und vermittelten weiterhin die Rekrutierung von weiteren Immunzellen durch die antigeninduzierte Freisetzung des jeweiligen Zytokins. Eine GMP-konforme Herstellung von TRUCKs, um diese für die potentielle Anwendung in zukünftigen klinischen Studien auf zu skalieren, erreichte am Beispiel von G_{D2}-spezifischen IL-18 TRUCKs eine große Ausbeute an hochfunktionellen Zellen, die alle T-Zelleffektorfunktionen widerspiegelten, die in der präklinischen Austestung ermittelt wurden. Die problemlose Implementierung der Herstellung von TRUCKs stimmt optimistisch, dass es auch möglich ist, weitere TRUCK-Produkte für eine potentielle klinische Verwendung zu generieren.

Zusammenfassend bringt dies die TRUCK-Technologie als Verbesserung für aktuelle CAR-T-Zellansätze und insbesondere für innovative Strategien zur spezifischen Adressierung von malignen Zellen durch intrazelluläre Antigenerkennung, einen Schritt näher zur klinischen Anwendung.

1 Introduction

1.1 T lymphocytes are part of the adaptive immune system

T lymphocytes, together with B lymphocytes, form the cellular component of the adaptive immune system [1]. In contrast to the first-line, innate immunity, this second part of the immune system enables reaction against structural parts of almost all pathogens, tumors or the environment, formation of an immunological memory and maintenance of immune homeostasis. T and B lymphocytes are capable of recognizing this wide variety of antigens through the expression of highly diverse receptors.

T cells originate from common lymphoid progenitors generated in the bone marrow, which then mature in the thymus. Within this maturation, by somatic recombination of large sets of gene segments a unique T-cell receptor (TCR) is generated for each T cell resulting in a giant repertoire of T cells with different specificities. Positive and negative selection ensures that only T cells with TCRs specific for a foreign peptide in context of own human leukocyte antigen (HLA) develop. HLA molecules are expressed on almost all cells and their key function is to continuously present all cellular peptides towards immune cells. Two major classes, HLA-I and HLA-II, can be distinguished, which particularly differ in the cell types on that they are expressed and the origin of antigens they present [2]. Depending on the class of HLA molecules their TCRs recognize during maturation in the thymus, T lymphocytes express either the co-receptor cluster of differentiation (CD)4 binding to HLA-I or CD8 binding to HLA-II and are therewith categorized into two major groups [1]. After maturation, T cells are released into the circulation, from where they traffic through secondary lymphoid organs (e.g. lymph nodes) for a constant immune surveillance.

Circulating naïve T cells are activated upon recognition of the appropriate peptide/HLA complex by their specific TCR, and the interaction is stabilized by the respective co-receptor binding to nonpolymorphic domains of the HLA molecule [1]. Associated to the TCR is the CD3 complex comprising γ, δ, ε and ζ chains. These molecules rapidly cluster upon target recognition by the TCR and adhesion molecules further mediate a tight connection between the T cell and antigen-presenting cell, an assembly that is termed as immunological synapse. After receiving further signals comprising co-stimulation and cytokine expression, and driven by phosphorylation of CD3 as well as recruited molecules, several transcription factors including nuclear factor of activated T cells (NFAT), nuclear factor κb (NF-κB) and activator protein 1 (AP-1) are expressed and

consequently fully activate the T cell. Depending on signals from cytokines and additional transcription factors, activated T cells differentiate into effector cells with various functions, such as cytotoxicity, helping signals or regulation of immune responses.

Activated $CD8^+$ T can differentiate into cytotoxic T lymphocytes (CTLs) [1]. These effector cells are able to directly kill virus-infected and transformed cells upon recognition of foreign intracellular peptides presented in context of HLA-I on the target-cell surface. This recognition induces the formation of an immunological synapse, in which CTLs can kill the target cell in a rapid process. This includes release of cytotoxic granules with granzyme B or perforin as well as expression of Fas ligand, both of which induce apoptosis of the malignant cell.

1.2 Adoptive therapy with genetic engineered T cells

Exploiting the cytotoxic function of T cells, an adoptive transfer of T cells to patients suffering from virus infections or cancer has emerged as therapy option [3]. For that purpose, T cells can be administrated at different degrees of previous selection and modification, including donor lymphocyte infusions, transfer of virus-specific T cells, tumor-infiltrating lymphocytes, TCR-engineered T cells or chimeric antigen receptor (CAR)-T cells.

1.2.1 Chimeric Antigen Receptor (CAR)-engineered T cells

In order to generate T cells with distinct antigen specificity and in sufficient numbers for an adoptive transfer, they can be genetically engineered. One strategy is the introduction of a recombinant TCR with known anti-virus- or anti-tumor-binding properties. Such TCR-T cells already showed promising results in clinical trials: targeting a peptide derived from the cancer-testis antigen New York esophageal squamous cell carcinoma 1 (NY-ESO-1) in context of HLA-A*02:01 could reach a response rate above 50% for patients with synovial cell sarcoma, melanoma and myeloma [4-6], and more clinical studies are currently performed (reviewed in [7]). However, careful evaluation is required for TCR-engineered T cells since introduction of a second TCR bears the risk of evolving unexpected specificities upon mispairing with the endogenous TCR or a low expression of the introduced TCR caused by competition with the endogenous TCR resulting in a decreased functionality [8-10]. Several strategies were developed to address these concerns

including disruption or silencing of the endogenous TCR or introduction of the endogenous TCR into alternative cells [11].

Another strategy to genetically modify T cells for defined antigen specificity that gained importance in the last years is the equipment with a CAR. CARs are artificial constructs consisting of one polypeptide chain that comprises antigen-binding properties of an antibody or natural ligand fused to the activating domains of cellular receptors, whereby formation of dimers with the endogenous TCR is prevented [12]. Mostly, a single-chain fragment variable (scFv), the specificity-determining region of an antibody, forms the antigen-recognition domain of the CAR [13, 14] and is able to mediate binding to extracellular antigens of all kinds with a considerably higher binding affinity than a TCR [15]. In the first generation of CARs, this scFv is connected to a hinge region, a transmembrane domain and the intracellular CD3ζ or Fc receptor γ chain (see Figure 1) [12]. Thereby, recognition of the appropriate target by the extracellular antigen-binding domain of the CAR results in intracellular activation of T-cell-signaling pathways. Considering that physiological T-cell activation and proliferation, additional to signaling by the TCR, requires co-stimulation and cytokine expression, further elements can be introduced into the CAR construct. Thus, second- and third-generation CARs were designed containing additional one or two signaling domains of co-stimulatory molecules such as 4-1BB, CD28 or OX40 and mostly exhibit a superior T-cell response and persistence. Fourth generation CAR-T cells, the so-called T cells redirected for universal cytokine-mediated killing (TRUCKs), are moreover capable to produce an additional effector molecule such as a cytokine [16]. Further strategies for fifth generation CARs incorporate the third synergistic T-cell activation signal by insertion of modules for antigen-dependent cytokine signaling by Janus kinase/signal transducer and activator of transcription 3/5 (JAK-STAT3/5) in the CAR construct [17].

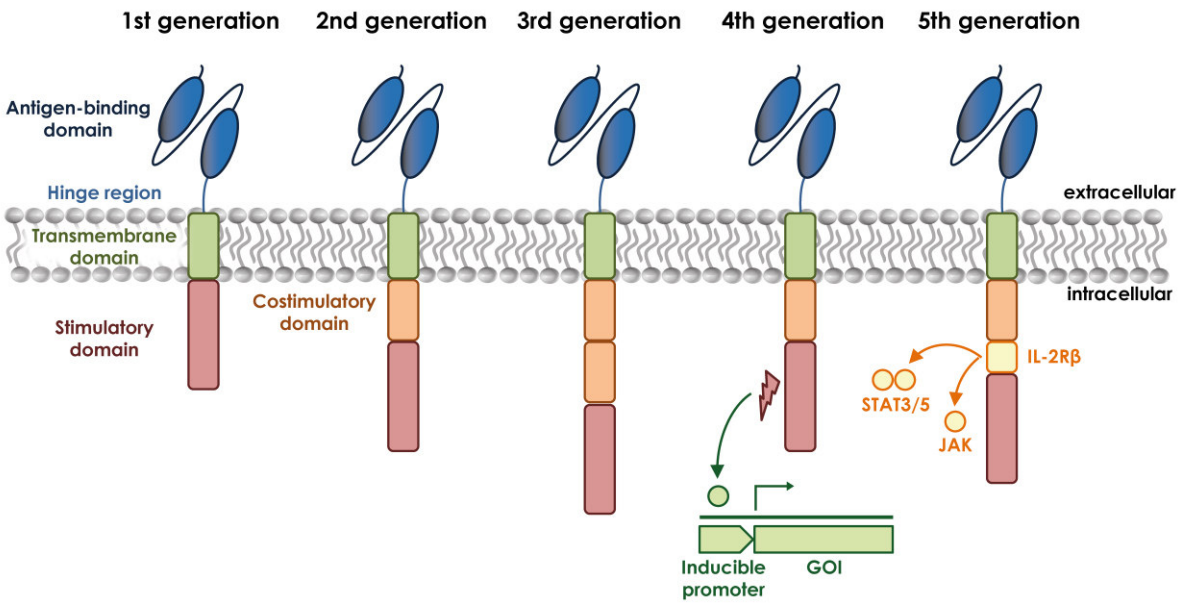


Figure 1. The structure of CAR constructs. First generation CARs consist of an antigen-binding domain (e.g. an antibody-derived scFv), a hinge region, a transmembrane domain (e.g. CD28-derived) and a stimulatory domain (e.g. the CD3ζ chain) [12]. In second and third generation CARs, one or two additional co-stimulatory domains (e.g. 4-1BB, CD28) are added. Fourth generation CARs, also known as TRUCKs, are further endowed with an inducible gene of interest (GOI) that is expressed upon signaling after CAR activation. Fifth generation CARs incorporate the IL-2 receptor subunit β (IL-2Rβ) for antigen-dependent activation of JAK-STAT3/5 signaling. Adapted from [18].

CAR-engineered T cells were shown to exhibit impressive anti-tumor activity in hematological malignancies [19-22] prompting the approval for three CD19-targeting CAR-T-cell therapies, Kymriah (Novartis Pharma, Basel, Switzerland), Yescarta (Kite Pharma, Los Angeles, USA), and Tecartus (Kite Pharma, Los Angeles, USA), for patients with relapsed or refractory B cell precursor acute lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), primary mediastinal large B-cell lymphoma (PMBCL), and mantle cell lymphoma (MCL) from 2017 to 2020 [23-25].

For implementation of further CAR-T-cell products into the clinics, manufacturing of the engineered cells under good manufacturing practice (GMP) is a critical prerequisite [26, 27]. The process starts with collection of peripheral blood mononuclear cells (PBMCs) from the patient, which is mostly realized by leukapheresis (see Figure 2). The first step of manufacturing is the isolation of T cells from PBMCs. Most often, $CD3^+$ T cells are selected, yet the feasibility of isolating subpopulations such as naïve, central memory or stem cell like memory T cells was shown, and studies attributed them to have superior functionality [28-30]. The isolated T cells are then activated via TCR and co-stimulation (e.g. anti CD3/CD28 beads or nanoparticles) for *ex vivo* expansion and introduction of the CAR [26]. The genetic information for the CAR is, most often, transferred into the cell by γ-retroviral or lentiviral vectors to obtain a stable CAR expression. Both

of them enable high transfer efficiency. Even though potential danger is minimized by the use of replication-incompetent virus, the CAR integration is a random process harboring the risk of insertional mutagenesis, and the production of GMP-grade lentiviral vectors is moreover time- and cost-intensive. To approach this, alternative shuttle systems using the transposon/transposase system and other non-viral strategies are currently under investigation. After the removal of residual CAR vector from the culture, expansion of the modified T cells to clinically usable numbers is performed. The final cell product is then formulated in the desired infusible media and controlled for several parameters including safety, purity, identity and quantity [27]. Together, CAR-T-cell manufacturing for clinical use is complex and thereby requires tight control of safety, quality and efficacy to ensure a defined and robust production at different manufacturing sites [26].

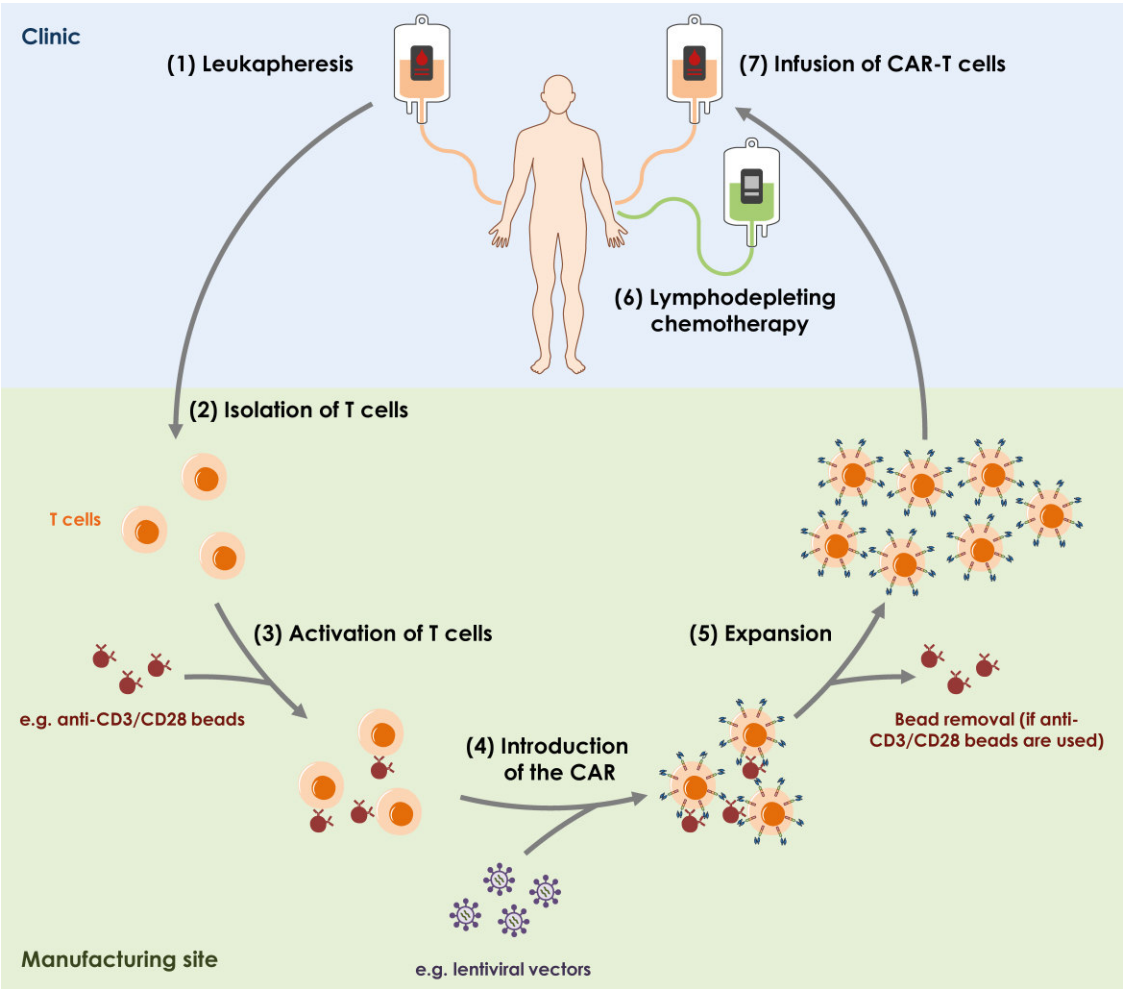


Figure 2. Manufacturing of CAR-T cells. Manufacturing of CAR-T cells for the clinical use is realized by performing the following steps [26]: leukapheresis of the patient, isolation of T cells, activation of T cells (e.g. by anti-CD3/CD28 beads), introduction of the CAR (e.g. via lentiviral vectors), expansion (whereby the beads are removed if CD3/CD28 activation beads are used), lymphodepleting chemotherapy of the patient and infusion of CAR-T cells. Adapted from [31].

1.2.2 TRUCKs, the next generation of CAR-T cells

CAR-T cells have been shown to be highly effective as therapy option for hematological malignancies [19-22]. However, in terms of targeting solid tumors, CAR-T-cell functionality and success of the treatment are limited [32]. This is, in most cases, due to the ability of solid tumors to form a tumor microenvironment (TME) consisting of a diversity of cells and soluble factors. The heterogeneity of solid tumors starts with the tumor cells themselves comprising cells with several genetic variants of different grades of differentiation, proliferation, vascularity or resistance to treatment [33]. Often, as mechanism of immune evasion, tumor cells display an impaired antigen processing and presentation, downregulation of surface antigens or insusceptibility towards killing mechanisms, which critically limits all kinds of target-specific or antibody-mediated therapies [34]. Further, among other cell types, especially immune cells located in the TME are reprogrammed by the tumor to acquire tumor-promoting abilities [35]. This process is mediated by the release of soluble mediators including regulatory cytokines, such as interleukin (IL)-10 and transforming growth factor β (TGF-β) [33], as well as induction of immunosuppressive cells such as regulatory T cells (Tregs). Thereby, an immune response towards the tumor is suppressed. Further, cells within the TME can even support the tumor. Especially tumor-associated macrophages (TAMs) secret growth factors, survival or proangiogenic factors, as well as reactive oxygen species or matrix-degrading enzymes to promote processes such as growth, angiogenesis and metastasis of the tumor. With this TME, tumors also suppress and hinder permission of lymphocytes including administrated adoptive T-cells that could otherwise fight tumor cells [32].

To overcome these barriers, TRUCKs, the fourth generation of CAR-T cells, were developed, which are able to produce an effector molecule in response to signaling of the CAR (see Figure 3) [36, 37]. Thereby, they can deliver molecules such as pro-inflammatory cytokines directly to the microenvironment of target-expressing cells, the tumor site, in a locally restricted manner [32]. This is of special interest, if the desired effector shows side effects upon systemic administration limiting the therapeutic dose. The targeted deposition is mediated by the combination of a constitutive expression of the CAR and an inducible expression of the recombinant protein in the T cell. For this, expression of the molecule is controlled by an NFAT-sensitive promotor that initiates transcription upon activation of the TCR/CD3 signaling pathway by CARs.

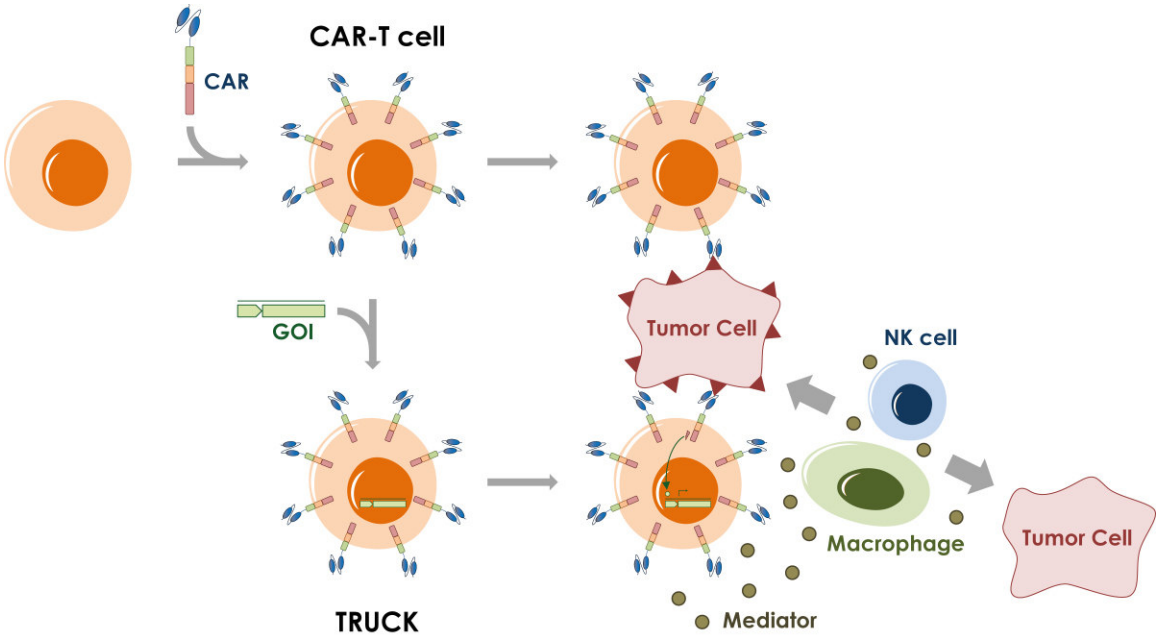


Figure 3. TRUCKs, the next generation of CAR-T cells. In TRUCKs, additionally to the CAR, an inducible GOI is introduced that is expressed upon CAR signaling after target-cell recognition [16]. The released mediator (e.g. IL-12) fulfils further functions, such as attraction and activation of immune cells (e.g. natural killer (NK) cells, macrophages) that can then also fight antigen-negative tumor cells. Adapted from [16].

The first molecule that was delivered by that strategy was IL-12 [36, 37]. Physiologically, this molecule is mainly produced by dendritic cells (DCs) and phagocytes (monocytes, macrophages and neutrophils) that are activated, e.g. in response to bacteria, fungi and intracellular pathogens such as viruses [38]. IL-12 mainly acts on natural killer (NK), natural killer T (NKT) and T cells, and promotes proliferation, an improved cytotoxic capacity as well as an enhanced production of cytotoxic mediators and cytokines (especially interferon-γ; IFN-γ) by these cells. Regarding T lymphocytes, IL-12 drives differentiation and improves functionality of type 1 helper T cells (T_H1) and CTLs, which are both essential for an effective response towards intracellular pathogens and tumor cells. The released IFN-γ, among other functions, particularly activates and improves the antimicrobial activity of macrophages, enabling a quick and T-cell-independent immune response in the first phase of an infection. Further, anti-angiogenesis factors are secreted by IL-12-activated cells. Overall, this indicates IL-12 as attractive agent to induce and improve the anti-tumor immune response for a potential use in cancer immunotherapy. Indeed, administrated IL-12 could elicit an effective antitumor activity in preclinical models. However, it mostly failed to mediate the same efficacy in clinical studies due to severe dose-limiting toxicities upon systemic delivery [39]. To circumvent this, innovative IL-12 delivery systems and locally-restricted administration routes are currently under investigation [40].

A persuasive strategy to deliver IL-12 directly to the tumor site and even otherwise not accessible metastases by exploiting the migratory abilities of T cells is the usage of TRUCKs [36, 37]. In these, CAR signaling upon encounter of the target promotes the inducible expression of IL-12 leading to an enhanced cytolytic capacity of the engineered T cells, attraction and activation of innate immune cells such as NK cells and macrophages, and re-modulation of the immunosuppressive TME [32]. With this, TRUCKs with an inducible IL-12 cassette were able to eradicate solid tumors with heterogeneous antigen expression in mice models, including elimination of antigen-loss tumor variants, a process that was critically dependent on the IL-12-mediated function of macrophages and activity of the cytokine tumor necrosis factor α (TNF-α) [36]. Further, TRUCKs were shown to change the immunosuppressive TME by altering and diminishing suppressor cells, which required both, the CAR and inducible IL-12, and additionally the presence of immune cells harboring the IL-12 receptor in the host [37].

An alternative cytokine that can be delivered by TRUCKs is IL-18. The rationale for the development of TRUCKs with this cytokine is that one of the major reasons for CAR-T-cell therapy failure in clinical trials is thought to be a reduced functionality and enhanced exhaustion of T cells [41]. Non-responding patients display T cells with an elevated expression of inhibitory receptors, a decreased expression of pro-inflammatory cytokines as well as an impaired proliferative capacity [42]. IL-18 released by TRUCKs was shown to convert T cells into effector cells with a pro-inflammatory signature in an early differentiation stage and moreover re-modulate the tumor-microenvironment towards an inflammation with increased numbers of activated DCs, NK cells and M1 macrophages and lower numbers of immunosuppressive DCs, M2 macrophages and Tregs. Thereby, they elicited superior functionality towards large solid tumor lesions in mice models when compared to conventional CAR-T cells. In another study, IL-18 secreting CAR-T cells were shown to augment proliferation and IFN-γ production in T cells, improve the $CD4^+$ T cell mediated helper effect towards $CD8^+$ T cells and thereby markedly enhance the antitumor response in mouse models [43].

1.3 The hurdle to choose an ideal target for CAR-T-cell therapy

While the physiological TCR repertoire is restricted by the selection process in the thymus that excludes all receptors recognizing antigens independent from HLA or self-antigens, CARs can be

designed to recognize basically all extracellular molecules on target cells including proteins, carbohydrate and glycolipid molecules [44]. With this HLA-independent binding, CAR-T cells are able to overcome the immune escape mechanism of tumor cells to downregulate HLA expression, but are also limited to the recognition of cell surface antigens representing only a minority of all cellular proteins. Moreover, further requirements restrict the choice of a disease-associated target. First, the antigen should exhibit a high coverage meaning that it is ideally expressed by all tumor cells. Given the heterogeneity of cells in solid tumors, this is, most often, only feasible by targeting multiple antigens or involving endogenous immune cells with various specificities. Further, specificity for tumor cells is an important determinant for the choice of target molecule. In order to prevent side effects, the target should optimally be exclusively expressed on tumor and not be present on healthy cells. Since most neoantigens, mutated proteins that are only expressed by tumor cells, are intracellular, they cannot be reached by a CAR, which restricts the selection of available targets to tumor-associated antigens that are overexpressed on cancer cells but also on healthy cells. To overcome resulting "on-target/off-tumor" toxicities when using these antigens, the CAR-T-cell dose can be reduced and adjusted to prevent organ damage, although this may also limit the effectiveness of the adoptive therapy. Lastly, the target molecule should be stably expressed as its downregulation or mutation by tumor cells would cause therapy failure. Thus, the target should play an essential role in tumor growth and survival. Taken together, the choice of an appropriate CAR target fulfilling as many mentioned attributes as possible is challenging and requires compromises and innovative strategies.

1.3.1 A virus-derived peptide as target for Post-Transplant Lymphoproliferative Disorder (PTLD)

1.3.1.1 Development of PTLD

One of the most severe complications after hematopoietic stem cell (HSCT) and solid organ transplantations (SOT) is the post-transplant lymphoproliferative disorder (PTLD) [45]. Although there are a few forms of PTLD caused by proliferating T and NK cells, the majority of PTLD cases originate from Epstein-Barr virus (EBV)-infected B cells [46]. Uncontrolled expansion of EBV^+ B cells after transplantations can either occur upon primary infection of EBV-seronegative patients through an EBV^+ graft or acquisition after transplantation, or by virus reactivation in EBV^+ patients. The likelihood to develop PTLD varies from 1% to 20% for different transplanted organs

with a higher risk for patients with a primary infection early after transplantation and receiving intensive immunosuppressive regimen [45, 47]. The three-year overall survival of PTLD patients ranges from 49% to 80% [48, 49].

EBV is a double-stranded DNA virus and belongs to the class of human gamma herpesviruses. More than 90% of all humans are infected with EBV, yet the vast majority does not exhibit clinical symptoms [46]. EBV is orally transmitted and, most likely, primarily infects cells of the oral mucosal epithelium. In these, the virus enters a lytic phase and induces production of further virions resulting in a spread of the infection into the throat. Further, mucosal B cells are infected in this stage of infection, in which so-called "growth-transforming" latency genes are expressed [50]. These drive proliferation in resting B cells and induce development of B cell blasts that can further circulate through the body. Although an effective T-cell response is induced in most immunocompetent individuals that diminishes the number of infected B cells, small numbers of 1 to 50 EBV^+ cells per 10^6 B lymphocytes are able to sustain in the memory B cell compartment by entering a regulated latency program [46]. EBV latency is characterized by four phases of controlled proliferation and differentiation of infected B cells. While several viral genes including Epstein-Barr nuclear antigens (EBNA) 1, 2, 3A, 3B, 3C, LP, latent membrane proteins (LMPs) 1 and 2A/B, the noncoding Epstein-Barr virus-encoded small RNAs (EBER) and a cluster of viral microRNAs, BamH1 rightwards transcripts (BART), are expressed in the first latency phase III, the following stages II and I are defined by decreasing viral gene expression ending in latency phase 0, in which no viral proteins are expressed. By this and further mechanisms including release of a viral IL-10 that results in an immunosuppressive microenvironment, downregulation of peptide presentation in context of HLA-I, and a reduced sensitivity towards apoptosis-inducing pathways, latently EBV-infected cells are able to evade the immune system and persist lifelong in the host.

Whereas primary EBV infection in childhood mostly does not show any symptoms, an acquisition in adolescence can cause an infectious mononucleosis, which is an acute process that is ended by a T-cell mediated immune response [46]. The subsequent latent EBV infection is mostly asymptomatic and characterized by an interplay of latently infected B cells that evade immune recognition and cells of the immune system that are able to restrict reactivation of viral lytic cycles and proliferation of EBV-infected cells. In this immune surveillance, especially $CD8^+$ T cells specific for the lytic proteins BamHI Z/R fragment leftward open reading frame 1 (BZLF1 and BRLF1), but also for latent proteins, play an important role. Moreover, $CD4^+$ T cells are able to

react upon recognition of presented viral peptides and NK cells can sense a potential HLA-I downregulation.

This finely tuned balance, however, is interfered upon delayed immune reconstitution or immunosuppressive treatment after HSCT or SOT [51]. Resulting reduced numbers or an impaired function of EBV-specific T cells can enable proliferation of transformed EBV^+ B cells and the development of PTLD, whereby cells display an expression pattern similar to EBV latency phase III. The important role of T-cell immunity in EBV immune surveillance is further shown by similar EBV^+ B cell lymphoproliferations that are described for patients with AIDS or primary immunodeficiencies and elder persons [46].

1.3.1.2 The T-cell-receptor-like monoclonal antibody TÜ165

Treatment options for PTLD include a reduction of immunosuppression and/or chemotherapy as well as depletion of B cells, all of which display varying response rates and side effects [52-54]. Considering the important role of T cells in controlling proliferation of EBV-infected B cells in healthy individuals, a highly specific approach is the adoptive transfer of EBV-specific T cells selected or expanded from EBV^+ donors [54]. These cells were shown to expand after infusion into PTLD patients, lyse EBV-infected lymphoma cells and persist for several years [54-57]. However, for this approach, the availability of at least partially HLA-matched T-cell donors with a high frequency of EBV-specific T cells is required.

A strategy to confer specificity of T cells to recognize cells that are infected with an intracellular pathogen is the introduction of a CAR that is based on the scFv of a so-called TCR-like monoclonal antibody (mAb). Analog to TCRs, these mAbs specifically recognize complexes of a defined peptide in the HLA context [58]. Considering B cell physiology, such TCR-like mAbs only rarely occur naturally, but can be generated utilizing hybridoma [59] and phage display technologies [58]. Their usage in immunotherapy is under investigation as it was shown that tumor and virus-infected cells can be recognized by TCR-like mAbs with a high specificity [60-66] and usually higher affinities than TCRs with the same target [67]. Using the migratory and memory-forming abilities of T cells and avoiding repeated administration of the antibody, CAR-T cells with scFvs of TCR-like mAbs as antigen-binding domain were developed and their efficiency in combating tumors cells based on the presentation of associated peptides in complex with an HLA molecule was demonstrated [68-70].

In 1993, Uchanska-Ziegler *et al.* [71] described one of the earliest TCR-like mAb against a human epitope. Using hybridoma technology, the group discovered the mAb TÜ165 recognizing an EBV-infected B-cell line, but the binding was abolished upon deletion of HLA-B*35 from the cell line or usage of HLA-B*35-positive cells that were not infected with EBV. They concluded that TÜ165 has a TCR-like binding specificity and recognizes a peptide/HLA complex composed of an EBV-encoded or -controlled peptide and HLA-B*35 as epitope. Later, it was found that TÜ165 recognizes the EBV-peptide LPPHDITPY derived from EBNA-3C and some structurally similar synthetic peptides when they are bound to HLA-B*35:01 [72].

1.3.2 The disialoganglioside G_{D2} as tumor-associated antigen

G_{D2} is a disialoganglioside, which is a glycosphingolipid with two sialic acid residuals [73]. Such molecules are located within microdomains in the plasma membrane and detectable on the cell surface. G_{D2} has restricted expression on neuronal cells of the CNS, peripheral nerves, skin melanocytes, and mesenchymal stroma cells. The exact biological function of G_{D2} is not fully understood, but being expressed on immature cells during embryogenesis, it contributes to processes such as cell adhesion and signal transduction [74]. Due to their neuroectodermal origin, G_{D2} is abundantly expressed on neuroblastoma and melanoma cells; additionally, G_{D2} is expressed in childhood tumors, such as retinoblastoma, diffuse intrinsic pons glioma, Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, and desmoplastic small round cell tumors, as well as in adult breast and small cell lung cancer (reviewed in [73]). In tumor cells, G_{D2} was shown to play an essential role in proliferation, invasion and motility of tumor cells, and to have a beneficial effect on immunosuppressive cells of the TME contributing to tumor immune evasion [75]. With its high coverage on several cancer entities, higher expression on tumor than on healthy cells, and expected stable expression due to the essential role in tumor survival, G_{D2} is an attractive target for immunotherapy.

Neuroblastoma is one of the most common solid tumors of childhood with a long-term survival of below 50% for high-risk patients despite aggressive therapy options and approved immunotherapeutic approaches [76]. Due to the high expression and coverage of G_{D2} on neuroblastoma cells, G_{D2}-targeting therapies were first evaluated in this tumor entity. Additional to G_{D2}-specific antibodies, one of which is approved for the treatment of high-risk neuroblastoma patients [77], G_{D2}-specific CARs based on the monoclonal antibody 14.G2a [78] showed

encouraging *in vitro* functionality [79] and were tested in clinical trials (reviewed in [73]). Although these trials showed safety of the engineered T-cell product without neurotoxic impairments due to "on-target/off-tumor" toxicity and even revealed impressive tumor lysing capabilities after administration, they failed to persist and act in the long-term to sustain remission [80].

In other tumor entities, such as Ewing sarcoma, an aggressive solid tumor of mesenchymal origin with a poor outcome, G_{D2} is only heterogeneously expressed among tumor cells in individual Ewing sarcomas [81]. However, a strategy to upregulate G_{D2} expression on all tumor cells by administration of an epigenetic agent is currently under investigation [82].

2 Project Aims

The adoptive transfer of CAR-T cells showed an impressive anti-tumor response in patients with hematological malignancies [19-22]. However, generation of similarly effective CAR-T-cell products targeting other tumor entities remains challenging. Particularly solid tumors often form an immunosuppressive TME, in which the release of suppressive soluble mediators as well as redirection of cells to acquire tumor-promoting and immune-regulatory properties inhibits an anti-tumor response by immune cells including adoptively transferred CAR-T cells [33]. To approach this, TRUCKs were developed, which, additionally to the constitutively expressed CAR, contain an inducible cassette for a soluble mediator such as a cytokine that is only expressed upon CAR activation following target recognition at the tumor site [36, 37].

The choice of ideal tumor targets for CAR-T cells and TRUCKs is challenging, because they should exhibit a high coverage on all malignant cells, distinct specificity for the tumor and stability of expression despite potential selection pressure [44]. An EBNA-3C-derived peptide of the EBV latency type III gene expression program persuasively fulfils the majority of requirements because of its tight affiliation with EBV-associated PTLD and lacking presentation on healthy cells [46]. Using the TCR-like mAb TÜ165 recognizing this peptide in context of HLA-B*35 [71] to confer antigen specificity to a CAR would facilitate targeting of an intracellular antigen, despite a CAR's inherent restriction to only recognize surface molecules [44]. Development of TRUCKs with a TÜ165-based CAR and an inducible IL-12 expression would further suggest an innovative approach to not only spare healthy cells but moreover mobilize additional immune cells to control PTLD (**research article 1**).

G_{D2} is an antigen that is highly expressed on various solid tumors including neuroblastoma [73], which is one of the most common solid tumors for children and bears a poor outcome for high-risk patients [76]. In clinical trials, G_{D2}-specific CAR-T cells failed to sustain remission in neuroblastoma patients because the administrated cells did not persist [80]. This could be approached by applying the TRUCK technology. Originally, TRUCKs were generated by co-transduction of two vectors encoding for the CAR and the inducible cytokine, respectively, which bears an increased risk for insertional mutagenesis and requires an enhanced production of GMP-grade viral vector [26]. To approach that, the second aim was to test, if G_{D2}-specific TRUCKs with inducible cytokine expression exhibit target specificity and functionality when they are generated by transduction with a modular, "all-in-one" vector comprising the constitutive expression of a

G_{D2}-targeting CAR and the NFAT-inducible expression of IL-12 or IL-18 in a single lentiviral vector (**research article 2**).

The third aim was to assess the feasibility to manufacture functional TRUCKs in a clinical scale and under GMP-compliant conditions by using the closed and automated CliniMACS Prodigy® (Miltenyi Biotec), whereby TRUCKs targeting G_{D2} with inducible IL-18 expression encoded in an "all-in-one" vector were used as proof-of-concept. Functional comparison of these cells with TRUCKs generated in the laboratory scale, as well as assessing the feasibility of cryopreservation of the manufactured TRUCKs, would reveal, if clinical-scale production of TRUCKs is easy to implement for a potential future use in clinical studies (**research article 3**).

3 Research Articles

3.1 CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation

Published October 30, 2020 in Journal of Immunotherapy of Cancer

Anna Christina Dragon[1], Katharina Zimmermann[2], Thomas Nerreter[3], Deborah Sandfort[1], Julia Lahrberg[1], Stephan Klöß[4,5], Christina Kloth[2], Caroline Mangare[1], Agnes Bonifacius[1], Sabine Tischer-Zimmermann[1], Rainer Blasczyk[1], Britta Maecker-Kolhoff[6], Barbara Uchanska-Ziegler[7], Hinrich Abken[8], Axel Schambach[2,9], Michael Hudecek[3], Britta Eiz-Vesper[1,*]

[1]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, 30625 Hannover, Germany

[2]Institute for Experimental Hematology, Hannover Medical School, 30625 Hannover, Germany

[3]Department of Internal Medicine II, Universitätsklinikum Würzburg, 97070 Wuerzburg, Germany

[4]Institute of Cellular Therapeutics, Hannover Medical School, 30625 Hannover, Germany

[5]Fraunhofer Institute for Cell Therapy and Immunology IZI, 04103 Leipzig, Germany

[6]Department of Pediatric Hematology and Oncology, Hannover Medical School, 30625 Hannover, Germany

[7]Ziegler Biosolutions, 79761 Waldshut-Tiengen, Germany

[8]Regensburg Center for Interventional Immunology (RCI), Department of Genetic Immunotherapy, Universitätsklinikum Regensburg, 93053 Regensburg, Germany

[9]Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA 02115, USA

*Correspondence: Eiz-Vesper.Britta@mh-hannover.de; Tel.: +49-511-532-9715

Author contribution statement

Anna Christina Dragon transduced and expanded primary TÜ165 CAR-T cells and TRUCKs and checked their phenotype (Fig. 2B-F). She planned and performed the reporter assay, co-culture experiments for the assessment of activation marker and intracellular cytokine expression, the release of cytokines and cytotoxic mediators, cytotoxicity and live cell imaging (Fig. 2G-K, 3, 4, 5, S2, S3, S4). She planned the migration assay and planned and performed transwell assays for the assessment of NK-92 functionality (Fig. 6D,E). She analyzed all data, wrote, reviewed and edited the manuscript.

All author contributions: K.Z. cloned the TÜ165 CAR iIL-12 and iEGFP constructs (summarized in Fig. 2A). T.N. cloned the TÜ165 CAR constructs with short and longe hinge regions. D.S. helped with performing and analyzing transduction and expansion of primary TÜ165 TRUCKs, checking

their phenotype (Fig. 2B-F) and functionality (Fig. 2G, H, 3B-D, F, H, 5A-C). J.L. helped in the development of TÜ165 CAR constructs and evaluated TÜ165 mAb binding to target cells (Fig. 1). S.K. helped with performing the live cell imaging. C.K. did the migration assays (Fig. 6A-C). C.M., A.B., S.T.-Z. and B.M.K. helped in data interpretation. R.B. helped with conceptualization, funding acquisition and supply of resources. B.U.-Z. provided the TÜ165 mAb and helped with data interpretation. H.A. and A.S. contributed to the conceptualization. M.H. developed the TÜ165 CAR concept. B.E.-V. developed the study design, acquired funding, supplied resources and helped in data interpretation. All authors reviewed and edited the manuscript.

Copyright

The article was published under the Creative Commons Attribution (CC BY) 4.0 license permitting unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Dragon, A.C., Zimmermann, K., Nerreter, T., Sandfort, D., Lahrberg, J., Klöß, S., Kloth, C., Mangare, C., Bonifacius, A., Tischer-Zimmermann, S., Blasczyk, R., Maecker-Kolhoff, B., Uchanska-Ziegler, B., Abken, H., Schambach, A., Hudecek, M., and Eiz-Vesper, B., *CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation.* J Immunother Cancer, 2020. **8**(2).

Open access Original research

Journal for ImmunoTherapy of Cancer

CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation

Anna Christina Dragon ,[1] Katharina Zimmermann,[2] Thomas Nerreter ,[3] Deborah Sandfort,[1] Julia Lahrberg,[1] Stephan Klöß,[4,5] Christina Kloth,[2] Caroline Mangare,[1] Agnes Bonifacius,[1] Sabine Tischer-Zimmermann,[1] Rainer Blasczyk,[1] Britta Maecker-Kolhoff,[6] Barbara Uchanska-Ziegler,[7] Hinrich Abken,[8] Axel Schambach,[2,9] Michael Hudecek,[3] Britta Eiz-Vesper[1]

To cite: Dragon AC, Zimmermann K, Nerreter T, *et al*. CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation. *Journal for ImmunoTherapy of Cancer* 2020;**8**:e000736. doi:10.1136/jitc-2020-000736

► Additional material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jitc-2020-000736).

AS, MH and BE-V contributed equally.

Accepted 18 September 2020

Check for updates

© Author(s) (or their employer(s)) 2020. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

For numbered affiliations see end of article.

Correspondence to
Professor Britta Eiz-Vesper; eiz-vesper.britta@mh-hannover.de

ABSTRACT

Background Immunosuppressive therapy or T-cell depletion in transplant patients can cause uncontrolled growth of Epstein-Barr virus (EBV)-infected B cells resulting in post-transplant lymphoproliferative disease (PTLD). Current treatment options do not distinguish between healthy and malignant B cells and are thereby often limited by severe side effects in the already immunocompromised patients. To specifically target EBV-infected B cells, we developed a novel peptide-selective chimeric antigen receptor (CAR) based on the monoclonal antibody TÜ165 which recognizes an Epstein-Barr nuclear antigen (EBNA)–3C-derived peptide in HLA-B*35 context in a T-cell receptor (TCR)-like manner. In order to attract additional immune cells to proximity of PTLD cells, based on the TÜ165 CAR, we moreover generated T cells redirected for universal cytokine-mediated killing (TRUCKs), which induce interleukin (IL)-12 release on target contact.

Methods TÜ165-based CAR-T cells (CAR-Ts) and TRUCKs with inducible IL-12 expression in an all-in-one construct were generated. Functionality of the engineered cells was assessed in co-cultures with EBNA-3C-peptide-loaded, HLA-B*35-expressing K562 cells and EBV-infected B cells as PTLD model. IL-12, secreted by TRUCKs on target contact, was further tested for its chemoattractive and activating potential towards monocytes and natural killer (NK) cells.

Results After co-cultivation with EBV target cells, TÜ165 CAR-Ts and TRUCKs showed an increased activation marker expression (CD137, CD25) and release of proinflammatory cytokines (interferon-γ and tumor necrosis factor-α). Moreover, TÜ165 CAR-Ts and TRUCKs released apoptosis-inducing mediators (granzyme B and perforin) and were capable to specifically lyse EBV-positive target cells. Live cell imaging revealed a specific attraction of TÜ165 CAR-Ts around EBNA-3C-peptide-loaded target cells. Of note, TÜ165 TRUCKs with inducible IL-12 showed highly improved effector functions and additionally led to recruitment of monocyte and NK cell lines.

Conclusions Our results demonstrate that TÜ165 CAR-Ts recognize EBV peptide/HLA complexes in a TCR-like manner and thereby allow for recognizing an intracellular EBV target. TÜ165 TRUCKs equipped with inducible IL-12 expression responded even more effectively and released IL-12 recruited additional immune cells which are generally missing in proximity of lymphoproliferation in immunocompromised PTLD patients. This suggests a new and promising strategy to specifically target EBV-infected cells while sparing and mobilizing healthy immune cells and thereby enable control of EBV-associated lymphoproliferation.

BACKGROUND

Epstein-Barr virus (EBV) infection or reactivation is a frequent complication of hematopoietic stem cell and solid organ transplantation. While EBV-infected B cells are under the control of EBV-specific T cells in immunocompetent individuals, delayed immune reconstitution and immunosuppressive treatment after transplantation can lead to the uncontrolled proliferation of malignantly transformed EBV-infected B cells, resulting in post-transplant lymphoproliferative disease (PTLD). The incidence of PTLD ranges from 1% to 20%, depending on the transplanted organ, the patient's EBV serostatus, and the intensity of immunosuppression.[1] PTLD has a 3-year overall survival rate of only 49% to 80% and significant side effects, emphasizing the need for novel treatment approaches.[2 3]

Current treatment options, such as reduction of immunosuppression, chemotherapy and B-cell depletion, exhibit varying response rates and are often limited by severe side effects.[4–6] Recently, it was shown that adoptively transferred EBV-specific T cells expanded or directly isolated from the blood of EBV-seropositive donors can restore

the EBV-specific T-cell immunity and persist for several years.[6–9]

An immunotherapeutic approach gaining increasing importance in the last years is to genetically redirect T cells by introducing chimeric antigen receptors (CARs) that combine antigen-binding properties with the activating signals of cellular receptors (eg, CD3ζ chain).[10] Most often, an antibody-based single-chain variable fragment (scFv) is used as the antigen recognition domain, which mediates binding to extracellular antigens.[11] Current limitations to CAR-T cell (CAR-T) applications, such as an immunosuppressive tumor microenvironment and heterogeneous antigen expression, prompted the development of T cells redirected for universal cytokine-mediated killing (TRUCKs), which further endow T cells with the ability to deliver a transgenic effector molecule that accumulates in the target tissue.[12] TRUCKs engineered to secrete a proinflammatory cytokine, such as interleukin (IL)-12 or IL-18, on tumor cell engagement exhibit improved effector functions; they act by recruiting and activating innate immune cells (eg, natural killer (NK) cells and macrophages), and by re-programming the immunosuppressive tumor microenvironment. When an inducible expression cassette is used, the cytokine is only released after CAR activation, which reduces systemic toxicity by locally restricting the presence of the cytokine.[13] Impressively, TRUCKs were shown to eliminate heterogeneous solid tumor lesions and antigen-loss variants that would otherwise remain invisible to conventional, antigen-dependent CAR-Ts.[14]

The major challenge in CAR design is to choose an appropriate target antigen only expressed on tumor and not on healthy cells to prevent on-target off-tumor toxicity.[15] In order to specifically address malignant EBV-positive PTLD cells and sparing healthy cells, so-called T-cell receptor (TCR)-like monoclonal antibodies (mAbs) can be utilized to direct CARs against an intracellular EBV antigen. TCR-like mAbs specifically bind to an epitope consisting of a defined peptide in complex with an HLA molecule and their usage as therapeutic agents is increasingly being investigated, since tumor or virus-infected cells could be targeted with a high specificity.[16–22] Using the binding specificity of TCR-like mAbs for the generation of CARs, researchers could circumvent the need for frequent antibody administration and enable a persisting in vivo response to tumor-associated peptides in the HLA context.[19 23–25]

In the present study, we generated a CAR based on the scFv of the TCR-like mAb TÜ165. TÜ165 is one of the first TCR-like mAbs with human specificity and exclusively binds to an EBV-infected, HLA-B*35-positive B-cell line but not to cell lines lacking HLA-B*35 or non-EBV-infected HLA-B*35-positive cells.[26] It was shown that TÜ165 recognizes LPPHDITPY when bound to HLA-B*35:01, a peptide derived from the Epstein-Barr nuclear antigen (EBNA)−3C, which is expressed in EBV latency type III associated with PTLD.[27] The peptide is predicted to bind to the majority of HLA-B*35 subtypes (www.cbs.dtu.dk/services/NetMHCpan; July 7, 2020[28]) and, depending on the geographic region, 11.3% to 37.9% of the population are positive for HLA-B*35 alleles (www.allellefrequencies.net; July 7, 2020[29]). The antibody's unique TCR-like binding specificity allows the CAR to address an intracellular EBV target antigen, thus sparing healthy B cells in the immunocompromised PTLD patients.

By additionally introducing an inducible IL-12 (iIL-12) expression cassette into the TÜ165 CAR construct, we developed iIL-12-secreting TÜ165 TRUCKs with improved effector functions that recruit and activate bystander immune cells, which otherwise occur rarely in close proximity to lymphoproliferative cells in PTLD patients. Our results demonstrate that TÜ165 CAR-Ts and TÜ165 CAR-based TRUCKs that are equipped with inducible IL-12 expression are able to specifically and effectively respond to an intracellular EBV target and may thereby enable control of EBV-associated lymphoproliferation in immunocompromised patients.

METHODS

Human sample materials

All experiments were performed with residual blood samples from routine platelet collection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Lymphosep (c.c.pro).

Cell lines and peptide loading

Cell lines used and their cultivation medium are listed in online supplemental table S1. K562 were transduced with a lentiviral vector to express HLA-A*35:01 (K562-B*35). B-lymphoblastoid cell lines (B-LCLs) were generated by infecting PBMCs with cell culture supernatant of B95-8 (#ACC-100, DSMZ) and adding 1 µg/mL ciclosporin A (Sigma).

Peptide loading was performed by incubating cells in serum-free RPMI 1640 with 10 µg/mL EBNA-3C-derived peptide LPPHDITPY (EZ Biolab) at 37°C and 5% CO_2. In TÜ165 mAb and CAR binding studies, the aforementioned peptide and CMV_pp65 IPSINVHHY, EBV_EBNA-3A VPATQPQY, EBV_BALF2 YPLREVATL and EBV_EBNA1 HPVGEADYFEY peptides (all EZ Biolab) were added at the specified concentrations and incubated as mentioned.

Construction of TÜ165 CAR, iIL-12-secreting TÜ165 CAR and iEGFP-expressing TÜ165 CAR vectors

The TÜ165 CAR was designed by synthesizing the scFv of TÜ165 (Thermo Fisher Scientific) and cloning it into the previously described ROR1-CAR-epHIV7 vectors.[30] To generate the iIL-12-secreting TÜ165 CAR and inducible enhanced green fluorescent protein (iEGFP)-expressing TÜ165 CAR constructs, we cloned the complete CAR backbone, including the co-expressed truncated epidermal growth factor receptor (EGFRt), into the newly developed pRRL.PPT.NFATenh.synTATA.IL12.PGK.newMCS.

GD2CAR.PRE and pRRL.PPT.NFATenh.synTATA.EGFP.PGK.newMCS.GD2CAR.PRE.[31] Further details as well as generation and titration of lentiviral vectors are provided in the online supplemental methods.

Generation of TÜ165 CAR-Ts and TÜ165 TRUCKs

Untouched $CD8^+$ T cells were isolated (Miltenyi Biotec) from PBMCs from HLA-B*35-positive donors and activated with anti-CD3/CD28 beads (Thermo Fisher Scientific) at a ratio of 1:1 in TexMACS (Miltenyi Biotec) with 3% human serum (c.c.pro; CTL medium) supplemented with 12.5 ng/mL IL-7 and IL-15 (PeproTech). After 1 day, T cells were transduced with lentiviral particles by spinoculation using an multiplicity of infection (MOI) of 3 and addition of 5 µg/mL Polybrene Infection/Transfection Reagent (Merck). Untransduced T cells were treated equally and served as controls. The anti-CD3/CD28 beads were removed on the following day. On day 7 or 9, $EGFRt^+$ T cells were enriched as described[30] or via fluorescence-activated cell sorting (FACS). After a total expansion time of 9 to 17 days, functionality of the transduced T cells was assessed by co-culturing them with target cells. For this, 5×10^4 target cells and effector cells according to the specified effector-to-target (E:T) ratios were seeded in 200 µL CTL medium. Since they do not express IL-12, iEGFP-expressing TÜ165 CAR-Ts (TÜ165 EGFP-TRUCKs) were expected to show the same functionality as TÜ165 CAR-Ts and were both used to compare functionality with iIL-12-secreting TÜ165 CAR-Ts (TÜ165 TRUCKs).

CAR reporter assay

A previously described reporter cell line based on the Jurkat T cell line JE6-1[32 33] was transduced with lentiviral particles by spinoculation using an MOI of 1 and addition of 5 µg/mL Polybrene Infection/Transfection Reagent. After two days, target cell recognition of these reporter cells was assessed by co-culturing them with unloaded K562, K562-B*35 or peptide-loaded K562-B*35 as target cells. For this, $1x10^5$ reporter and target cells, respectively, were seeded in an E:T ratio of 1:1 in 200 µL RPMI 1640, 10% fetal bovine sereum (FBS; Sigma-Aldrich) and 2 mM L-glutamine. JE6-1 cell activation was assessed after 24 hours by measurment of EGFP and enhanced cyan fluorescent protein (ECFP) by flow cytometry.

Flow cytometry

Antibodies used are listed in online supplemental table S2. TÜ165 was purified from the supernatant of a TÜ165 hybridoma cell line. Intracellular staining was performed by using the IntraPrep Permeabilizaton Reagent (Beckman Coulter). Samples were analyzed on a BD FACSCanto Flow Cytometer (Becton Dickinson).

Specific activation marker and intracellular cytokine upregulation in response to EBNA-3C-peptide-loaded K562-B*35 (K562-B*35/pEBV) as target cells was calculated by subtracting the frequency or mean fluorescence intensity (MFI) of effector T cells co-cultured with unloaded K562-B*35 from the respective frequency or MFI of those co-cultured with K562-B*35/pEBV (**Equation 1**).

$$\text{Specific upregulation} = \text{read-out (K562-B*35/pEBV)} - \text{read-out (K562-B*35)} \quad (1)$$

Multiplex cytokine analyis

Cytokine concentrations in the supernatant were determined using a customized LEDGENDplex Multi-Analyte Flow Assay (BioLegend), which allowed for the detection of human IL-2, IL-12 (p70), granzyme B, interferon (IFN)-γ, perforin and tumor necrosis factor (TNF)-α. Samples were analyzed with LEGENDplex V.8.0 software (BioLegend). In experiments with K562-B*35 target cells, specific cytokine release was calculated by subtracting the amount of secreted cytokine of effector T cells cultured with K562-B*35 from the respective amount in those cultured with K562-B*35/pEBV (**Equation 1**).

Determination of cytotoxicity by LDH assay and flow cytometry

The release of lactate dehydrogenase (LDH) into the cell culture supernatant was assessed by using the Cytotoxicity Detection Kit (Roche). Cells lysed by adding Triton X-100 (Merck) to a final concentration of 1% to all control wells served as maximum controls. Absorbance was assessed at a wavelength of 490 nm with a reference wavelength of 690 nm on a Synergy 2 Multi-Mode Microplate Reader (Biotek). LDH release was calculated using **Equation 2**:

$$\text{LDH release [\%]} = 100\% \times (\text{absorbance}_{490-690}/\text{absorbance}_{490-690}, \text{maximum control}) \quad (2)$$

B-LCL killing was assessed by flow cytometry and by gating target cells as $CD3^-CD8^-$ cells. Killing was calculated with **Equation 3**:

$$\text{Killing [\%]} = 100\% \times (1 - (\text{frequency}_{\text{afterco-culture}}/\text{frequency}_{\text{before co-culture, according to E:T ratio}})) \quad (3)$$

Live cell imaging

To visualize interactions, untransduced T cells labeled with 0.25 µM carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific), TÜ165 CAR-Ts stained using 2 µM CellTrace Violet (Thermo Fisher Scientific), and unstained K562-B*35/pEBV were mixed at a ratio of 1:1:2 in CTL medium and monitored every 30 s for 2 hours with an Olympus IX81 microscope. Analysis was performed using 20X short-distance objective lenses and Olympus ScanR analysis software.

Migration and activation of innate immune cells

The influence of TÜ165 TRUCKs on innate immune cells was assessed by measuring their chemoattractant effect on monocytes (Mono-Mac-6 (MM6) cells) and NK cells (NK-92 cells). The migration of those cells towards prediluted (1:3) 48 hours culture supernatants of the engineered T cells with and without target cells or prediluted (1:3) IL-12 standard in the indicated concentrations

was investigated in a Boyden chamber as previously described.[31]

The effect of TÜ165 TRUCKs on the killing capacity of NK-92 cells was measured using 0.4 µm transwell inserts (Greiner Bio-One) that allowed the exchange of soluble mediators but not cell migration. T cells were co-cultured with K562-B*35/pEBV in the transwell above a co-culture of NK-92 cells with CellTrace Violet-stained SPI-801. After 48 hours, SPI-801 killing by NK-92 cells was measured by flow cytometry and gating on dying cells SPI-801 based on the forward and side scatter properties of CellTrace Violet-positive cells.

The influence of IL-12 released by TÜ165 TRUCKs on IFN-γ expression by NK-92 was also assessed. NK-92 cells were co-cultured with SPI-801 cells in 48 hours culture supernatants of the engineered T cells with and without target cells. The supernatants were pooled from two donors, respectively, and prediluted 1:2.5 in RPMI 1640 with 10% FBS and 2 mM L-glutamine. After 4 hours, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and complementary DNA (cDNA) was reverse-described with the High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Expression of IFN-γ was quantified by inventoried mixes (Thermo Fisher Scientific) whereby Glyceraldehyde-3-phosphate dehydrogenase served as internal control.

Statistical analysis

Statistical analysis was performed with GraphPad Prism V.5.02 using the one-tailed Mann-Whitney U test. Values are shown as mean with SEM. ***p≤0.001; **p≤0.01; *p≤0.05; ns>0.05.

RESULTS

TÜ165 specifically binds to EBNA-3C-derived peptide/HLA-B*35 complexes

B-LCLs expressing EBV latency type III proteins similar to malignant EBV-PTLD cells[6] were generated by infecting PBMCs with EBV. In the binding specificity assay, EBV-positive B-LCLs and PBMCs from the same donor were stained with TÜ165 culture supernatant, which recognized B-LCLs from HLA-B*35-positive donors at a frequency of 87%, but did not recognize HLA-B*35-negative B-LCLs and non-EBV-infected PBMCs (figure 1A,B). HLA-B*35:01-typed donors were used for these binding studies; however, all other HLA-B*35 alleles are also predicted to present the EBNA-3C-derived peptide LPPHDITPY enabling TÜ165 binding, which could be shown for HLA-B*35:03-positive B-LCLs (online supplemental figure S1). Binding of TÜ165 to PBMCs was enabled by exogenously loading HLA-B*35-positive PBMCs with the EBNA-3C-derived peptide (referred to as PBMCs/pEBV); this indicates that presentation of the EBNA-3C peptide in the HLA context is mandatory for antibody binding (figure 1C). Conversely, loading with an HLA-B*35-restricted control peptide derived from CMV_pp65 (IPSINVHHY; referred to as PBMCs/pCMV) resulted in significantly lower antibody binding.

In an additional model, K562 cells endogenously lacking HLA expression were lentivirally transduced to express HLA-B*35:01 (referred to as K562-B*35), resulting in HLA expression by 96% of cells (figure 1D). Binding assays showed that TÜ165 weakly recognized unloaded K562-B*35, whereas exogenous loading of K562-B*35 with the EBNA-3C-derived peptide LPPHDITPY (referred to as K562-B*35/pEBV) resulted in TÜ165 binding to 99% of the cells (figure 1E). Binding of TÜ165 to different peptide/HLA-B*35 complexes was peptide concentration-dependent (figure 1F). TÜ165 recognized K562-B*35/pEBV in a highly specific manner, with half-maximal binding at a low peptide loading concentration of 1.5×10^{-7} M, whereas K562-B*35 loaded with peptides derived from EBV_BALF2, EBV_EBNA1 and EBV_EBNA-3A or CMV_pp65 were bound at low levels of 9% to 12% when used at the same loading concentration.

TÜ165 CAR-Ts and TRUCKs with inducible IL-12 generated using a clinical manufacturing and expansion protocol have a favorable phenotype

A second-generation TÜ165 CAR was generated based on the TÜ165 scFv (figure 2A). TÜ165 TRUCKs were developed by inserting a nuclear factor of activated T-cells (NFAT)-driven inducible IL-12 expression module into the same vector (CAR iIL-12), as was previously described for a different CAR.[31] A TÜ165 CAR with an iEGFP expression module was used as control (CAR iEGFP). As the extracellular spacer between the scFv and the transmembrane domain can strongly influence CAR functionality, we tested two different CAR constructs with a short (12 AA) and a long (228 AA) spacer, respectively, to determine the optimal distance between effector-cell and target-cell epitope, as was previously described for CARs with other specificities.[30 34 35] T cells transduced with the TÜ165 CAR long-spacer construct showed lower functionality (no increase in CD137 and markedly lower cytotoxicity towards target cells) than the corresponding short-spacer construct with TÜ165 CAR-Ts (online supplemental figures S2A,B). For this reason, all constructs used for further experiments contained the short spacer.

$CD8^+$ T cells were lentivirally transduced and expanded under conditions similar to those used for clinical manufacturing (figure 2B).[36] As with the untransduced T cells, we performed 76-fold to 86-fold expansion of TÜ165 CAR-Ts and iIL-12-secreting TÜ165 CAR-based TRUCKs (referred to as TÜ165 TRUCKs) in 9 days (figure 2C). The transduction efficiency was 33% for the CAR without and 16% for the CAR construct with iIL-12, which was enriched to 93% and 91%, respectively, by using the co-expressed marker EGFRt for purification (figure 2D). Both untransduced and transduced T cells showed no sign of exhaustion after 9 days of expansion as PD-1 expression, an indicator of exhaustion and activation, decreased to 5% (figure 2E). LAG-3 and TIM-3 were shown to be upregulated following anti-CD3/CD28 stimulation but

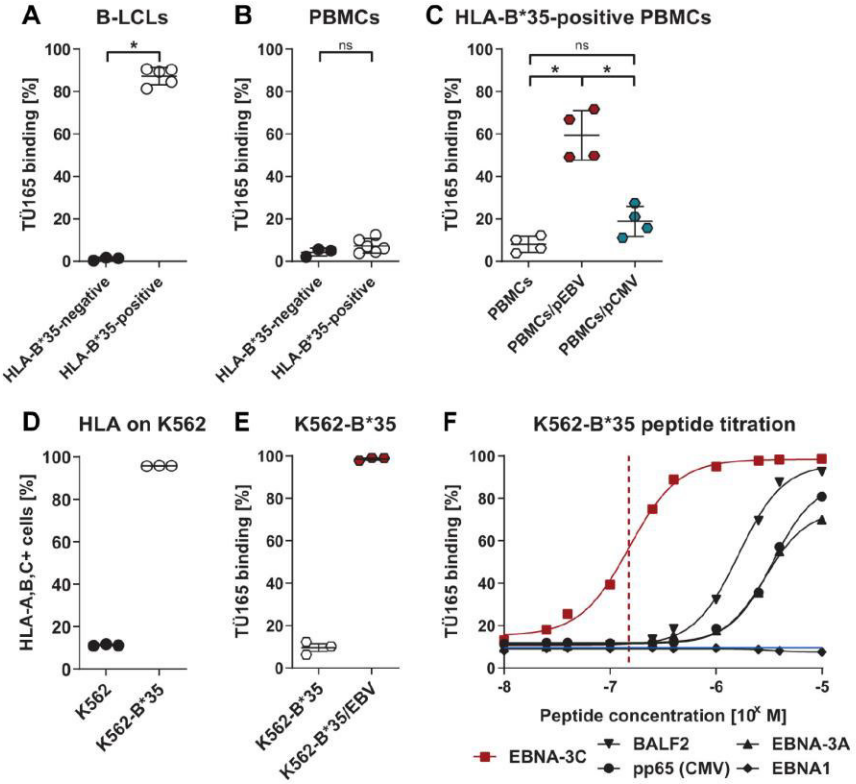


Figure 1 TÜ165 specifically binds to EBNA-3C-derived peptide/HLA-B*35 complexes. TÜ165 binding to (A) B-LCLs and (B) PBMCs from HLA-B*35-negative and HLA-B*35-positive donors. (C) TÜ165 binding to PBMCs from HLA-B*35-positive donors loaded with 10 µg/mL of EBNA-3C peptide LPPHDITPY or CMV pp65 peptide IPSINVHHY, respectively. (D to F) Studies of K562 cells lentivirally transduced to express HLA-B*35:01 (K562-B*35). (D) Confirmation of HLA class I expression. (E) TÜ165 binding to K562-B*35 loaded with 10 µg/mL EBNA-3C-derived peptide or (F) the indicated concentrations of peptides derived from EBV proteins EBNA-3C, BALF2 (YPLREVATL), EBNA-3A (VPATQPQY), and EBNA1 (HPVGEADYFEY) and CMV protein pp65 (IPSINVHHY). The red dashed line indicates the half-maximal binding of TÜ165 to K562-B*35/pEBV. The blue line indicates background binding of TÜ165 to unloaded K562-B*35. Data are shown as mean of 3 donors with a sigmoidal regression curve. (A to E) Data are shown as mean±SEM on scatter plots where each point represents one donor (n≥3). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *p≤0.05. B-LCLs, B-lymphoblastoid cell lines; EBNA, Epstein-Barr nuclear antigen; ns, not significant; PBMCs, peripheral blood mononuclear cells; PBMCs/pCMV, pp65-peptide-loaded PBMCs; PBMCs/pEBV, EBNA-3C-peptide-loaded PBMCs.

also decreased again until day 16 (data not shown). The T-cell phenotypes largely shifted to an increased proportion of central memory (T_{CM}; 67% to 88%) and lower frequencies of naïve (T_N; 8% to 17%), effector memory (T_{EM}; 3% to 14%) and effector memory RA (T_{EMRA}; 0 to 4%) T cells after expansion (figure 2F).

To confirm CAR-induced cytokine release in TÜ165 TRUCKs, T cells harboring the TÜ165 CAR iEGFP control (referred to as TÜ165 EGFP-TRUCKs) were co-cultured with K562-B*35/pEBV as target cells for 48 hours before EGFP expression measurement. EGFP expression was significantly upregulated (45%) in TÜ165 EGFP-TRUCKs cultured with the target cells, but remained low (8.7%) in those cultured without them (figure 2G). IL-12 assays showed that iIL-12-equipped TÜ165 TRUCKs cultured with the same target cells released much higher amounts of IL-12 (447 pg/mL) than those cultured without them (21.1 pg/mL), and that TÜ165 CAR-Ts without the iIL-12 cassette did not express IL-12 (figure 2H). These findings demonstrate that the inducible cassette was activated in TÜ165 TRUCKs after target-cell engagement.

Specificity of the TÜ165-based CAR was determined by using a previously described reporter assay and the Jurkat T cell line JE6-1.[32 33] TÜ165 CAR iEGFP-transduced Jurkat cells did not respond to co-cultivation with K562, K562-B*35 or K562-B*35 loaded with peptides derived from EBV_BALF2, EBV_EBNA1 and EBV_EBNA-3A or CMV_pp65, whereas both, the reporter for NFAT as well as the indicator for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), were induced upon recognition of K562-B*35/pEBV in a concentration-dependent manner (figure 2I,J). The same peptide specificity could be observed for the induction of the inducible EGFP cassette in primary EGFP TRUCKs (figure 2K). Background activation of these cells following co-cultivation with K562 and K562-B*35 can, most likely, be attributed to an endogenous TCR-mediated,

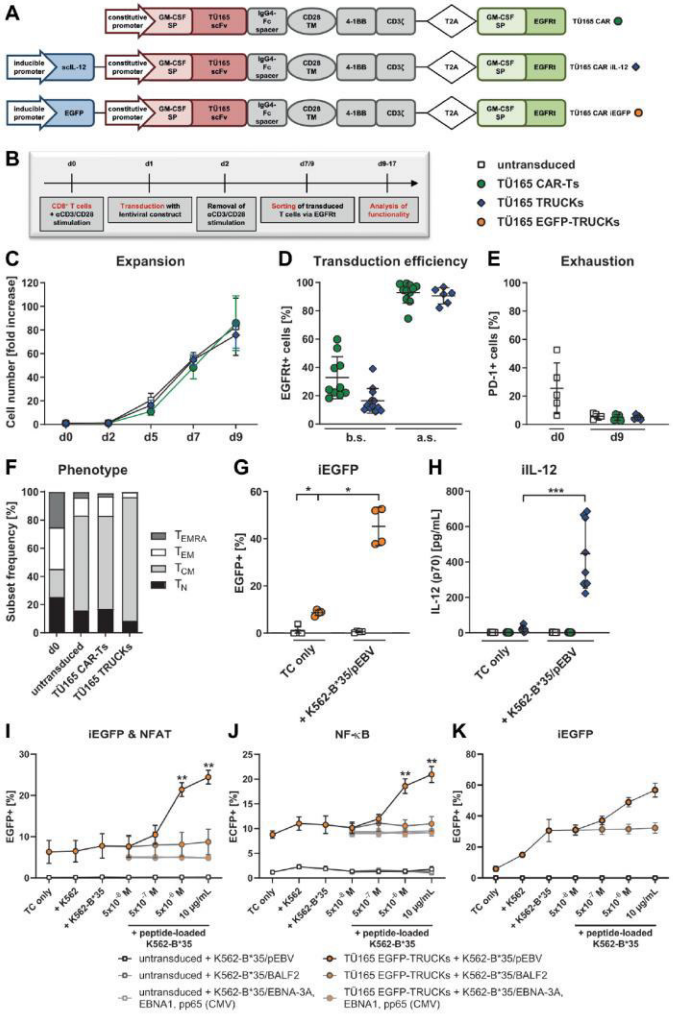


Figure 2 TÜ165 CAR-Ts and TRUCKs generated using a clinical manufacturing protocol have a favorable phenotype. (A) The TÜ165 CAR construct contained a constitutive promoter for the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) signal peptide (SP), a single-chain variable fragment (scFv) of TÜ165, a 12 AA short spacer based on IgG4-Fc, a CD28 transmembrane domain (TM), the cytoplasmic signaling domains of 4-1BB and CD3ζ, and a GM-CSF signal peptide with a truncated epidermal growth factor receptor (EGFRt) sequence connected by a T2A ribosomal skip element. TÜ165 CAR constructs with iIL-12 and iEGFP additionally contained an inducible promotor element with six NFAT response elements and a synthetic promoter (NFATsyn) in addition to human IL-12 or EGFP, respectively. (B) Experimental layout for $CD8^+$ T-cell transduction with lentiviral constructs, sorting and expansion. (C) T cell expansion, as assessed by cell counting: The fold increase in cells was calculated by dividing the cell numbers on a given day by the respective cell numbers on day 0 (d0). Data are shown as mean±SEM of 4 donors. (D) Transduction efficiency, as measured by staining T cells with biotin-anti-EGFRt and PE-streptavidin antibody on d9 before sorting (b.s.) and after sorting (a.s.). (E) PD-1 expression during expansion served as an indicator of exhaustion. (F) T cells were phenotyped on d0 and d9 and characterized as either naïve (T_N, $CD45RA^+CD62L^+$), central memory (T_{CM}, $CD45RA^-CD62L^+$), effector memory (T_{EM}, $CD45RA^-CD62L^-$) or terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}, $CD45RA^+CD62L^-$). Data are shown as mean of 5 donors. (G) Induction of EGFP expression and (H) IL-12 release by T cells cultured alone (TC only) or with the specified target cells for 48 hours. (I, J, K) A reporter JE6-1 Jurkat T cell line (I, J) or primary T cells (K) were transduced with the TÜ165 CAR iEGFP construct to generate TÜ165 EGFP-TRUCKs. They were co-cultured with the indicated unloaded target cells or K562-B*35 loaded with the specified concentrations of peptides derived from EBV proteins EBNA-3C, BALF2 (YPLREVATL), EBNA-3A (VPATQPQY), and EBNA1 (HPVGEADYFEY) and CMV protein pp65 (IPSINVHHY) at an E:T ratio of 1:1. After 24 hours (I, J) or 48 hours (K), induction of the iEGFP cassette (I, K) and the NFAT EGFP reporter (I) as well as the NF-κB ECFP reporter (J) was assessed by flow cytometry. Data are shown as mean±SEM of 2–5 donors. (D, E, G, H) Data are shown as mean±SEM on scatter plots where each point represents one donor ($n \geq 4$). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *$p \leq 0.05$, ***$p \leq 0.001$. CAR-T, chimeric antigen receptor-T cells; iEGFP, inducible enhanced green fluorescent protein; iIL-12, inducible interleukin-12; NFAT, nuclear factor of activated T-cells; TRUCK, T cells redirected for universal cytokine-mediated killing.

allogeneic response towards K562, since the JE6-1 cell line harboring the TÜ165 CAR but lacking any TCR did not respond towards unloaded K562.

TÜ165 CAR-T-cell response to HLA/EBV peptide recognition, characterized by activation marker and cytokine upregulation, is improved by inducible IL-12

Target recognition by engineered T cells was assessed by measuring activation marker and cytokine expression in response to co-culture with K562-B*35/pEBV versus K562-B*35. Untransduced CD8$^+$ T cells did not show CD137 expression (<2%) in response to either target-cell type, whereas TÜ165 CAR-Ts showed increased CD137 expression (4.7%) in response to the EBV peptide-loaded target cells (figure 3A). TÜ165 TRUCKs showed markedly increased CD137 expression (17.7%) in response to K562-B*35/pEBV. Specific CD137 upregulation was defined as the difference between expression levels in T cells cultured with K562-B*35/pEBV and unloaded K562-B*35 (individual data sets are shown in online supplemental figures S3A–G). CAR-Ts and TRUCKs

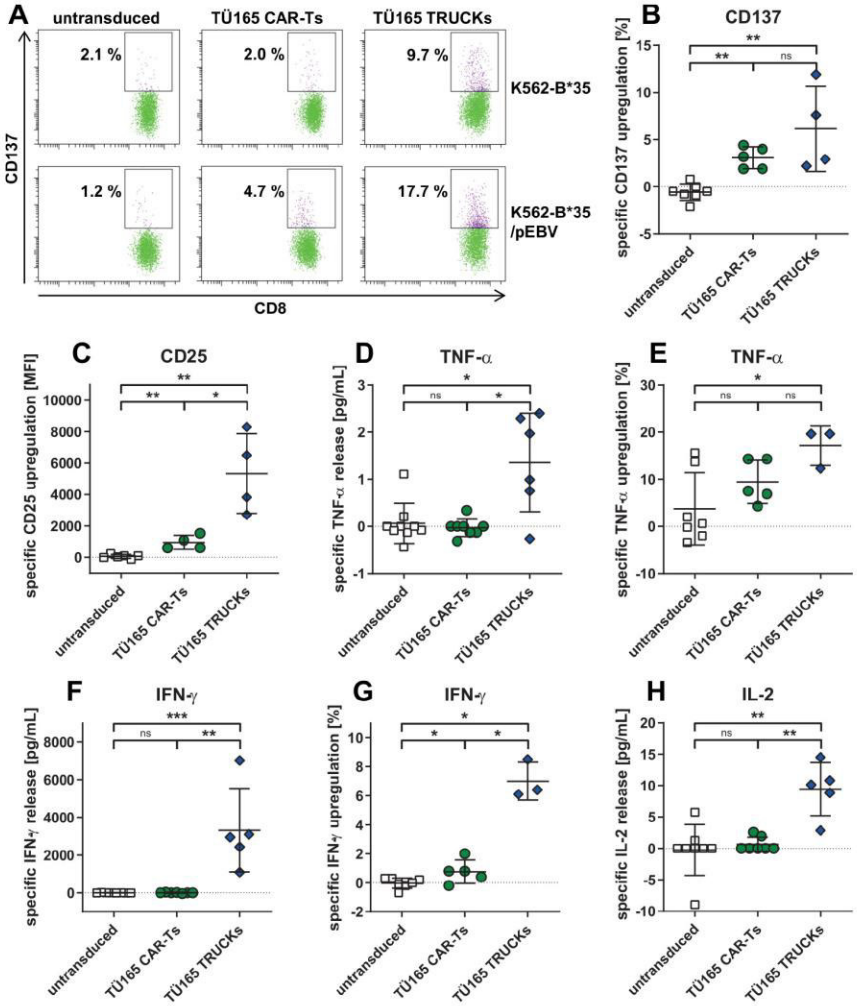


Figure 3 TÜ165 CAR-T-cell activation and cytokine response to HLA/EBV peptide recognition is improved by inducible IL-12. Engineered T cells were co-cultured with EBNA-3C peptide-loaded K562-B*35 (K562-B*35/pEBV) at an effector-to-target (E:T) ratio of 1:1 for 48 hours. (A) CD137 expression after co-culture of the specified CD8$^+$ T cells with unloaded (K562-B*35) or loaded (K562-B*35/pEBV) target cells (representative plots). (B) Specific upregulation of CD137 (%), (C) upregulation of CD25 mean fluorescence intensity (MFI), (E) TNF-α upregulation (%), and (G) IFN-γ upregulation (%) on CD8$^+$ T cells as well as specific release of (D) TNF-α, (F) IFN-γ and (H) IL-2 (pg/ml) into cell culture supernatants were calculated by subtracting the respective read-outs after co-culture with unloaded K562-B*35 from those after co-culture with K562-B*35/pEBV. (B to H) Data are shown as mean±SEM on scatter plots where each point represents one donor (n≥3). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *p≤0.05, **p≤0.01, ***p≤0.001. CAR-T, chimeric antigen receptor-T cells; EBV, Epstein-Barr virus; IFN-γ, interferon-γ; IL, interleukin; TNF, tumor necrosis factor; ns, not significant; TRUCK, T cells redirected for universal cytokine-mediated killing.

showed significant specific CD137 upregulation (3% and 6%, respectively), whereas untransduced T cells did not (−0.5%) (figure 3B). The specific upregulation of activation markers was further confirmed by detecting the MFI of CD25 on T cells. Compared with levels in untransduced T cells, CD25 MFIs in both TÜ165 CAR-Ts and TRUCKs increased significantly in response to the EBV peptide (MFI of 960 and 5300, respectively), and TÜ165 TRUCKs showed a significantly stronger response than TÜ165 CAR-Ts (figure 3C).

The release of TNF-α into the cell culture supernatant increased significantly (by 1.4 pg/mL) in TÜ165 TRUCKs co-cultured with target cells, whereas untransduced T cells did not secrete more TNF-α on contact with EBV-loaded versus unloaded K562-B*35 (figure 3D). This finding was confirmed by intracellular staining of TNF-α, which revealed a slight upregulation in TÜ165 CAR-Ts in response to the EBV peptide (9.5%) and a significant upregulation in TÜ165 TRUCKs (17%) (figure 3E). IFN-γ was released similarly: TÜ165 TRUCKs secreted significantly more (3300 pg/mL) IFN-γ as target response than both, untransduced T cells (2.2 pg/mL) and TÜ165 CAR-Ts (4.5 pg/mL), and intracellular staining confirmed this (figure 3F,G). TÜ165 TRUCKs also showed a proliferative response to the EBV target: IL-2 secretion by TÜ165 TRUCKs was upregulated by 9.4 pg/mL, whereas no measurable increase was detected in untransduced T cells or TÜ165 CAR-Ts (figure 3H).

TÜ165 CAR-T response to EBV-infected cells, characterized by activation marker and cytokine upregulation, is improved by inducible IL-12

Further, mimicking an EBV lymphoproliferation model, we used autologous EBV-infected B-LCLs as target cells and the respective uninfected PBMCs as controls. The results confirmed activation of the inducible expression cassette in TÜ165 TRUCKs on B-LCL engagement: EGFP expression was upregulated (18% to 22%) in TÜ165 EGFP-TRUCKs co-cultured with the EBV-infected B-LCLs at different E:T ratios, but remained at background levels (7% to 8%) when the TÜ165 EGFP-TRUCKs were cultured alone or with uninfected PBMCs (figure 4A). TÜ165 TRUCKs equipped with the inducible IL-12 cassette responded to B-LCLs by releasing higher amounts of IL-12 (45 to 216 pg/mL), but showed no significant increase in response to the uninfected target cells (figure 4B). As proof of target recognition and activation response, we measured CD69 expression on engineered T cells as MFI. After co-culture with B-LCLs at different E:T ratios, MFI CD69 values for TÜ165 CAR-Ts increased (840 to 870) compared with those for TÜ165 CAR-Ts cultured alone or with PBMCs (<42) (figure 4C,D). TÜ165 TRUCKs showed the same increase pattern, but the effect was even higher. CD25 MFI analysis revealed a reduced pre-activation of TÜ165 TRUCKs after expansion, but confirmed upregulation of activation marker expression on TÜ165 CAR-Ts and TÜ165 TRUCKs co-cultured with B-LCLs, but not on those co-cultured with PBMCs (figure 4E). However, CD25 upregulation most likely can be attributed to a TCR-mediated response of EBV-specific memory T cells as also untransduced T cells reacted equally and only EBV-seropositive donors were utilized. As specific response to B-LCLs, TRUCKs released IFN-γ: IFN-γ levels were threefold higher in TÜ165 TRUCKs co-cultured with B-LCLs than in those co-cultured with PBMCs or cultured alone (figure 4F). TÜ165 CAR-Ts showed a similar response to a smaller extent, equally to untransduced T cells. Moreover, IL-2 release in TÜ165 TRUCKs cultured with B-LCLs was enhanced; TÜ165 CAR-Ts showed only slight and non-significant increases in IL-2 (figure 4G).

TÜ165 CAR-Ts kill EBV-positive target cells and release effector molecules

Cytotoxicity of TÜ165 CAR-Ts and TRUCKs towards target cells was determined by measuring the release of LDH into cell culture supernatants. When co-cultured with unloaded K562-B*35, untransduced T cells and TÜ165 CAR-Ts exhibited similarly low cytotoxicities of 11% and 12% to 13%, respectively (figure 5A). Whereas untransduced T cells showed the same level of cytotoxicity towards K562-B*35/pEBV, TÜ165 CAR-Ts and TÜ165 TRUCKs showed significantly higher cytotoxicity (20% and 25%, respectively) after target-cell recognition. The amount of released effector molecules (perforin and granzyme B) in the supernatant confirmed these results since target-specific secretion of both mediators was significantly higher in both TÜ165 CAR-Ts and TÜ165 TRUCKs compared with levels in untransduced T cells (figure 5B,C; individual data sets in online supplemental figures S3H,I). TÜ165 TRUCKs secreted more perforin and significantly more granzyme B than TÜ165 CAR-Ts, indicating that the induced IL-12 resulted in improved effector T-cell function.

Using the EBV lymphoproliferation model, we assessed the number of B-LCLs after 48 hours of co-culture with TÜ165 TRUCKs by FACS. An initial experiment showed killing of B-LCLs by TÜ165 TRUCKs, whereas B-LCLs expanded when co-cultured with untransduced T cells (figure 5D,E). This was confirmed by an increased release of granzyme B in co-cultures of TÜ165 TRUCKs with autologous B-LCLs (figure 5F).

Live cell imaging revealed specific target-cell contact of TÜ165 CAR-Ts. For this experiment, TÜ165 CAR-Ts and untransduced T cells were added in equal amounts and co-cultured with K562-B*35/pEBV. After 45 min of culture, transduced TÜ165 CAR-Ts became active and elongated on contact and were recruited by the target cells, whereas untransduced T cells remained mainly inactive and round (figure 5G, online supplemental figure S4). After 2 hours of culture, large clusters of TÜ165 CAR-Ts and target cells were detected.

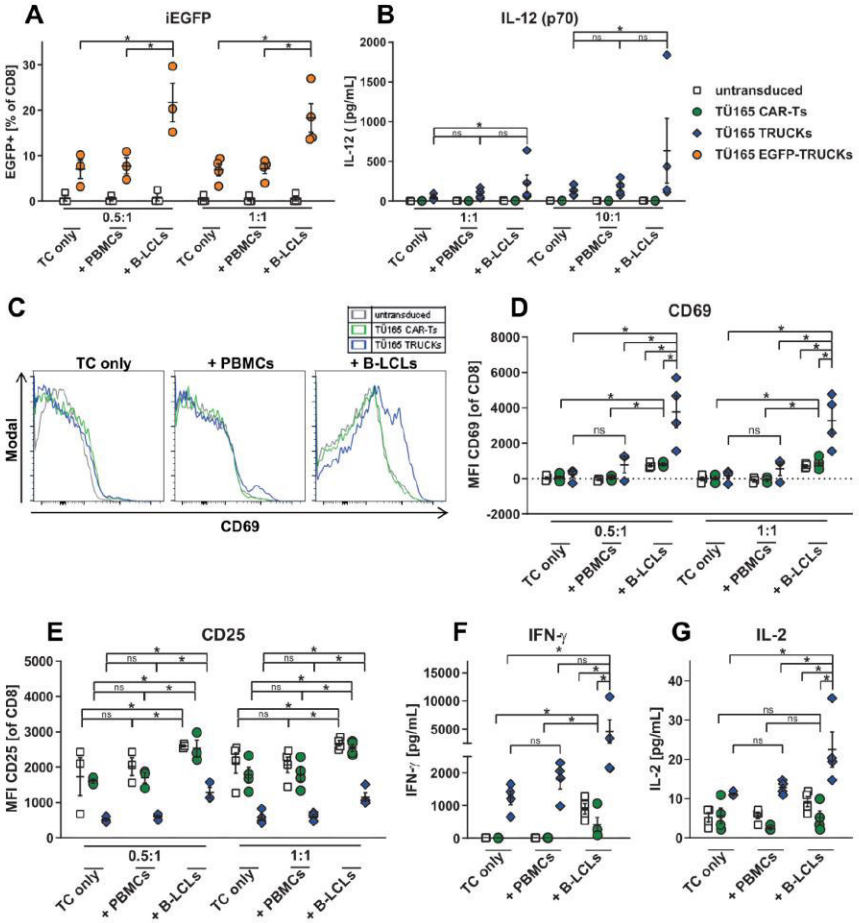


Figure 4 TÜ165 CAR-T activation and cytokine response to EBV-infected cells is improved by inducible IL-12. Engineered T cells were cultured for 48 hours alone (TC only), with autologous EBV-infected B-LCLs (+BLCLs) or with uninfected PBMCs (+PBMCs), respectively. (A) Expression levels of EGFP and (B) release of IL-12 were determined in T cells cultured alone and with the specified target cells at the indicated E:T ratios for proof of induction of the NFAT-driven inducible expression cassette. (C, D) CD69 median fluorescence intensity (MFI) values for $CD8^+$ T cells cultured alone and with PBMCs or B-LCLs, respectively, at an E:T ratio of 0.5:1 in representative histograms (C) and in scatter plots at E:T ratios of 0.5:1 and 1:1 (D). (E) CD25 MFI values for the aforementioned cells and E:T ratios. (F) IFN-γ and (G) IL-2 concentrations (pg/mL) were measured in cell culture supernatants at an E:T ratio of 10:1 by Legendplex analysis. (A, B, D–G) Data are shown as mean±SEM on scatter plots where each point represents one donor (n≥3). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *p≤0.05. B-LCLs, B-lymphoblastoid cell lines; CAR-T, chimeric antigen receptor-T cells; iEGFP, inducible enhanced green fluorescent protein; IFN-γ,interferon-γ; IL, interleukin; ns, not significant; NFAT, nuclear factor of activated T-cells; PBMCs, peripheral blood mononuclear cells; TRUCK, T cells redirected for universal cytokine-mediated killing.

TÜ165 TRUCKs facilitate the recruitment of MM6 and NK-92 cells and improve effector functions of NK-92 cells

To determine if the IL-12 released by TÜ165 TRUCKs after target-cell contact specifically recruits and activates macrophages and NK cells as described for other TRUCKs,[13] we tested supernatants of engineered T cells co-cultured with K562-B*35/pEBV for 48 hours for their chemoattractive potential towards monocytes (Mono-Mac-6 (MM6) cells) and NK cells (NK-92 cells) by using a Boyden chamber. MM6 cells migrated through a membrane with a pore size of 8 µm at a density of 170 cells/cm^2 when attracted by supernatants of TÜ165 TRUCKs co-cultured with K562-B*35/pEBV compared with a density of only 16 to 18 cells/cm^2 when attracted by supernatants of TÜ165 TRUCKs cultured alone or with unloaded K562-B*35 (figure 6B). Similarly, NK-92 cells migrated towards supernatants of TÜ165 TRUCKs co-cultured with peptide-loaded K562-B*35 at a higher density (figure 6A,C). Supernatants of untransduced T cells and TÜ165 CAR-Ts did not attract MM6 or NK-92 cells.

To measure the effect of the released IL-12 on NK-cell activity, we cultured engineered T cells in a transwell allowing cytokine exchange but not cell-cell contact with an NK-92 co-culture with HLA-class I-negative SPI-801 cells. The target-induced release of IL-12 by TÜ165 TRUCKs resulted in an increased killing capacity of

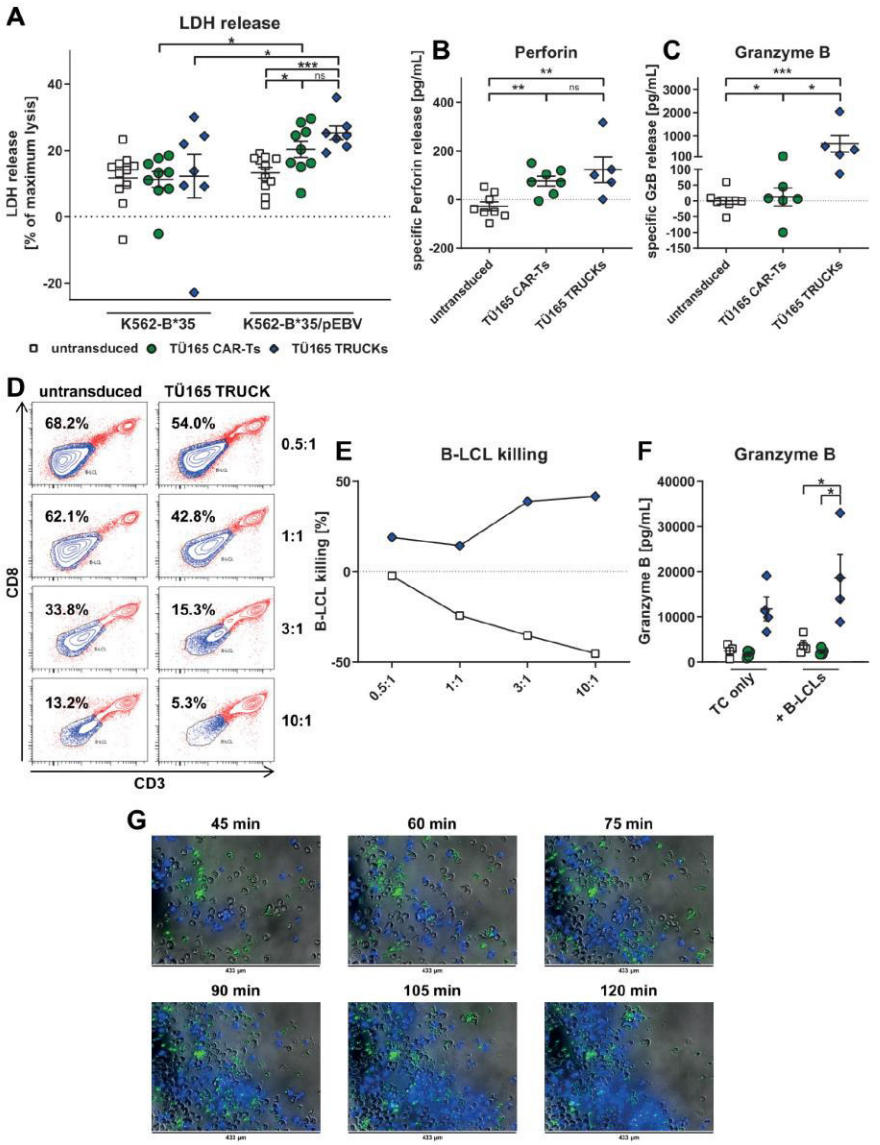


Figure 5 TÜ165 CAR-Ts kill EBV-positive target cells and release effector molecules. (A to C) Results for engineered T cells co-cultured with K562-B*35/pEBV for 48 hours at an effector-to-target (E:T) ratio of 1:1. (A) The release of lactate dehydrogenase (LDH) into the cell culture supernatant was measured as an indicator of the killing of target cells (unloaded K562-B*35 or K562-B*35/pEBV) by T cells. LDH levels are expressed as a percentage of the maximum lysis level obtained using controls lysed with 1% Triton X-100. (B) Specific release of perforin and (C) specific release of granzyme B; specific release into the co-culture supernatant was calculated by subtracting the value measured after co-culture with K562-B*35 (unloaded) from the value after co-culture with K562-B*35/pEBV. (D to F) Engineered T cells were co-cultured with autologous B-LCLs for 48 hours at (D,E) the indicated E:T ratios in an representative experiment or (F) an E:T ratio of 1:1. (D) B-LCLs were gated as $CD3^-$ $CD8^-$ cells. (E) B-LCL killing was calculated by dividing the frequency after 48 hours by the respective frequency on day 0 and subtracting it from 100%. (F) Release of granzyme B into the cell culture supernatant of the indicated T cells cultured alone (TC only) or in co-cultures with B-LCLs (+B LCLs). (G) Carboxyfluorescein succinimidyl ester (CFSE)-labeled untransduced T cells (green), CellTrace Violet-stained TÜ165 CAR-Ts (blue), and unlabeled K562-B*35/pEBV were set at a ratio of 1:1:2 and monitored under the microscope (Olympus IX81) at the indicated time points. Tracking experiments were performed using 20X short-distance objective lenses, and the results were analyzed using Olympus ScanR analysis software. Cell sizes of effector and target cells were estimated using a digital field of view of 433×330 µm and a resolution of 1344×1024 pixels instead of analog scale bars. (A to C, F) Data are shown as mean±SEM on scatter plots where each point represents one donor (n≥4). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *p≤0.05, **p≤0.01, ***p≤0.001. B-LCLs, B-lymphoblastoid cell lines; CAR-T, chimeric antigen receptor-T cells; EBV, Epstein-Barr virus; ns, not significant; TRUCK, T cells redirected for universal cytokine-mediated killing.

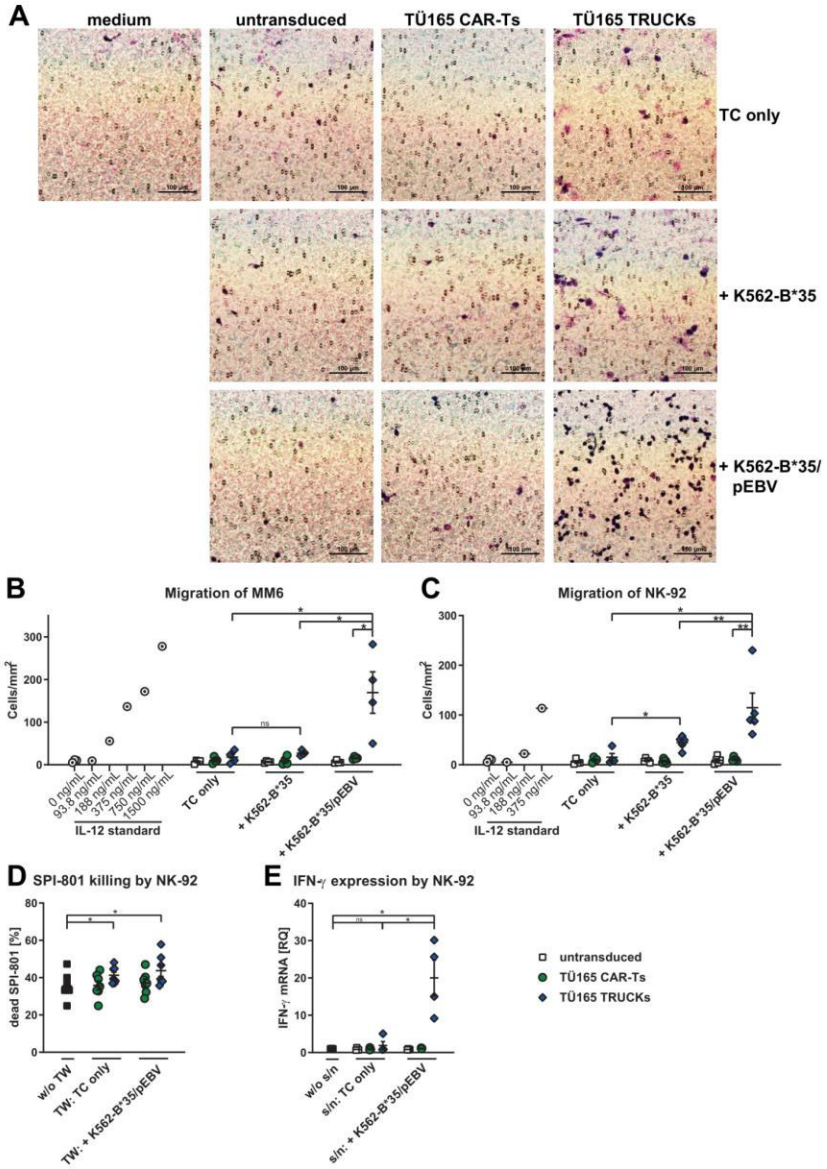


Figure 6 TÜ165 TRUCKs recruit MM6 and NK-92 cells and improve effector functions of NK-92 cells. (A to C) Chemoattractive potential of IL-12 released by TÜ165 TRUCKs on target recognition in terms of the migration of (A,C) NK cells (NK-92 cells) and (B) monocytes (MM6 cells). Supernatant of engineered T cells cultured alone (TC only) or together with unloaded K562-B*35 or K562-B*35/pEBV, respectively, for 48 hours were diluted 1:3, placed in a Boyden chamber, covered with an 8 µm polycarbonate membrane and incubated for another 4 hours. Medium served as the control supernatant. The membranes were Giemsa stained. (A) Representative pictures of Giemsa-stained NK-92 cells (violet) on the bottom of the membrane. (B, C) The number of cells that migrated through the membrane was determined. Different concentrations of IL-12 served as control supernatants. (D) Engineered T cells were cultured alone (TC only) or with K562-B*35/pEBV at an E:T ratio of 1:1 in a transwell (TW) insert with a pore size of 0.4 µm above a co-culture of NK-92 cells with CellTrace Violet-stained SPI-801 cells at an E:T ratio of 1:1. NK-92 cells co-cultured with SPI-801 cells with no cells in the transwell (w/o TW) served as controls. Dead SPI-801 cells were detected based on forward and side scatter properties of CellTrace Violet$^+$ cells. (E) Supernatants (s/n) of engineered T cells cultured alone or together with K562-B*35/pEBV for 48 hours were diluted 1:2.5 and utilized as culture medium for a co-culture of NK-92 cells with SPI-801 at an E:T ratio of 1:1. A co-culture in RPMI 1640 with 10% FBS and 2 mM L-glutamine (w/o s/n) served es control. After 4 hours, total RNA was isolated, reverse-described and quantified by quantitative PCR whereby Glyceraldehyde-3-phosphate dehydrogenase served as internal control for calculation of RQ values. (B to E) Data are shown on scatter plots where each point represents one donor (n≥4; IL-12 standards n=1). Statistical analysis was performed using the one-tailed Mann-Whitney U test. *p≤0.05, **p≤0.01. CAR-T, chimeric antigen receptor-T cells; IFN-γ, interferon-γ; MM6, Mono-Mac-6; mRNA,messenger RNA; NK, natural killer; ns, not significant; TRUCK, T cells redirected for universal cytokine-mediated killing.

NK-92 cells towards SPI-801 cells: killing increased from 35% to 41% in transwell-conditions without induced IL-12 to 44% in transwell conditions with target-activated TRUCKs (figure 6D). Further, the effect of IL-12 containing supernatants of engineered T cells co-cultured with K562-B*35/pEBV was measured in co-cultures of NK-92 and SPI-801. Quantitative PCR revealed a significantly increased expression of IFN-γ after 4 hours when the cells were cultivated in prediluted supernatants of TÜ165 TRUCKs co-cultured with K562-B*35/pEBV (RQ of 20) compared with cultivation in the control supernatants (RQ of 1 to 2) (figure 6E). This indicates that the released target-induced IL-12 by TÜ165 TRUCKs was biologically active. TÜ165 CAR-Ts had no impact on the functionality of NK-92 cells.

DISCUSSION

Currently, the two dominant strategies for engineering T cells are to introduce either a recombinant TCR or a CAR into T cells. TCR-like CAR-Ts combine the advantages of TCR- and CAR-engineered T cells. Major concerns when generating TCR-engineered T cells (TCR-Ts) are the development of unexpected specificities due to mispairing of the introduced TCR with the endogenous TCR and low expression of the introduced TCR; however, some strategies were recently developed to circumvent these limitations.[37] The use of CAR-Ts, on the other hand, prevents dimer formation with the endogenous TCR. Some studies already showed impressive anti-tumor responses, leading to the recent approval of two CD19-targeting CAR-T therapies for the treatment of different B-cell malignancies.[38] Since the depletion of all B cells includes EBV-infected lymphoma cells, CD19 CAR-T transfer for the treatment of PTLD is conceivable. However, as is the case for current PTLD treatment options like chemotherapy and antibody-mediated B-cell depletion, this strategy would be limited by the patient's poor condition and the high level of immunosuppression needed to prevent organ rejection.[4–6] Thus, an approach targeting only EBV-infected B cells and sparing healthy B cells to treat PTLD without diminishing the already low numbers of immune cells in these patients would be very desirable. Adoptively transferred EBV-specific T cells address this problem, but their use is often limited by time constraints or by the lack of an appropriate donor.[6] Building on this strategy in the present study, we utilized the TCR-like mAb TÜ165, which binds to a PTLD-associated, EBNA-3C-derived peptide presented in the HLA-B*35 context. TCR-like antibodies have shown promise as therapeutic agents designed for the targeting of tumor and virus-infected cells.[16–22] Otherwise, for the HLA-B*35/LPPHDITPY epitope no naturally occurring TCRs could be found (online supplemental figure S5). The absence of T cells with natural specificity against the epitope can be explained by the fact that the epitope is not immunodominant, respective T-cell frequencies are too low to induce a detectable response or that specific T cells have been deleted via tolerance mechanisms due to self-reactivity.

In the present study, TÜ165 bound to EBV-positive B-LCLs from HLA-B*35-positive donors expressing similar EBV proteins as PTLD cells.[6] B-LCLs were generated by using the laboratory EBV strain B95.8, but also naturally occurring strains C666-1 (GenBank no. AB828190.1), HKNPC1 (GenBank no. JQ009376.2) and M81 (GenBank no. KF373730.1) encode the LPPHDITPY peptide indicating the recognized sequence is conserved in different EBV strains. Only the laboratory strain AG867 (GenBank no. DQ279927.1) has an amino acid exchange at the first position of the peptide (leucine → phenylalanine; L → F; FPPHDITPY), to which the TÜ165 antibody and CAR binding would need to be assessed in further studies. Besides B-LCLs, the mAb TÜ165 bound to EBNA-3C peptide-loaded PBMCs and equally loaded HLA-B*35-transduced K562s (K562-B*35/pEBV). This confirms the reported capability of TÜ165 to bind to EBNA-3C peptide/HLA-B*35:01 epitopes.[26 27] The peptide was predicted to be presented by a majority of HLA-B*35 subtypes (www.cbs.dtu.dk/services/NetMHCpan; 07.07.2020[28]) and HLA-B*35 is a prevalent allele (www.allellefrequencies.net; 07.07.2020[29]). HLA-B*35:03-positive B-LCLs could be stained with the mAb TÜ165 with the same or slightly lower frequency confirming the predicted peptide binding and indicating TÜ165 is also able to recognize the EBNA-3C peptide in context of other HLA-B*35 subtypes than the reported HLA-B*35:01. It would be interesting to assess the binding capability of the TÜ165 antibody and CAR to the EBV peptide in complex with different HLA-B*35 subtypes in further studies. TÜ165 binding was moreover peptide concentration-dependent. EBNA-3C peptide-loaded K562-B*35 were recognized at low loading concentrations, indicating a high binding specificity, whereas other EBV/CMV peptides were not. Importantly, unloaded K562-B*35 and PBMCs can be expected to present a variety of peptides derived from self-proteins on their HLA-B*35 molecules,[39] but TÜ165 did not bind to them. Consequently, we conclude that TÜ165 binding is restricted to the EBNA-3C-derived peptide when presented by HLA-B*35 and that binding to other peptides will occur only at unphysiologically high peptide loading concentrations. Overall, the observed differential recognition of B-LCLs and PBMCs as well as the recognition of EBNA-3C peptide-loaded versus unloaded K562-B*35 confirms the suitability of TÜ165 to specifically distinguish between EBV-associated target and healthy cells.

TÜ165-based CAR-Ts and TRUCKs showed a favorable phenotype with low PD-1 expression, indicating that the cells were not exhausted despite being expanded around 100-fold in the first 9 days using an expansion protocol similar to the one used for clinical manufacturing. Only following activation with CD3/CD28 beads, expression of PD-1, TIM-3 and LAG-3 was transiently increased on T cells (data not shown). However, since these markers are also activation markers, only long-term expression would demonstrate an exhausted state.[40] Here, after cultivation for 16 days, expression of all markers markedly decreased again (data not shown) and cells were shown to be functional. Therefore, we deduced that T-cell dysfunction might not be a major problem in TÜ165 CAR-T and TRUCK generation. Previous studies

have shown that the phenotype of adoptively transferred T cells predicts their long-term persistence and engraftment in vivo.[41 42] In this context, less differentiated T_{CM} cells, which formed the main population after expansion in the present study, showed superior functionality *in vivo*: they exhibited long-term persistence and occupied memory T-cell niches.

When TCR-like CAR-Ts were first introduced as a new strategy, various problems had to be addressed. For example, one study using CARs based on high-affinity TCR-like antibodies recognizing Wilms tumor suppressor gene 1 (WT1) peptide/HLA-A02 complexes showed a loss of specificity and elimination of HLA-A*02-positive, peptide-negative cells, whereas the low-affinity TCR-like CAR-Ts maintained potent cytotoxicity and specificity.[43] Maus *et al*[44] later confirmed that lowering the affinity of a TCR-like CAR to the TCR affinity range ensures epitope specificity and effective cytotoxicity of the CAR. This is most likely due to the ability of the CAR-Ts to sequentially engage peptide/HLA complexes, as is reportedly necessary for proper T-cell activation. In the present study, the TÜ165 CAR was shown to be activated highly specifically only on recognition of K562-B*35/pEBV in a concentration-dependent manner, whereas unloaded K562, K562-B*35 or K562-B*35 loaded with other peptides did not activate the CAR. Only when transduced into primary T cells, a background activation of TÜ165 CAR-Ts and TRUCKs following stimulation with unloaded K562 and K562-B*35 was detected. A major drawback of this model is allogenicity. Although K562 cells only express low levels of HLA (mainly HLA-C),[45] untransduced T cells mildly recognize the cell line, which is shown by a very slight upregulation of CD137 and CD25 and release of TNF-α and IFN-γ in co-cultures with unloaded K562-B*35 (online supplemental figure S3). TÜ165 CAR-Ts exhibit a response towards the unloaded control cells to a similar extent showing that this response most likely is mediated by the TCR and not unspecifically by the introduced CAR towards empty HLA-B*35. TÜ165 TRUCKs, however, showed an even enhanced response to unloaded K562-B*35, which might be due to the fact that allogeneic recognition of HLA on the surface of K562 cells by the TCR induces NFAT expression and a subsequent induction of the IL-12 cassette. This hypothesis could be confirmed by testing the TÜ165 EGFP-TRUCK in JE6-1 Jurkat cells as effector cells. By the use of these cells lacking endogenous TCRs, no background response towards either K562 or K562-B*35 cells was observed. The expression of EGFP and ECFP is only induced upon loading with the EBNA-3C-derived peptide. These findings showed that the recognition of the epitope by the scFv of the CAR is highly specific.

When using primary T cells as effector cells, substracting the activation and cytokine data obtained for co-cultures with unloaded K562-B*35 from the ones in co-cultures with K562-B*35/pEBV could indicate the LPPHDITPY-specific component of the TÜ165 CAR-T-cell recognition. Here, TÜ165 CAR-Ts showed functionality by upregulating the expression of activation markers (CD137 and CD25) and by slightly increasing intracellular IFN-γ production in specific response to K562-B*35/pEBV but not to unloaded K562-B*35 controls. Moreover, they exhibited cytotoxicity by upregulating perforin and granzyme B when co-cultured with EBV peptide-loaded target cells and formed large clusters around them. When cultured with autologous B-LCLs in a model of lymphoproliferation, they increased the expression of activation markers (CD69 and CD25) and released IFN-γ in a range similar to that of untransduced T cells. Of note, expansion and off-target cytotoxicity of TÜ165 CAR-Ts towards unloaded K562 cells and uninfected PBMCs was equal to that of untransduced T cells. This indicates that they did not recognize HLA-B*35 without EBNA-3C peptide or when complexed with other naturally occurring peptides. The maintenance of specificity in TÜ165 CAR-Ts can be explained by the fact that the CAR is based on an immunoglobulin M (IgM) antibody, which normally has 10 antigen-binding sites. When reduced to a single antigen-binding site in the TÜ165 CAR, a lower avidity and weaker binding might be the consequence. This, in turn, allows the aforementioned sequential peptide/HLA engagement and yields functional CAR-Ts. In general, effector functions of the TCR-like TÜ165 CAR were rather mild when compared with other CARs, such as the well-described CD19 CAR. However, CAR activation is known to be highly dependent on the expression levels of target antigen[46] explaining that the target of the TÜ165 CAR, a peptide presented in HLA context, induces less CAR activation as compared with other highly-expressed surface molecules such as CD19. Considering their weak target binding and mild functionality, it would need to be evaluated, if TÜ165 CAR-Ts are able to effectively control EBV-associated lymphoproliferation for a potential future clinical use. For this, detailed *in vivo* testing is now required. Regarding possible models to study TÜ165 CAR-T function, immunocompromised mice previously engrafted with human B-LCLs are a model of EBV-associated PTLD that was recently used to test an EBV-specific, TCR-like mAb for the treatment of PTLD.[20] Further, generation and testing of other TCR-like CARs and TRUCKs recognizing EBV epitopes in context of PTLD would be desirable to extend the therapy to HLA-B*35-negative patients. As source for the scFv, TCR-like mAbs were described that recognize EBV-derived peptides in context of HLA-A*02:01,[20 47] and more can be generated using conventional hybridoma and phage display technology.[16 48]

Furthermore, we generated TÜ165 TRUCKs which induced IL-12 secretion only on recognition of the TÜ165 target. We encoded constitutive CAR expression and inducible cytokine expression in a novel 'all-in-one' vector to further facilitate the manufacturing of these cells for clinical applications and to reduce the risk of insertional mutagenesis when introduced into other cells as previously described.[31] To evaluate functionality of the inducible cassette, TRUCKs with inducible EGFP expression were used. When transduced into JE6-1 reporter cells, EGFP expression was only induced after specific target recognition. It was not induced in 100% of cells; however we only measured it after 48 hours and kinetics of CAR activation and signaling are not fully understood yet.[49] There is evidence that EGFP expression might not be completely upregulated in some cells at that time point or even already be downregulated again.

Furthermore, Zimmermann *et al* showed that T cells transduced with a GD_2-specific CAR and the same inducible EGFP cassette did upregulate EGFP expression on 60% of the cells after specific target recognition.[31] The slightly reduced EGFP induction of 45% in TÜ165 EGFP-TRUCKs can, most likely, again be explained by the aforementioned more rare expression of the target antigen. IL-12 has been reported to induce T helper 1 differentiation of $CD4^+$ T cells and to enhance cellular immunity by increasing IFN-γ release, augmenting granzyme and perforin production, and improving T and NK-cell proliferation.[50] In the present study, we used an inducible delivery route of IL-12 by TÜ165 TRUCKs on target recognition. This resulted in highly improved CAR function in specific response to EBV peptide as well as to malignant EBV-infected cells. Also the abovementioned allogeneicity towards unloaded K562-B*35 was enhanced, because, most likely, signaling by the endogenous TCR lead to an induction of IL-12. For a potential future clinical application, this would imply that TRUCKs should not be utilized in the allogeneic setting as they would probably cause an even more severe graft-versus-host disease. However, this should not be a problem in the autologous use and, moreover, attempts to generate universal CAR-Ts with disrupted TCR expression are currently under investigation.[51] Compared with CAR-Ts, TÜ165 TRUCKs showed augmented upregulation of CD25 expression, TNF-α and IFN-γ release, and IL-2 secretion. This is in line with previous findings that other IL-12-engineered T cells show substantially increased IFN-γ and TNF-α release, which is mandatory for the accumulation of activated macrophages in the tumor lesion and enables the elimination of antigen-loss cancer cells.[14] Both IFN-γ and TNF-α promote resistance to tumor-derived immunosuppressive factors often released in EBV-positive Hodgkin's disease.[52] TRUCKs with other specificities have not shown enhanced cytotoxicity[14] or superior CD107a or granzyme B expression[53] compared with the respective CAR-engineered or TCR-engineered T cells. Here, interestingly, TÜ165 TRUCKs released slightly more perforin and significantly more granzyme B on recognition of the EBV-associated target than TÜ165 CAR-Ts. Moreover, TÜ165 TRUCKs tended to be more cytotoxic and exhibited cytotoxicity and granzyme B release towards autologous B-LCLs. Thus, besides their usage in the treatment of PTLD, we here provide a proof of principle how to improve the functionality of weakly binding CAR-Ts with mild effector function. As described above, TCR-like CAR-Ts were shown to maintain epitope specificity and effective cytotoxicity by lowering the affinity of a TCR-like CAR to the TCR affinity range.[44] Furthermore, for clinical use, one major concern is tonic CAR-T signaling leading to exhaustion.[54] Hence, using low affinity CARs in combination with an inducible cytokine might provide a safer and more enduring T cell product with the same or even increased functionality, which would need to be examined in further CAR studies.

The rationale for developing TRUCKs with inducible IL-12 was not only to improve functionality but also, and even more importantly, to modulate the tumor microenvironment. In a study with TCR-Ts against EBV-associated Hodgkin's disease, the released IL-12 mediated resistance to the anti-proliferative and -cytotoxic effects of TGF-β that normally limit T-cell functionality against these tumors.[52] Moreover, IL-12 results in the recruitment of NK cells and macrophages to the tumor lesion, thereby promoting antitumor effects.[55] In the present study, TÜ165 TRUCKs releasing IL-12 on target engagement recruited both monocyte and NK-cell lines and enhanced target-cell killing as well as IFN-γ expression of NK-92 cells. PTLD patients display a heterogeneous immune deficiency associated with their immunosuppressive treatment regimen.[56] However, the importance of NK cells in EBV-associated malignancies was demonstrated since primary immunodeficiencies in NK cells predispose patients to EBV-driven pathologies[57] and a distinct subpopulation of tonsillar NK cells could restrict B-cell transformation by EBV.[58] Thus, the use of IL-12-secreting TÜ165 TRUCKs to attract and activate NK cells, which are not normally located in close proximity to PTLD cells, is a new approach to treating immunocompromised patients that not only spares healthy B cells, but also attracts as many other innate immune cells as possible to induce a broad anti-EBV response. An immunocompetent mouse model is required to study attraction and involvement of endogenous immune cells by the engineered T cells *in vivo*. IL-12-induced toxicities are of particular interest and should be evaluated in future studies. Most of the available studies of inducible IL-12-secreting TRUCKs report that safe adoptive transfer of those cells into mice is possible, but one group observed severe toxicities associated with increased systemic levels of TNF-α and IFN-γ due to the non-specific release of IL-12.[59] Although the amounts of IL-12 secreted by unstimulated TRUCKs in vitro in the present study were lower than those in the aforementioned study, evaluation of possible toxicities should definitely be a major focus of prospective preclinical *in vivo* testing of TÜ165 TRUCKs.

CONCLUSIONS

In conclusion, the results of this study demonstrated the suitability of CAR-Ts based on the peptide-selective and TCR-like mAb TÜ165 to specifically recognize and react to an intracellular EBV target associated with PTLD. Equipping the TÜ165 CAR with inducible IL-12 expression to generate TÜ165 TRUCKs improved effector functions and, importantly, led to the recruitment and activation of surrounding immune cells, which normally are not found in close proximity to lymphoproliferative cells. Thus, we suggest this method of specifically targeting EBV-infected cells while sparing and mobilizing the healthy immune cells in already immunocompromised patients as a new and promising approach to PTLD treatment, thereby enabling the control of EBV-associated lymphoproliferation.

Author affiliations
[1]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Niedersachsen, Germany
[2]Institute for Experimental Hematology, Hannover Medical School, Hannover, Niedersachsen, Germany
[3]Department of Internal Medicine II, Universitätsklinikum Würzburg, Wuerzburg, Bayern, Germany

[4]Institute of Cellular Therapeutics, Hannover Medical School, Hannover, Niedersachsen, Germany
[5]Fraunhofer Institute for Cell Therapy and Immunology IZI, Leipzig, Sachsen, Germany
[6]Department of Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Niedersachsen, Germany
[7]Ziegler Biosolutions, Waldshut-Tiengen, Baden-Wuertemberg, Germany
[8]Regensburg Center for Interventional Immunology (RCI), Department of Genetic Immunotherapy, Universitätsklinikum Regensburg, Regensburg, Bayern, Germany
[9]Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts, USA

Acknowledgements We would like to thank Andreas Ziegler (Ziegler Biosolutions, Waldshut-Tiengen, Germany) for his helpful suggestions concerning the use of TÜ165 and for providing the TÜ165-producing hybridoma cell line. We also thank Aline Häcker and Winfried Wels, Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany, for their help in designing the experimental layout of the transwell assays. We further thank Peter Steinberger, Medical University of Vienna, Institute of Immunology, Vienna, Austria, for providing the reporter JE6-1 Jurkat T cell line and the respective protocols. Thanks also to Marina Kramer, Sarina Lukis and Elvira Schulde for technical support and to Dr Matthias Ballmeier (Core Facility for Cell Sorting, MHH, Germany) for FACS-based cell sorting of transduced T cells.

Contributors Conceptualization: ACD, JL, HA, MH, AS, BM-K and BE-V. Methodology: ACD, BU-Z, KZ, TN, DS, JL, SK, CK and ST-Z. Software: ACD. Validation: ACD and BE-V. Formal analysis: ACD and BE-V. Investigation: ACD and BE-V. Resources: BU-Z, RB, AS and BE-V. Data curation: ACD and BE-V. Writing — original draft preparation: ACD. Writing — review and editing: All authors. Visualization: ACD. Supervision: BM-K and BE-V. Project administration: BE-V. Funding acquisition: RB, MH, AS and BE-V.

Funding This research was funded by "From CARs to TRUCKs" grants (Krebshilfe/German Cancer Aid-Priority Program in Translational Oncology, to BE-V, MH, HA, AS), the Comprehensive Cancer Center Hannover (Claudia van Schilling-Center), Member of the CCC Lower Saxony (ZN3481 CCC), the German Federal Ministry of Education and Research (reference numbers: 01EO0802 IFB-Tx and ZN3327 CORE), and, in part, by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; project ID 158989968, SFB 900/B11 to BE-V and 324392634, TRR 221 to MH).

Disclaimer The funders played no role in designing the study, in collecting, analyzing or interpreting the data, in writing the manuscript, or in the decision to publish the results.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All experiments were performed with residual blood samples from routine platelet collection. According to standard donation requirements, the respective donors had no signs of acute infection and no previous history of blood transfusion. Written informed consent was obtained from all donors as approved by the Ethics Committee of Hannover Medical School (2519–2014, 3639–2017).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The data sets and protocols used and/or analysed during the current study are available from the corresponding author (eiz-vesper.britta@mh-hannover.de) on reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iDs
Anna Christina Dragon http://orcid.org/0000-0002-5228-7627
Thomas Nerreter http://orcid.org/0000-0002-0493-1369

REFERENCES

1 Petrara MR, Giunco S, Serraino D, *et al*. Post-transplant lymphoproliferative disorders: from epidemiology to pathogenesis-driven treatment. *Cancer Lett* 2015;369:37–44.
2 Dierickx D, Tousseyn T, Sagaert X, *et al*. Single-center analysis of biopsy-confirmed posttransplant lymphoproliferative disorder: incidence, clinicopathological characteristics and prognostic factors. *Leuk Lymphoma* 2013;54:2433–40.
3 Trappe RU, Dierickx D, Zimmermann H, *et al*. Response to rituximab induction is a predictive marker in B-cell post-transplant lymphoproliferative disorder and allows successful stratification into rituximab or R-CHOP consolidation in an international, prospective, multicenter phase II trial. *J Clin Oncol* 2017;35:536–43.
4 Reshef R, Vardhanabhuti S, Luskin MR, *et al*. Reduction of immunosuppression as initial therapy for posttransplantation lymphoproliferative disorder(★). *Am J Transplant* 2011;11:336–47.
5 Elstrom RL, Andreadis C, Aqui NA, *et al*. Treatment of PTLD with rituximab or chemotherapy. *Am J Transplant* 2006;6:569–76.
6 Bollard CM, Rooney CM, Heslop HE. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. *Nat Rev Clin Oncol* 2012;9:510–9.
7 Schultze-Florey RE, Tischer S, Kuhlmann L, *et al*. Dissecting Epstein-Barr virus-specific T-cell responses after allogeneic EBV-specific T-cell transfer for central nervous system posttransplant lymphoproliferative disease. *Front Immunol* 2018;9:1475.
8 Tischer S, Priesner C, Heuft H-G, *et al*. Rapid generation of clinical-grade antiviral T cells: selection of suitable T-cell donors and GMP-compliant manufacturing of antiviral T cells. *J Transl Med* 2014;12:336.
9 Priesner C, Esser R, Tischer S, *et al*. Comparative analysis of Clinical-Scale IFN-γ-Positive T-cell enrichment using partially and fully integrated platforms. *Front Immunol* 2016;7:393.
10 Eshhar Z, Waks T, Gross G, *et al*. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* 1993;90:720–4.
11 Clambey ET, Davenport B, Kappler JW, *et al*. Molecules in medicine mini review: the αβ T cell receptor. *J Mol Med* 2014;92:735–41.
12 Chmielewski M, Abken H. Trucks: the fourth generation of cars. *Expert Opin Biol Ther* 2015;15:1145–54.
13 Chmielewski M, Hombach AA, Abken H. Of cars and trucks: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma. *Immunol Rev* 2014;257:83–90.
14 Chmielewski M, Kopecky C, Hombach AA, *et al*. Il-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Res* 2011;71:5697–706.
15 Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. *Cancer Discov* 2013;3:388–98.
16 Andersen PS, Stryhn A, Hansen BE, *et al*. A recombinant antibody with the antigen-specific, major histocompatibility complex-restricted specificity of T cells. *Proc Natl Acad Sci U S A* 1996;93:1820–4.
17 Dao T, Yan S, Veomett N, *et al*. Targeting the intracellular WT1 oncogene product with a therapeutic human antibody. *Sci Transl Med* 2013;5:176ra33.
18 Sergeeva A, Alatrash G, He H, *et al*. An anti-PR1/HLA-A2 T-cell receptor-like antibody mediates complement-dependent cytotoxicity against acute myeloid leukemia progenitor cells. *Blood* 2011;117:4262–72.
19 Zhao Q, Ahmed M, Tassev DV, *et al*. Affinity maturation of T-cell receptor-like antibodies for Wilms tumor 1 peptide greatly enhances therapeutic potential. *Leukemia* 2015;29:2238–47.
20 Lai J, Tan WJ, Too CT, *et al*. Targeting Epstein-Barr virus-transformed B lymphoblastoid cells using antibodies with T-cell receptor-like specificities. *Blood* 2016;128:1396–407.
21 Hülsmeyer M, Chames P, Hillig RC, *et al*. A major histocompatibility complex-peptide-restricted antibody and t cell receptor molecules recognize their target by distinct binding modes: crystal structure of

human leukocyte antigen (HLA)-A1-MAGE-A1 in complex with FAB-HYB3. *J Biol Chem* 2005;280:2972–80.
22 Chames P, Hufton SE, Coulie PG, *et al*. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. *Proc Natl Acad Sci U S A* 2000;97:7969–74.
23 Zhang G, Wang L, Cui H, *et al*. Anti-Melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor. *Sci Rep* 2014;4:3571.
24 Rafiq S, Purdon TJ, Daniyan AF, *et al*. Optimized T-cell receptor-mimic chimeric antigen receptor T cells directed toward the intracellular Wilms tumor 1 antigen. *Leukemia* 2017;31:1788–97.
25 Stewart-Jones G, Wadle A, Hombach A, *et al*. Rational development of high-affinity T-cell receptor-like antibodies. *Proc Natl Acad Sci U S A* 2009;106:5784–8.
26 Uchańska-Ziegler B, Nössner E, Schenk A, *et al*. Soluble T cell receptor-like properties of an HLA-B35-specific monoclonal antibody (TU165). *Eur J Immunol* 1993;23:734–8.
27 Menssen R, Orth P, Ziegler A, *et al*. Decamer-like conformation of a nona-peptide bound to HLA-B*3501 due to non-standard positioning of the C terminus. *J Mol Biol* 1999;285:645–53.
28 Reynisson B, Alvarez B, Paul S, *et al*. NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic Acids Res* 2020;48:W449–54.
29 Gonzalez-Galarza FF, McCabe A, Santos EJMD, *et al*. Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. *Nucleic Acids Res* 2020;48:D783–8.
30 Hudecek M, Lupo-Stanghellini M-T, Kosasih PL, *et al*. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res* 2013;19:3153–64.
31 Zimmermann K, Kuehle J, Dragon AC, *et al*. Design and characterization of an "all-in-one" lentiviral vector system combining constitutive anti-GD2 CAR expression and inducible cytokines. *Cancers* 2020;12:375.
32 Jutz S, Leitner J, Schmetterer K, *et al*. Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: simultaneous measurement of NF-κB, NFAT and AP-1. *J Immunol Methods* 2016;430:10–20.
33 Rydzek J, Nerreter T, Peng H, *et al*. Chimeric antigen receptor library screening using a novel NF-κB/NFAT reporter cell platform. *Mol Ther* 2019;27:287–99.
34 Hudecek M, Sommermeyer D, Kosasih PL, *et al*. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer Immunol Res* 2015;3:125–35.
35 Hombach A, Heuser C, Gerken M, *et al*. T cell activation by recombinant FcepsilonRI gamma-chain immune receptors: an extracellular spacer domain impairs antigen-dependent T cell activation but not antigen recognition. *Gene Ther* 2000;7:1067–75.
36 Lock D, Mockel-Tenbrinck N, Drechsel K, *et al*. Automated manufacturing of potent CD20-Directed chimeric antigen receptor T cells for clinical use. *Hum Gene Ther* 2017;28:914–25.
37 Zhang Y, Li Y. T cell receptor-engineered T cells for leukemia immunotherapy. *Cancer Cell Int* 2019;19:2.
38 Lamprecht M, Dansereau C. Car T-cell therapy: update on the state of the science. *Clin J Oncol Nurs* 2019;23:6–12.
39 Escobar H, Crockett DK, Reyes-Vargas E, *et al*. Large scale mass spectrometric profiling of peptides eluted from HLA molecules reveals N-terminal-extended peptide motifs. *J Immunol* 2008;181:4874–82.
40 Catakovic K, Klieser E, Neureiter D, *et al*. T cell exhaustion: from pathophysiological basics to tumor immunotherapy. *Cell Commun Signal* 2017;15:1.
41 Berger C, Jensen MC, Lansdorp PM, *et al*. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 2008;118:294–305.
42 Klebanoff CA, Gattinoni L, Torabi-Parizi P, *et al*. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A* 2005;102:9571–6.
43 Oren R, Hod-Marco M, Haus-Cohen M, *et al*. Functional comparison of engineered T cells carrying a native TCR versus TCR-like antibody-based chimeric antigen receptors indicates affinity/avidity thresholds. *J Immunol* 2014;193:5733–43.
44 Maus MV, Plotkin J, Jakka G, *et al*. An MHC-restricted antibody-based chimeric antigen receptor requires TCR-like affinity to maintain antigen specificity. *Mol Ther Oncolytics* 2016;3:16023–9.
45 Ziegler A, Laudien D, Heinrichs H, *et al*. K562 cells express human major histocompatibility antigens. *Immunogenetics* 1981;13:359–65.
46 Srivastava S, Riddell SR. Engineering CAR-T cells: design concepts. *Trends Immunol* 2015;36:494–502.
47 Ahmed M, Lopez-Albaitero A, Pankov D, *et al*. TCR-mimic bispecific antibodies targeting LMP2A show potent activity against EBV malignancies. *JCI Insight* 2018;3. doi:10.1172/jci.insight.97805. [Epub ahead of print: 22 Feb 2018].
48 Froscher BG, Klinman NR. Immunization with SV40-transformed cells yields mainly MHC-restricted monoclonal antibodies. *J Exp Med* 1986;164:196–210.
49 Sheih A, Voillet V, Hanafi L-A, *et al*. Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. *Nat Commun* 2020;11:219.
50 Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;3:133–46.
51 Zhao J, Lin Q, Song Y, *et al*. Universal cars, universal T cells, and universal CAR T cells. *J Hematol Oncol* 2018;11:132.
52 Wagner H-J, Bollard CM, Vigouroux S, *et al*. A strategy for treatment of Epstein-Barr virus-positive Hodgkin's disease by targeting interleukin 12 to the tumor environment using tumor antigen-specific T cells. *Cancer Gene Ther* 2004;11:81–91.
53 Zhang L, Kerkar SP, Yu Z, *et al*. Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. *Mol Ther* 2011;19:751–9.
54 Salter AI, Ivey RG, Kennedy JJ, *et al*. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Sci Signal* 2018;11. doi:10.1126/scisignal.aat6753. [Epub ahead of print: 21 aug 2018].
55 van Herpen CM, Looman M, Zonneveld M, *et al*. Intratumoral administration of recombinant human interleukin 12 in head and neck squamous cell carcinoma patients elicits a T-helper 1 profile in the locoregional lymph nodes. *Clin Cancer Res* 2004;10:2626–35.
56 Tan GW, Visser L, Tan LP, *et al*. The microenvironment in Epstein-Barr virus-associated malignancies. *Pathogens* 2018;7. doi:10.3390/pathogens7020040. [Epub ahead of print: 13 Apr 2018].
57 Münz C. Epstein-Barr virus-specific immune control by innate lymphocytes. *Front Immunol* 2017;8:1658.
58 Lünemann A, Vanoaica LD, Azzi T, *et al*. A distinct subpopulation of human NK cells restricts B cell transformation by EBV. *J Immunol* 2013;191:4989–95.
59 Kunert A, Chmielewski M, Wijers R, *et al*. Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors. *Oncoimmunology* 2017;7:e1378842.
60 Sukdolak C, Tischer S, Dieks D, *et al*. CMV-, EBV- and ADV-specific T cell immunity: screening and monitoring of potential third-party donors to improve post-transplantation outcome. *Biol Blood Marrow Transplant* 2013;19:1480–92.

3.2 Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-G_{D2} CAR Expression and Inducible Cytokines

Published February 6, 2020 in Cancers

Katharina Zimmermann[1], Johannes Kuehle[2], **Anna Christina Dragon**[3], Melanie Galla[1], Christina Kloth[1], Loreen Sophie Rudek[1], I. Erol Sandalcioglu[4], Belal Neyazi[4], Thomas Moritz[1], Johann Meyer[1], Claudia Rossig[5], Bianca Altvater[5], Britta Eiz-Vesper[3], Michael Alexander Morgan[1], Hinrich Abken[6] and Axel Schambach[1,7,*]

[1]Institute of Experimental Hematology, Hannover Medical School, 30625 Hannover, Germany

[2]Center for Molecular Medicine Cologne, University of Cologne, and Department I of Internal Medicine, University Hospital Cologne, 50931 Cologne, Germany

[3]Institute for Transfusion Medicine, Hannover Medical School, 30625 Hannover, Germany

[4]Department of Neurosurgery, Medical Faculty, Otto-von-Guericke University, 39120 Magdeburg,

[5]Department of Pediatric Hematology and Oncology, University Children's Hospital Muenster, 48149 Muenster, Germany

[6]Regensburg Centre for Interventional Immunology (RCI), Department of Genetic Immunotherapy, and University Hospital Regensburg, 93053 Regensburg, Germany;

[7]Division of Hematology/Oncology, Boston Children´s Hospital, Harvard Medical School, Boston, MA 02115, USA

* Correspondence: Schambach.Axel@mh-hannover.de; Tel.: +49-511-532-5170

Author contribution statement

Anna Christina Dragon provided primary T cells for the transduction with the lentiviral vectors, acquired and analyzed parts of the data summarized in Figure 6 – in detail: in part transduced and expanded the primary T cells and performed parts of the co-culture experiments with provided target cells under similar conditions as used for clinical manufacturing for the assessment of activation marker expression, cytokine release and cytotoxicity and performed cytokine measurements by LEGENDplex (Fig. 6, S4). A.C.D. reviewed and edited the manuscript.

All author contributions: K.Z. in part cloned the lentiviral vectors (summarized in Fig. 1A, 2A), acquired and analyzed all data for Figures 1-5, S1-S3 and in parts for Figures 6, 7 and S4. K.Z. planned and designed the experimental setups and overall concept, created Figures and wrote the manuscript. J.K. in part cloned and designed the lentiviral vectors summarized in Figure 2A, helped in conceptualization and critically reviewed the manuscript. M. G. in part designed and cloned the inducible NFATsyn promoter element and took part in the "all-in-one" TRUCK vector design, critically discussed and interpreted data. C. K. generated and analyzed data for the migration assay (Fig. 7). L.S.R. helped with lentiviral vector production and T-cell transduction. I.E.S. and B.N. provided the primary glioblastoma samples from glioblastoma tumor resections and provided their

clinical expertise. T.M. took part in funding acquisition and helped with data interpretation for Figure 7. J.M. helped to critically analyze and interpret data. C.R. and B.A. provided the G_{D2}CAR sequence and GD3S/GD2S transgene for the generation of the G_{D2}^{+} HT1080 target cells. C.R. reviewed the manuscript. B.E.V. interpreted data for Figure 6 and S4 and provided resources for data sets seen in Figure 6 B-G and S4. M.A.M. aided critically data interpretation and discussion, participated in manuscript writing, proof-read and revised the manuscript. H.A. developed the inducible NFATmIL2 promoter and participated in development of the "all-in-one" vector design. H.A. critically revised the manuscript. A.S. developed the overall study concept, took part in funding acquisition, designed the "all-in-one" TRUCK concept and in part cloned the lentiviral vectors (summarized in Fig. 1A, 2A), helped in data interpretation and participated in manuscript conceptualization and manuscript writing. All authors reviewed and edited the manuscript.

Copyright

The article was published under the Creative Commons Attribution (CC BY) 4.0 license permitting unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Zimmermann, K., Kuehle, J., Dragon, A.C., Galla, M., Kloth, C., Rudek, L.S., Sandalcioglu, I.E., Neyazi, B., Moritz, T., Meyer, J., Rossig, C., Altvater, B., Eiz-Vesper, B., Morgan, M.A., Abken, H., and Schambach, A., *Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-GD2 CAR Expression and Inducible Cytokines.* Cancers, 2020. **12**(2): p. 375.

cancers

MDPI

Article

Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-G_{D2} CAR Expression and Inducible Cytokines

Katharina Zimmermann [1], Johannes Kuehle [2], Anna Christina Dragon [3], Melanie Galla [1], Christina Kloth [1], Loreen Sophie Rudek [1], I. Erol Sandalcioglu [4], Belal Neyazi [4], Thomas Moritz [1], Johann Meyer [1], Claudia Rossig [5], Bianca Altvater [5], Britta Eiz-Vesper [3], Michael Alexander Morgan [1], Hinrich Abken [6] and Axel Schambach [1,7,*]

1 Institute of Experimental Hematology, Hannover Medical School, 30625 Hannover, Germany; zimmermann.katharina@mh-hannover.de (K.Z.); galla.melanie@mh-hannover.de (M.G.); Kloth.Christina@mh-hannover.de (C.K.); Loreen.S.Rudek@stud.mh-hannover.de (L.S.R.); moritz.thomas@mh-hannover.de (T.M.); meyer.johann@mh-hannover.de (J.M.); morgan.michael@mh-hannover.de (M.A.M.)

2 Center for Molecular Medicine Cologne, University of Cologne, and Department I of Internal Medicine, University Hospital Cologne, 50931 Cologne, Germany; johannes.kuehle@uk-koeln.de

3 Institute for Transfusion Medicine, Hannover Medical School, 30625 Hannover, Germany; Dragon.Anna@mh-hannover.de (A.C.D.); Eiz-Vesper.Britta@mh-hannover.de (B.E.-V.)

4 Department of Neurosurgery, Medical Faculty, Otto-von-Guericke University, 39120 Magdeburg, Germany; erol.sandalcioglu@med.ovgu.de (I.E.S.); belal.neyazi@med.ovgu.de (B.N.)

5 Department of Pediatric Hematology and Oncology, University Children's Hospital Muenster, 48149 Muenster, Germany; rossig@ukmuenster.de (C.R.); bianca.altvater@ukmuenster.de (B.A.)

6 Regensburg Centre for Interventional Immunology (RCI), Department of Genetic Immunotherapy, and University Hospital Regensburg, 93053 Regensburg, Germany; Hinrich.Abken@klinik.uni-regensburg.de

7 Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

* Correspondence: schambach.axel@mh-hannover.de; Tel.: +49-511-532-5170

Received: 6 December 2019; Accepted: 4 February 2020; Published: 6 February 2020

Abstract: Genetically modified T cells expressing chimeric antigen receptors (CARs) so far have mostly failed in the treatment of solid tumors owing to a number of limitations, including an immunosuppressive tumor microenvironment and insufficient CAR T cell activation and persistence. Next-generation approaches using CAR T cells that secrete transgenic immunomodulatory cytokines upon CAR signaling, known as TRUCKs ("T cells redirected for universal cytokine-mediated killing"), are currently being explored. As TRUCKs were engineered by the transduction of T cells with two separate vectors, we developed a lentiviral modular "all-in-one" vector system that combines constitutive CAR expression and inducible nuclear factor of activated T cells (NFAT)-driven transgene expression for more efficient production of TRUCKs. Activation of the G_{D2}-specific CAR via $GD2^+$ target cells induced NFAT promoter-driven cytokine release in primary human T cells, and indicated a tight linkage of CAR-specific activation and transgene expression that was further improved by a modified NFATsyn promoter. As proof-of-concept, we showed that T cells containing the "all-in-one" vector system secrete the immunomodulatory cytokines interleukin (IL)12 or IL18 upon co-cultivation with primary human $GD2^+$ tumor cells, resulting in enhanced effector cell properties and increased monocyte recruitment. This highlights the potential of our system to simplify application of TRUCK-modified T cells in solid tumor therapy.

Keywords: glioblastoma; "all-in-one" lentiviral vector; TRUCK; G_{D2}CAR; NFAT; inducible cytokines; IL12; IL18

1. Introduction

Adoptive cell therapy using chimeric antigen receptor (CAR)-modified T cells is one of the greatest breakthroughs in tumor therapy in recent years [1–3]. The authorization of Kymriah® (Novartis) and Yescarta® (Kite Gilead) underline CAR T cell efficacy and feasibility, especially of CD19-CAR T cells for the treatment of $CD19^+$ B cell malignancies [4–8]. However, further improvements using CAR T cells are needed to address solid tumors. Heterogeneous antigen expression, insufficient tissue homing, and limited persistence of CAR T cells at the tumor site, as well as the immunosuppressive tumor microenvironment (TME), are major barriers that need to be overcome in the context of solid tumors [9–12]. Systemic application of interleukins, for example, interleukin (IL)12, provided some anti-tumor activity, as demonstrated by tumor regression and prolonged survival in murine studies [13,14], but was also linked with severe systemic toxicities [15,16].

In this direction, the TRUCK ("T cell redirected for universal cytokine-mediated killing") concept was established, which equips CAR-redirected T cells with an additional NFAT (nuclear factor of activated T cells) inducible cytokine response that results in the restricted locoregional release of a transgenic cytokine upon CAR engagement of cognate target cells. The TRUCK approach can strengthen T cell activation and shape the TME by attracting innate immune cells to provide a more successful attack against tumor cells [17–21]. CAR T cells designed to release transgenic, inducible IL12 had improved cytolytic activity against solid tumors and increased the accumulation of activated macrophages and innate immune cells in the tumor tissue without inducing systemic toxicity in a murine model [19].

Of note, the TRUCK concept can also be transferred to other cytokines and transgenes of choice. IL18 was found to create a pro-inflammatory environment and increase T cell persistence in murine xenograft models [22]. In mouse models, IL18-secreting CAR T cells designed to target the carcinoembryonic antigen (CEA) showed superior anti-tumor activity against solid tumors in a pancreatic cancer model [18]. Additional studies indicated improved tumor control in leukemia and melanoma models using CD19-CAR T cells that constitutively express IL18 [23,24].

Current clinical trials underline the principle of CAR T cells with cytokine-enhanced activity. For example, CAR T cells engineered to secrete IL12 and target MUC16ecto-expressing solid tumors (NCT02498912), as well as CAR T cells targeting G_{D2} and secreting IL15 for cytokine support for the treatment of relapsed/refractory neuroblastoma, are being currently investigated in early phase trials (NCT03721068) (www.clinicaltrials.gov).

The generation of TRUCKs was originally accomplished by the transduction of two vector constructs, that is, one vector for constitutive CAR expression and one vector for inducible cytokine expression. The transduction of two vectors per T cell might enhance the risk of insertional mutagenesis by various genetic integrations and, thus, hinder manufacturing for clinical application. To overcome these limitations, T cell transduction protocols with single vectors were described, which mostly led to constitutive cytokine expression via an internal ribosomal entry site (IRES) or 2A site [14,23]. Regarding these aspects, we developed a modular "all-in-one" vector system that combines constitutive CAR expression and inducible CAR activation-induced cytokine secretion in a single vector. Additionally, the cytokine response is tightly linked to CAR expression and activation, so that cytokine expression would ideally be induced in the tumor upon recognition of the targeted solid tumor antigen. Thus, the "all-in-one" system should improve the safety and efficacy of T cell therapy, while avoiding systemic toxicity of anticancer principles.

As proof-of-concept, we generated a modular "all-in-one" vector system based on clinically applied third generation lentiviral self-inactivating (SIN) vectors designed to constitutively express a CAR in combination with NFAT-inducible cytokine expression, for example, IL12 or IL18. Therefore, we further codon-optimized a well-established second generation CAR that targets the cell-surface disialoganglioside G_{D2} [25], which has restricted expression in normal tissues, but is expressed on several solid tumors, including glioblastoma [26], neuroblastoma [27], and sarcomas [28]. Clinical trials already support the safety and efficacy of G_{D2}-redirected CAR T cells without undesirable adverse

effects [29–31]. However, response rates of neuroblastoma patients treated with G_{D2}CAR T cells were lower than the successful treatment rates for CD19-CAR T cell therapies in leukemia patients [32], which reflects the need for improved therapies in solid tumor settings.

Here, we demonstrated the reliability of the established "all-in-one" vector construct regarding tight and effective induction of reporter genes and anti-cancer cytokines in primary human T cells that led to a potent anti-tumor effect in vitro, with the potential to reshape the TME in the case this system would be applied in vivo to treat solid tumors. The NFAT promoter was effectively and selectively induced after G_{D2}CAR-specific activation by $GD2^+$ target cells and patient-derived primary glioblastoma cells, which led to a strict CAR-activation-dependent cytokine secretion. Importantly, we showed that CAR T cell-secreted cytokines IL12 and IL18 led to increased recruitment of monocytes, as tested in a migration assay.

Thus, our "all-in-one" vector represents a useful tool for targeted applications to enhance the efficacy for future treatment of solid tumors.

2. Results

2.1. Specificity and Function of the G_{D2}CAR

We first reassessed the well-established second generation CAR targeting the cell-surface ganglioside G_{D2} with respect to specific target recognition. The G_{D2}CAR contains a single-chain variable fragment (scFv) derived from the monoclonal antibody 14.G2a that has been approved for therapeutic use in children with neuroblastoma and has been used in previous clinical trials [33], an IgG1 (HCH_2CH_3) hinge domain followed by the CD28 transmembrane (TM) domain, and the intracellular 4-1BB and CD3ζ signaling domains [25] (Figure 1A). To verify the specificity and function of the G_{D2}CAR, the CAR was initially cloned into a third generation lentiviral self-inactivating (SIN) vector [34] under the control of the human phosphoglycerate kinase (hPGK) promoter (LV-G_{D2}CAR) and tested in a murine T cell hybridoma reporter cell line. The T cell hybridoma cells, which stably express a reporter construct consisting of an NFAT-sensitive minimal IL-2 promoter that induces enhanced green fluorescent protein (EGFP) expression upon CAR-specific activation, were transduced with the LV-G_{D2}CAR vector construct [35] with a transduction efficiency of approximately 60%. NFAT-driven EGFP was upregulated after co-culture with HT1080 cells engineered to express the target antigen G_{D2} (~96%) (Figure 1B), while little EGFP upregulation was observed upon co-culture with wild-type HT1080 cells that mostly lack GD2 expression (~8%) (Figure 1C,D). Although only 15% of G_{D2}CAR-transduced hybridoma cells upregulated EGFP upon G_{D2}CAR-specific stimulation with HT1080 GD2 cells, this effect was sufficient to demonstrate the specificity of the G_{D2}CAR for the GD2 antigen and that the CAR activation led to NFAT signaling in the modified T cells. Moreover, these results indicated that the CAR transmits an activation signal through NFAT that is entirely due to G_{D2}CAR signaling, as the hybridoma cells do not express any endogenous human T cell receptor (TCR). These data provided the rationale for engineering the "all-in-one" vector based on the lentiviral G_{D2}CAR vector.

Cancers **2020**, *12*, 375

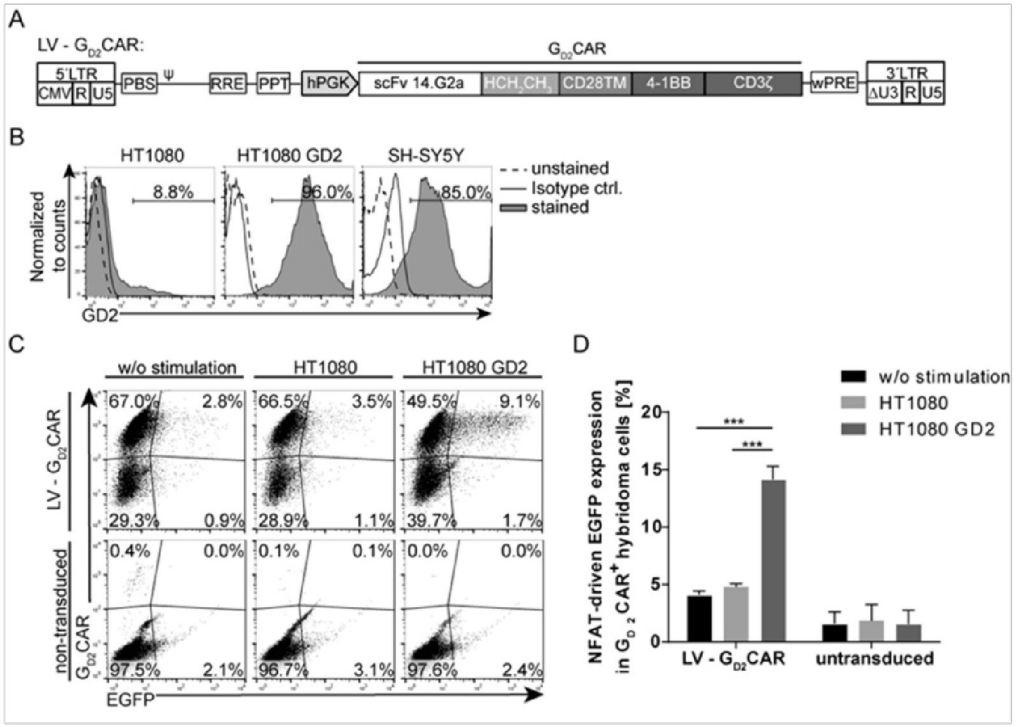


Figure 1. G_{D2} chimeric antigen receptor (G_{D2}-CAR)-specific activation leads to nuclear factor of activated T cells (NFAT) promoter-driven enhanced green fluorescent protein (EGFP) upregulation. (**A**) Modular scheme of lentiviral self-inactivating (SIN) vector encoding a second generation G_{D2}CAR expression cassette driven by the human phosphoglycerate kinase (hPGK) promoter. G_{D2}CAR consists of the single chain variable fragment (scFv) 14.G2a, the HCH_2CH_3 hinge domain, the CD28 transmembrane domain (TM), and the 4-1BB/CD3ζ signaling domain. Depicted is the configuration after integration. Indicated are the long-terminal repeat (LTR) (ΔU3, R, and U5), primer binding site (PBS), packaging signal (ψ), rev-responsive element (RRE), central poly-purine tract (cPPT), and woodchuck hepatitis virus post-transcriptional regulatory element (wPRE). (**B**) Flow cytometric analysis of G_{D2}-expressing target cell lines (HT1080 GD2 and neuroblastoma cell line SH-SY5Y) used for co-culture experiments. (**C**) Representative flow cytometric analysis of G_{D2}CAR-transduced hybridoma cells after co-culture with indicated target cells. NFAT-driven EGFP upregulation was observed after co-culture with $GD2^+$ target cells. Gated on $G_{D2}CAR^+$ hybridoma cells. (**D**) Summary of three independent co-culture experiments showing NFAT promoter-driven EGFP expression in G_{D2}CAR-transduced hybridoma cells after stimulation with or without $GD2^+$ target cells. Shown are mean values ± SD (n = 3). Indicated significance was determined by two-way analysis of variance (ANOVA) with Tukey's multiple comparison test; *** $p \leq 0.001$). CMV, cytomegalovirus.

2.2. Generation of "All-in-One" Vector Constructs for Targeted Therapy

To realize the TRUCK concept in a technically more applicable fashion, we combined inducible NFAT-driven cytokine secretion and constitutive CAR expression in a single vector and generated an "all-in-one" construct based on a clinically applied lentiviral SIN vector backbone (Figure 2A). To achieve inducible co-expression, an NFAT-driven cytokine cassette was inserted 3' of the central polypurine tract (cPPT) followed by the G_{D2}CAR expression cassette under control of the hPGK promoter, which enabled ubiquitous G_{D2}CAR expression in nearly every transduced immune cell. For CAR-induced expression, we generated and tested two different NFAT-inducible promoters, namely NFATmIL2 and NFATsyn. Six NFAT consensus response elements (6xNFAT) were either fused to a minimal IL2 promoter to generate NFATmIL2 [36] or to a synthetic promoter element in addition

Cancers **2020**, *12*, 375

to a synthetic TATA box to generate NFATsyn [37]. Both NFAT-responsive promoters were linked to an expression cassette for EGFP as an initial control module to optimize the vector configuration or the cytokine of interest. Each module of the vector was flanked by unique restriction sites to allow easy exchange of the respective modules. The designed architecture of the "all-in-one" vector constructs allowed independent secretion of the inducible gene of interest (iGOI), for example, EGFP control module, hIL12, or hIL18, driven by the CAR-activated NFATmIL2 or NFATsyn promoters. Compared with two-vector systems, direct linkage of CAR and iGOI within the "all-in-one" vector concept enables improved generation and control of genetically modified T cells and their inducible transgene expression.

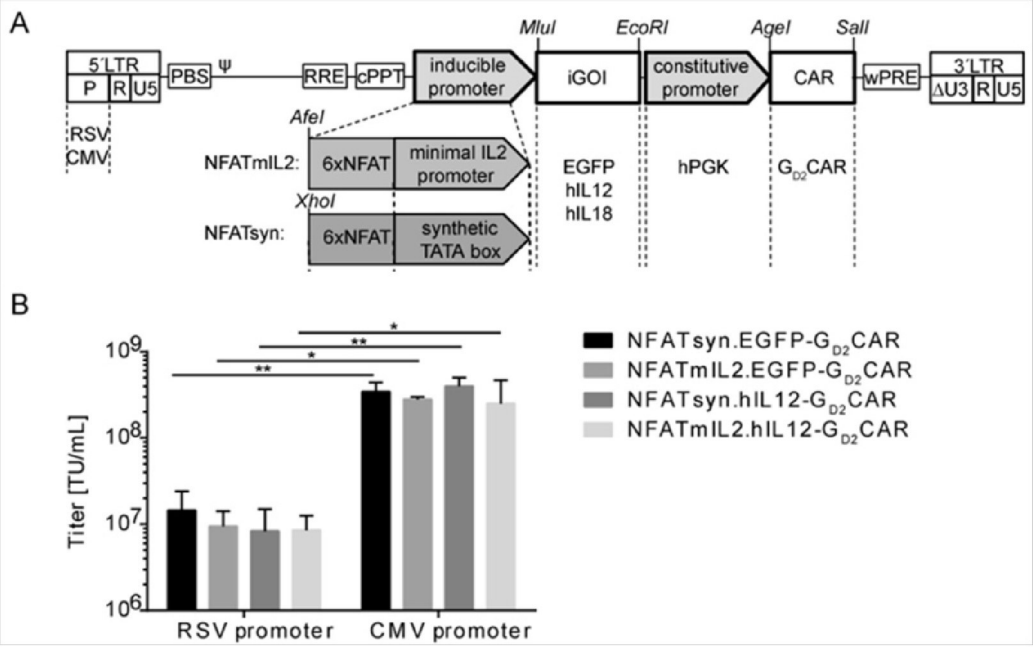


Figure 2. Schematic depiction of the modular "all-in-one" vector construct. (**A**) Lentiviral SIN vector scheme representing the main "all-in-one" construct elements and additional modifications. The vector plasmid configurations with indicated restriction sites are shown. The promoter sequence (P) in the 5'Long Terminal Repeat (LTR) is derived from Rous sarcoma virus (RSV) or cytomegalovirus (CMV). The construct is composed of a constitutively expressed G_{D2}CAR element driven by a human phosphoglycerate kinase (hPGK) promoter and an inducible element consisting of six consensus NFAT enhancer repeats and the corresponding promoter (minimal IL2 promoter (mIL2) or synthetic TATA box (syn)) followed by the inducible gene of interest, respectively. (**B**) CMV-driven lentiviral "all-in-one" vector constructs led to improved lentiviral vector production. Constructs were pseudotyped with vesicular stomatitis virus glycoprotein (VSVg). Titration was performed in human HT1080 fibroblasts based upon G_{D2}CAR expression as analyzed via flow cytometry three days post transduction. Bar graphs depict mean values ± SD (n = 3) of viral vector titers determined as described in Section 4.3. Indicated significance was determined by one-way ANOVA with Tukey's multiple comparison test; ** $p \leq 0.01$; * $p \leq 0.05$; ns > 0.05). iGOI, inducible gene of interest.

Initial lentiviral particle productions using a vector configuration with the Rous sarcoma virus (RSV) promoter in the 5'Long Terminal Repeat (LTR) region resulted in low virus vector titers and required further improvement in order to generate titers relevant for clinical applications (Figure 2B). Thus, we generated and tested different vector configurations for improved production of viral vector particles by transduction and analyses of the human fibrosarcoma cell line HT1080. The exchange of the RSV promoter for the cytomegalovirus (CMV) promoter in the 5'LTR region of the SIN lentiviral vector

plasmid resulted in a significant increase in lentiviral vector particle production to levels sufficient for clinical translation of "all-in-one" constructs (Figure 2B).

2.3. Promoter Choice of the Inducible Construct Determines the Extent of Activation

To evaluate the potency and safety of the designed "all-in-one" vector constructs for adoptive T cell therapy, primary human T cells were transduced (vectors shown in Figure 2A), sorted, and tested for CAR-specific activation. Mechanistically, T cell activation via signaling domains of the constitutively expressed CAR leads to NFAT-mediated transcription, which results in the expression of the NFAT-responsive iGOI cassette. CAR surface expression in transduced T cells was confirmed by staining with a G_{D2}CAR-specific antibody that recognizes the extracellular scFv domain. The application of viral vector particles at a multiplicity of infection (MOI) of 10 on T cells led to transduction rates of 30–50% for all tested constructs (Figure 3A). To test the proof-of-concept for the function of "all-in-one" vector constructs, we compared vectors harboring an EGFP expression cassette under control of two different NFAT-inducible promoters, NFATsyn and NFATmIL2. Marginal background EGFP expression was observed in unstimulated primary T cells expressing the "all-in-one" constructs (Figure 3B). Comparably moderate background EGFP expression was also observed in transduced primary T cells co-cultured with HT1080 (GD2-negative) target cells (Figure S1). In contrast, EGFP expression was highly induced in these T cells after G_{D2}CAR-mediated activation upon co-culture with $GD2^+$ target cells (HT1080 GD2 and SH-SY5Y) for 24 h (see Figure 1B for GD2 expression levels), and thus demonstrated the CAR-specific induction of the inducible expression cassette. Importantly, the NFATsyn promoter led to a ~3-fold higher EGFP expression compared with the NFATmIL2 promoter in transduced primary T cells. After stimulation with $GD2^+$ target cells, the NFATsyn promoter led to a 60% EGFP upregulation in $G_{D2}CAR^+$ T cells compared with approximately 20% EGFP upregulation driven by the NFATmIL2 promoter (Figure 3B), with a similar density of EGFP expression as recorded by the mean fluorescent intensity (MFI) (Figure 3C).

The data indicate a tightly controlled EGFP expression upon G_{D2}CAR-specific activation after recognition of its target antigen. Moreover, the choice of the promoter unit within the inducible cassette is a critical factor for the control of inducible transgene expression and leakiness. Owing to the higher induction capacity, the NFATsyn promoter-driven "all-in-one" construct variants were used in further analyses

2.4. Induction of the NFATsyn Promoter after G_{D2}CAR-Specific Activation with Patient-Derived Primary Glioblastoma Cells

To further characterize the function of T cells modified with NFATsyn.EGFP.G_{D2}CAR "all-in-one" vector constructs on primary target cells, we co-cultured "all-in-one" modified primary T cells with patient-derived $GD2^+$ glioblastoma cells. After isolation, the patient-derived primary glioblastoma cells form typical spheroid structures (Figure 4A). Flow cytometric analysis revealed high expression of the GD2 antigen in these tumor cells in vitro (Figure 4B). Sorted primary T cells transduced with the NFATsyn.EGFP-G_{D2}CAR and co-cultured with patient-derived glioblastoma cells specifically recognized the GD2-antigen expressed on glioblastoma. This became apparent by the NFAT-driven EGFP induction upon G_{D2}CAR signal transduction, as shown by fluorescence microscopy (Figure 4C) and EGFP quantification by flow cytometry (Figure 4D). Compared with non-transduced T cells that did not show EGFP induction after cultivation with glioblastoma cells, NFATsyn.EGFP-G_{D2}CAR transduced T cells specifically expressed EGFP and were enriched on or near the spheroid-forming glioblastoma cells that abundantly expressed the GD2-antigen (Figure 4C). Of note, the number of $G_{D2}CAR^+/EGFP^+$ cells after co-culture with patient-derived glioblastoma cells (Figure 4D) correlated well with the EGFP upregulation after co-culture with HT1080 $GD2^+$ and SH-SY5Y $GD2^+$ cell lines (Figure 3B).

Cancers **2020**, *12*, 375

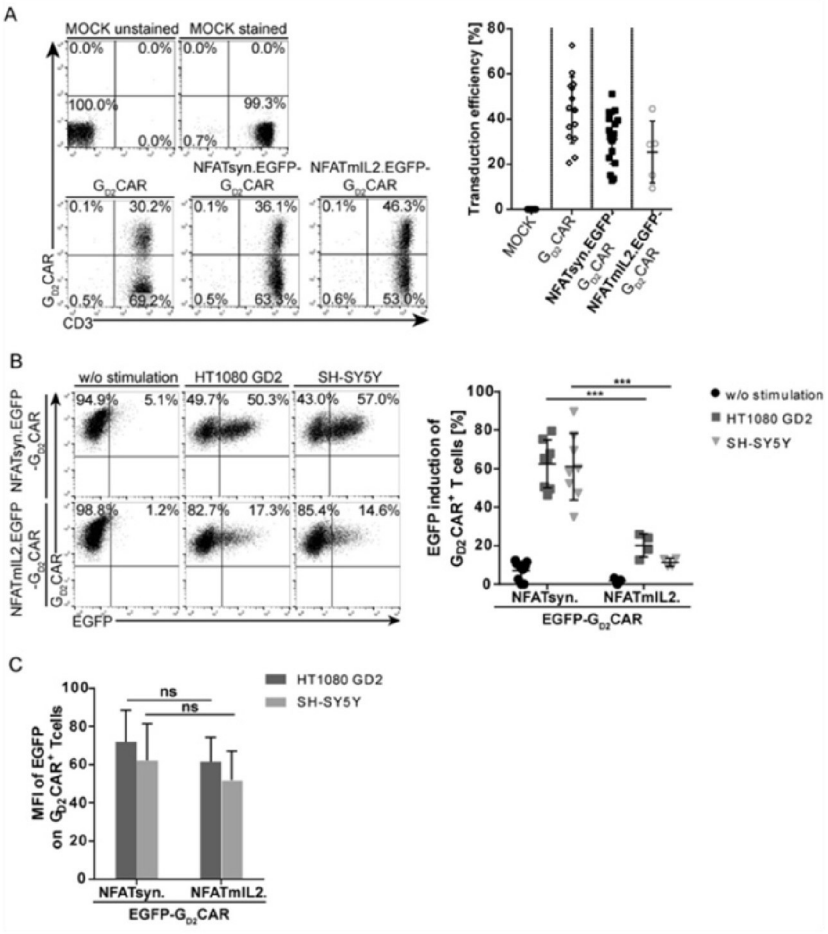


Figure 3. Promoter choice is critical for the amount of inducible transgene expression. (**A**) Representative flow cytometric analysis of primary human transduced CD3$^+$ T cells. Transduction efficiencies were determined six days post transduction via a G_{D2}CAR-specific antibody that recognizes the scFv region. The graph summarizes transduction efficiencies of the individual "all-in-one" constructs (NFATsyn.EGFP-G_{D2}CAR or NFATmIL2.EGFP-G_{D2}CAR) in primary human T cells (multiplicity of infection (MOI) 10). Shown are mean values ± SD (symbols indicate individual donors; n = 5–18). (**B**) Comparison of inducible promoters, namely NFATsyn and NFATmIL2. Primary human T cells were transduced with "all-in-one" EGFP constructs (NFATsyn.EGFP-G_{D2}CAR or NFATmIL2.EGFP-G_{D2}CAR), sorted, and co-cultured with GD2$^+$ target cells. Both constructs drive NFAT promoter-regulated EGFP expression upon G_{D2}CAR-specific activation. Representative flow cytometric analysis of NFAT-driven EGFP expression in transduced T cells after 24 h co-culture with a 6:1 effector/target (E/T) ratio. The graph summarizes multiple experiments of NFAT promoter-driven EGFP expression in "all-in-one" construct-positive (G_{D2}CAR$^+$) T cells post stimulation with GD2$^+$ target cells. Shown are mean values ± SD; symbols indicate individual co-culture experiments with individual donors; n = 4–8. Indicated statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test; *** $p \leq 0.001$. (**C**) Bars show mean fluorescent intensity (MFI) of inducible NFAT promoter-driven EGFP in "all-in-one" construct-positive (G_{D2}CAR$^+$) T cells after 24 h co-culture with GD2$^+$ target cells from three independent experiments (E/T 6:1). Shown are mean values ± SD. Indicated statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test; ns > 0.05. MOCK: untransduced T cells.

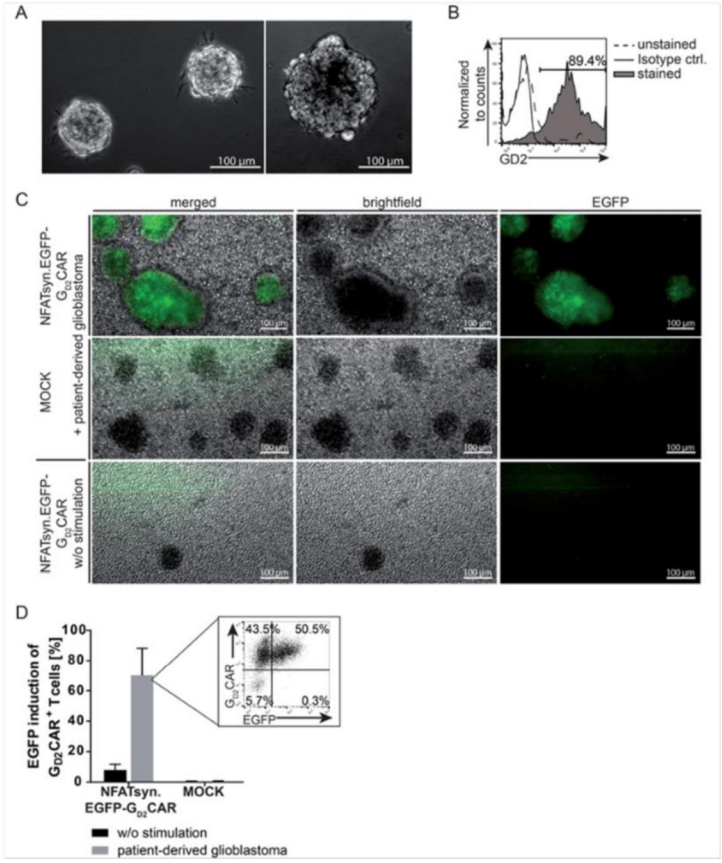


Figure 4. Specific induction of the NFATsyn promoter after CAR-specific activation with patient-derived primary glioblastoma cells. (**A**) Brightfield microscopy images of primary glioblastoma spheroids. (**B**) Expression of G_{D2} in primary glioblastoma cells analyzed via flow cytometric staining compared with isotype control (ctrl.) (representative patient sample). (**C–D**) Primary T cells from two independent donors were transduced with "all-in-one" NFATsyn.EGFP-G_{D2}CAR construct and co-cultured with or without patient-derived glioblastoma cells for 24 h. MOCK represents non-transduced T cells. (**C**) Representative images of NFATsyn.EGFP-G_{D2}CAR T cell co-culture experiments. The specific NFAT-driven EGFP upregulation after stimulation with patient-derived glioblastoma cells is shown in green. (**D**) Graph summarizes inducible NFAT-driven EGFP expression in "all-in-one" construct-positive ($G_{D2}CAR^+$) T cells post stimulation with $GD2^+$ target cells and a representative flow cytometric analysis in transduced T cells after 24 h is depicted. Shown are mean values ± SD; symbols indicate individual co-culture experiments with individual donors; $n = 2$.

2.5. The Modular Design Facilitates Exchange of the Inducible Transgene and Allows Incorporation of the Promising Anticancer Cytokines IL12 and IL18

As the modular design of the "all-in-one" vector construct also allows easy exchange of the iGOI, we next exchanged EGFP with cDNAs coding for human IL12 or IL18 (Figure 5A). Primary human T cells were transduced (MOI of 10) with the NFATsyn.hIL12-G_{D2}CAR construct or the NFATsyn.hIL18-G_{D2}CAR construct (Figure 5B) at similar efficiencies as the NFATsyn.EGFP-G_{D2}CAR (see Figure 3A). Furthermore, G_{D2}CAR-specific activation with $GD2^+$ target cells induced NFAT-driven IL12 secretion after 24 h to levels of 150 pg/mL (Figure 5C) or IL18 (Figure 5D) secretion after 48 h

to 100 pg/mL. In addition, unstimulated primary T cells expressing the "all-in-one" constructs as well as transduced primary T cells co-cultured with HT1080 (GD2-negative) cells induced marginal background human IL12 or human IL18 secretion (Figure S2), indicating a G_{D2}CAR-specific induction of cytokines.

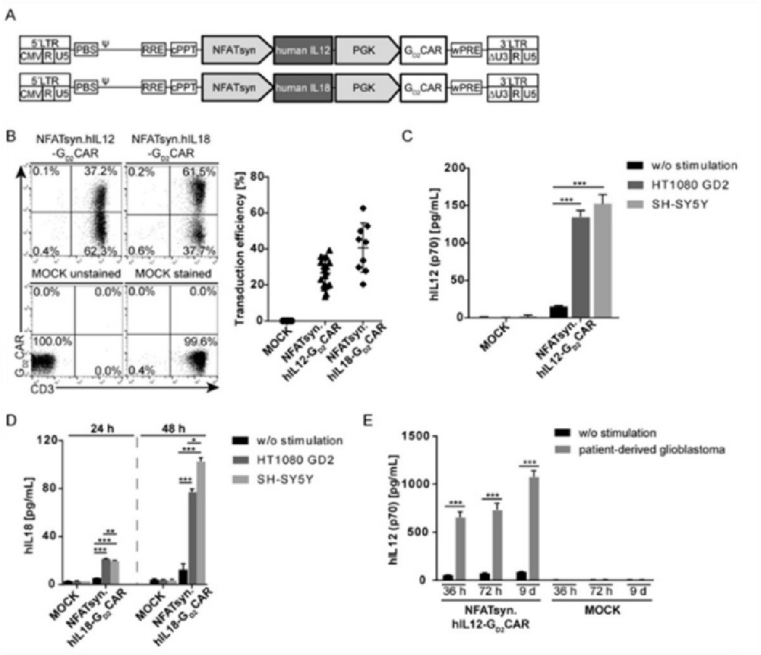


Figure 5. Effective human interleukin (IL)12 and human IL18 secretion after CAR-specific stimulation. (**A**) Schematic design of the "all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR constructs. Indicated is the inducible promoter (NFATsyn). (**B**) Representative flow cytometric analysis of primary human $CD3^+$ T cells that were transduced with the "all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR constructs at an MOI of 10. Transduction efficiency was determined six days post transduction via a G_{D2}CAR-specific antibody that recognizes the scFv region. The graph summarizes individual transduction efficiencies of the "all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR constructs in primary human T cells. Shown are mean values ± SD (symbols indicate individual donors; $n = 9$; MOI 10). (**C**) Human IL12 expression and (**D**) human IL18 expression in cell culture supernatants. Primary T cells were transduced with "all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR constructs and co-cultured with $GD2^+$ target cells for 24 h (and 48 h). Inducible hIL12 or hIL18 secretion was determined via enzyme-linked immunosorbent assay (ELISA). ELISAs were performed in triplicates in two (hIL18) or three (hIL12) independent experiments; unsorted cells; 10:1 effector/target (E/T) ratio. Shown are mean values ± SD. Indicated significance was determined by one-way ANOVA with Tukey's multiple comparison test; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns > 0.05. (**E**) Human IL12 secretion after co-culture of primary T cells transduced with "all-in-one" NFATsyn.hIL12-G_{D2}CAR with or without patient-derived glioblastoma cells for 36 h, 72 h, and 9 d. Inducible IL12 was determined via ELISA. ELISA was performed in duplicates. Shown are mean values ± SD. Indicated significance was determined by one-way ANOVA with Tukey's multiple comparison test; (*** $p \leq 0.001$).

The higher induction capacity of the NFATsyn promoter compared with the NFATmIL2 promoter was also observed in co-culture experiments with the hIL12-G_{D2}CAR (>100 pg/mL vs. <30 pg/mL) and hIL18-G_{D2}CAR (30–80 pg/mL vs. <20 pg/mL) transduced T cells and G_{D2}^+ target cells (Figure S3).

To mimic a more clinically relevant setting, patient-derived glioblastoma cells were co-cultured with T cells transduced with the NFATsyn.hIL12-G_{D2}CAR. Specific secretion of human IL12 after co-culture was observed and the amount of human IL12 in the cell culture supernatant increased over time to >1000 pg/mL (Figure 5E).

In conclusion, these results emphasize that the "all-in-one" vector constructs function properly in T cells co-incubated with primary patient-derived material.

2.6. "All-in-One" Vector Construct-Transduced Primary T Cells Exhibit a Superior Response with Regard to Cytotoxicity, Activation, and Immune Cell Recruitment

Primary human T cells transduced with the "all-in-one" constructs or the G_{D2}CAR construct were analyzed for cytotoxicity against $GD2^+$ target cells, T cell phenotype, and cytokine secretion profile.

To investigate whether the generated constructs enhance the cytotoxic properties of the T cells, we recorded the cytolytic activity of transduced (MOI of 3) and sorted T cells co-cultured with indicated target cells (Figure 6A). All T cells expressing a G_{D2}CAR showed higher cytotoxicity against $GD2^+$ target cells than non-transduced T cells, as measured by lactate dehydrogenase (LDH) release (Figure 6A). The similar level of in vitro cytotoxicity induced by the G_{D2}CAR T cells regardless of cytokine expression/secretion capacity was expected, as these experimental conditions do not account for the potential beneficial recruitment of additional immune cells via inducible cytokine secretion.

We determined expression of T cell activation markers after co-culture with $GD2^+$ target cells to investigate the G_{D2}CAR-related activation of the engineered T cells. Here, we observed that co-culture with $GD2^+$ target cells caused upregulation of $CD3^+CD69^+$ (Figure 6B) and $CD8^+CD137^+$ (Figure 6C) subpopulations in all T cells transduced with the constructs that contain a G_{D2}CAR. Moreover, T cells transduced with the NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR had increased expression levels of CD25 (based on MFI) upon co-culture with HT1080 GD2 cells (Figure 6D). In contrast, CD154 expression on $CD4^+$ T cells was expressed at similar levels in all analyzed T cell groups, except for a slight increase of $CD4^+CD154^+$ NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR T cells after co-culture with HT1080 GD2 cells (Figure 6E). In accordance with the cytotoxicity analyses, G_{D2}CAR containing T cells had upregulated expression of the analyzed activation markers, which underlines the specific activation potential of the transduced T cells compared with non-transduced T cells.

In addition to the activation markers, we also analyzed the expression levels of pro-inflammatory cytokines in supernatants from co-cultures of "all-in-one" vector transduced T cells in response to indicated target cells. Human primary T cells transduced with the NFATsyn.hIL12-G_{D2}CAR "all-in-one" construct showed increased hIL12 cytokine levels upon co-culture with $GD2^+$ target cells and upregulation of pro-inflammatory cytokines like tumor necrosis factor (TNF)α, Interferon (IFN)γ, and IL2, as well as cytolytic effector cytokines like perforin and granzyme B (Figure 6F). These results indicated a pro-inflammatory potential compared with non-transduced or other transduced T cells, as indicated (Figure S4). We also observed an increase in hIL18 secretion by NFATsyn.hIL18-G_{D2}CAR T cells upon co-culture with $GD2^+$ target cells (Figure 6G).

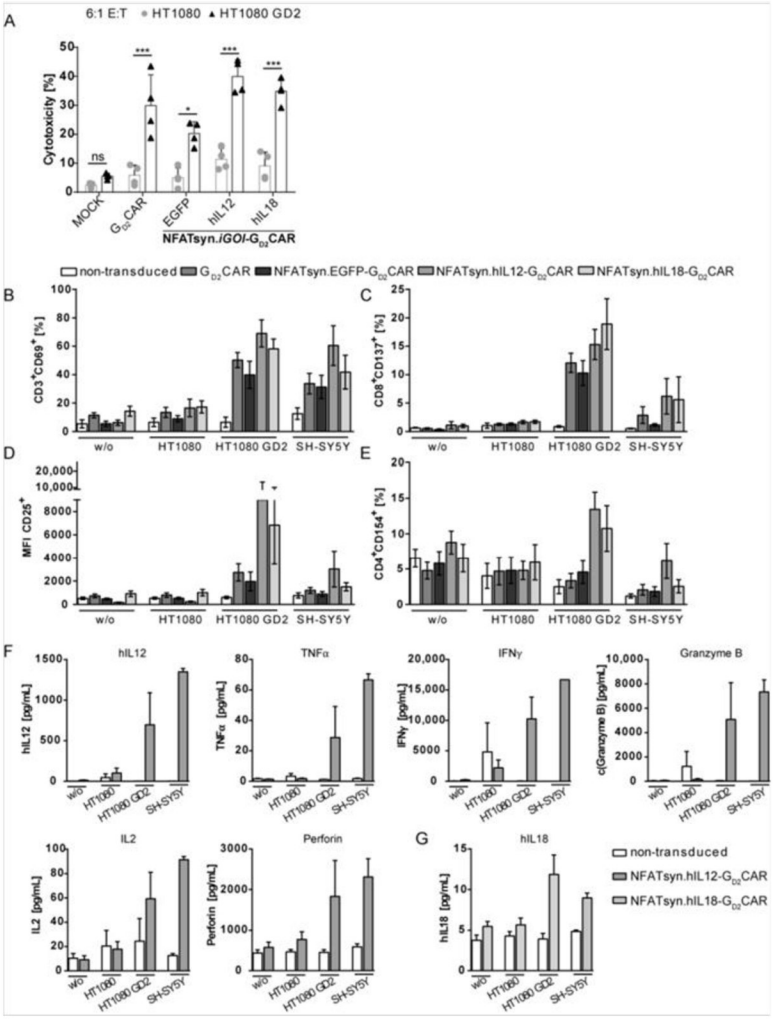


Figure 6. Cytotoxicity and activation marker analyses of "all-in-one" NFATsyn.iGOI-G_{D2}CAR constructs-transduced primary T cells. (**A**) G_{D2}CAR-specific signaling leads to target cell-specific killing. Lactate dehydrogenase (LDH) assay with supernatants of distinct "all-in-one" vector construct co-cultures. Cytotoxicity of sorted transduced primary human $CD4^+$/$CD8^+$ T cells (MOI 3) after 48 h co-culture with HT1080 and HT1080 GD2 target cells in a 1:6 effector/target (E/T) ratio. Shown are mean values ± SD (symbols indicate individual donors; $n = 4$). Indicated significance was determined by two-way ANOVA with Bonferroni's multiple comparison test; *** $p \leq 0.001$;; * $p \leq 0.05$; ns > 0.05. (**B**–**E**) Flow cytometric analysis of T cell-specific activation marker of sorted "all-in-one" vector construct transduced primary human T cells after 48 h co-culture with indicated target cell lines in a 6:1 E/T ratio. (**B**) Percentage of $CD3^+CD69^+$ T cells; (**C**) percentage of $CD8^+CD137^+$ T cells; (**D**) mean fluorescent intensity (MFI) of $CD25^+$ T cells; (**E**) percentage of $CD4^+CD154^+$ T cells after co-culture. Shown are mean values ±SD (symbols indicate individual donors; $n = 5$). (**F**) Cytokine analysis of co-culture supernatants harvested after G_{D2}CAR-specific activation of human $CD4^+$/$CD8^+$ T cells transduced with "all-in-one" vector constructs. Cytokine concentrations were determined after 48 h co-culture experiments of transduced primary T cells stimulated with indicated target cells in a 6:1 E/T ratio using a customized LEDGENDplex™ Multi-Analyte Flow Assay (BioLegend), which allowed simultaneous detection of human IL12, tumor necrosis factor (TNF)α, Interferon (IFN)γ, granzyme B, IL2, and perforin. (**G**) Detection of human IL18. Shown are mean values ±SD (symbols indicate individual donors; $n = 5$).

To demonstrate the biological activity of G_{D2}CAR-induced cytokines with respect to the recruitment of additional immune cells, we used a modified Boyden chamber assay to compare the migration potential of cell supernatants collected from co-culture experiments of transduced primary T cells and GD2$^+$ target cells (HT1080 GD2) to promote the recruitment of monocytes. The capacity of IL12 and IL18 containing supernatants to recruit the monocytic cell line MONO-MAC-6 (MM6) was shown by positive Giemsa staining of the transwell membrane through which migrating cells were recruited (Figure 7A). Giemsa staining of migrated monocytes revealed that greater numbers of MM6 cells were recruited by supernatants collected from "all-in-one" NFATsyn.hIL12-G_{D2}CAR and NFATsyn.hIL18-G_{D2}CAR transduced T cells co-cultured with HT1080 GD2 target cells as a result of induced cytokine secretion (IL12 and IL18). Moreover, supernatants from NFATsyn.hIL12-G_{D2}CAR transduced T cells showed a statistically significant effect for improved monocyte recruitment. Supernatants from NFATsyn.hIL18-G_{D2}CAR transduced T cells (Figure 7B) indicated a trend for a better recruitment potential. Taken together, supernatants from T cell subsets expressing "all-in-one" constructs designed to deliver cytokines in an inducible fashion upon CAR activation by antigen recognition exhibited an enhanced immune cell recruitment potential in vitro.

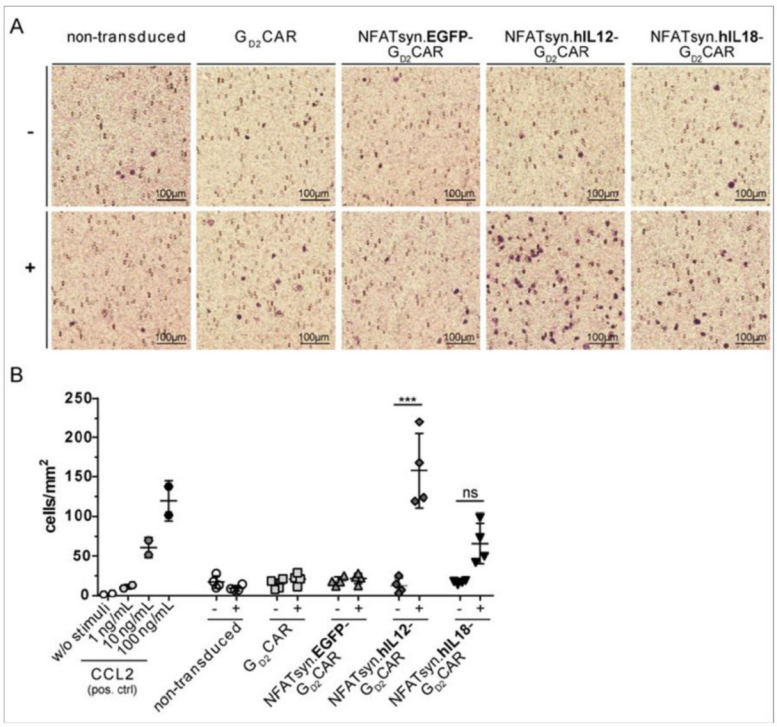


Figure 7. "All-in-one" NFATsyn.IL12-G_{D2}CAR and NFATsyn.IL18-G_{D2}CAR vector constructs exhibit higher recruitment potential of monocyte cell line MONO-MAC-6 (MM6) shown in a cell migration assay. Cell culture supernatants from co-culture experiments performed with "all-in-one" vector/G_{D2}CAR constructs transduced primary T cells (sorted) and GD2$^+$ target cells (E/T 6:1) were collected after 48 h and used in a cell migration assay with modified Boyden chambers and 5×10^5 MM6 cells/well. Supernatants from cells w/o stimulation served as controls. **(A)** Membranes were fixed and Giemsa stained after 4 h incubation. (**B**) Graph summarizes four independent experiments (each performed in duplicates) of migrated cells influenced by the different cell culture supernatants of the "all-in-one" constructs harvested after co-culture with (+) or without (−) HT1080 GD2$^+$ target cells. Different concentrations of C-C motifchemokine ligand 2 (CCL2) were used as positive control. Shown are mean values ± SEM. Indicated significance was determined by one-way ANOVA with Tukey's multiple comparison test; *** $p \leq 0.001$; ns > 0.05.

3. Discussion

We describe a method to generate TRUCKs using an "all-in-one" vector system for targeted adoptive cell therapy that combines constitutive CAR expression and inducible cytokine secretion. The system is therapeutically relevant, especially with regard to the treatment of solid tumors, as it redirects T cells to the target cells through the scFv of the constitutively expressed CAR and allows NFAT-driven cytokine induction only upon tumor target cell binding. This allows TRUCK T cells to shape the local tumor microenvironment and create a pro-inflammatory milieu by recruiting additional innate immune cells to help further eliminate the tumor. Previously published concepts of CAR T cells that secrete additional effector molecules are mostly based on transduction protocols that involve the delivery of two-vectors [19,38]. Recently, Liu et al. described a one-vector system for inducible cytokine expression driven by an NFAT promoter element with six NFAT response elements that is activated following activation of a constitutively expressed Glypican-3 (GP3)-CAR, and they nicely demonstrated the functionality of their one-vector system in in vivo mouse models for hepatocellular carcinoma (HCC) [21]. Similar to the one-vector system by Liu et al., the vector system described here offers a versatile cell modification strategy. In contrast to Liu et al., we used a different vector configuration for our "all-in-one" approach and basically focused on the establishment of an "all-in-one" vector system to further develop and improve the TRUCK concept. Moreover, the modular one-vector design that we describe here allows the easy exchange of the different cassettes, and thus facilitates adaptation for application in various settings. Transduction efficiencies of about 30–60% in primary human T cells were achieved with low MOIs. This was obtained after the introduction of a CMV promoter into the 5'LTR region of lentiviral transfer plasmids. This modification enabled the production of lentiviral "all-in-one" constructs with clinically applicable titers, with viral vector titers that were up to 15-fold higher than those achieved with previous vector systems containing the RSV promoter. This advance facilitates the production of high numbers of gene-modified T cells as the basis for further adoptive CAR T cell therapies. In contrast to previous procedures, only a single vector integration event is needed with this one-vector system, which also reduces the risk of insertional mutagenesis that was described as a potential side effect of retroviral gene transfer [39]. Although no severe adverse events have been reported in the context of lentiviral gene therapy studies in hematopoietic stem cells or human primary T cells, these studies did not test the safety of cells modified with more than one vector [40,41]. Furthermore, dual transductions also require two viral vectors produced under good manufacturing practices (GMPs), which is cost-intensive and laborious. Therefore, our developed "all-in-one" vector system simplifies the application of TRUCK modified T cells for tumor therapy and simplifies translation of this therapeutic concept for use by the scientific community. The tight linkage of CAR activation and cytokine secretion was also demonstrated for the "all-in-one" vector, as the induction of the desired protein of interest only occurred after G_{D2}CAR-specific activation, for example, by co-cultivation of the "all-in-one" transduced CAR T cells with $GD2^+$ target cells, which resulted in NFAT promoter-driven cytokine induction and secretion. This is not the case for CAR T cells that constitutively express an immune modulatory cytokine via an IRES or 2A site.

Stably integrated reporter cell lines for transgene expression harboring activation-inducible promoters were already described for functional testing of T or natural killer (NK) cell activation, as well as in the context of CAR T and NK cell therapy [42,43]. These reporters are mostly based on NFAT-, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB)-, and activator protein (AP)-1-based promoters, as activation pathways in T and NK cells are mediated by these transcription factors. Several studies used reporter constructs driven by NFAT response elements to analyze activation and signal transduction pathways in primary T cells [43,44]. In this regard, synthetic NFAT promoters were previously used for inducible transgene expression [13,19], but mainly in a separate vector that was transduced in addition to the CAR vector system. Here, we compared two different inducible NFAT-based promoters fused to the transgene of interest within the lentiviral "all-in-one" vector system in primary human T cells to improve the TRUCK concept. These promoters both included six NFAT response consensus enhancer elements, but differed in their fused core promoters

and TATA boxes, namely the minimal IL2 promoter and the synthetically designed core promoter plus TATA box, which was limited to sequences essential for expression [36,37]. Although both promoters were able to specifically induce transgene expression after CAR-specific activation, the NFATsyn promoter more potently induced transgene expression with significantly higher levels of EGFP, hIL12, or hIL18 production after CAR-specific activation in primary human T cells. These data indicated that, in addition to the number of response elements, the choice of the fused promoter is also of major importance for high-level inducible transgene expression. Moreover, the percentage of modified cells and the transgene expression levels could be titrated to the needed extent by modulation of the promoter. However, cell-type dependency and the feasibility of tight induction would need to be evaluated for further use of "all-in-one" vectors in other therapeutic cell types.

The pro-inflammatory cytokines IL12 and IL18 were studied in the context of our "all-in-one" vector system as they are promising therapeutic candidates for the treatment of solid tumors. IL12 has been explored to treat several malignancies in preclinical models and clinical applications, including melanoma and ovarian cancer. However, several cytokine-related toxicities were described and represent one of the main limitations of systemic delivery or constitutive expression via retroviral vectors in tumor therapy [15,45,46]. The "all-in-one" vector system represents a potential solution to overcome these toxicities as the inducible cytokine secretion is tightly linked to the activation of the CAR T cell, and thus ensures local and temporal cytokine expression only at the tumor site. One important aspect that needs to be addressed, but is difficult to evaluate, is a maximal baseline-expression level of the applied cytokines to allow local therapeutic effects without systemic side effects. We observed that the NFATsyn promoter provides a combination of high induction and low-baseline expression levels. However, most CAR T cell-targeted antigens expressed on solid tumors are not exclusively expressed on tumors, but are also present in normal tissues, which might result in unwanted "on target/off tumor" toxicities [10]. Thus, low-baseline expression without CAR signaling is clearly of benefit in this respect.

The G_{D2}CAR-specific activation after stimulation with $GD2^+$ target cells resulted in the upregulation of T cell-specific activation markers, without the additional increase of activation markers observed in T cells containing an inducible cytokine cassette. The effect of the induced IL12 cytokine expression in target antigen-activated NFATsyn.hIL12-G_{D2}CAR transduced T cells was also indicated by increased expression levels of several pro-inflammatory cytokines released by the engineered T cells. This underlines the potential benefit of the designed construct to recruit additional immune cells. The described benefit of the additional cytokine secretion by T cells was also observed in the in vitro cell migration assays with MM6 cells. The resulting cytokine release recruited monocytes, and thus provided proof-of-concept that our system could contribute to the creation of a pro-inflammatory environment in the treatment of solid tumors. These promising in vitro results should be confirmed in appropriate in vivo models to further address the pro-inflammatory potential of the anticancer cytokines to combat solid tumors. Thus, T cells transduced with "all-in-one" constructs should be tested in a humanized, clinically relevant mouse model, to determine their in vivo potency regarding attraction and recruitment of additional immune cells and long-term cytotoxic effects of the secreted cytokines.

Another potential application for the "all-in-one" constructs is to screen CARs and TCRs in reporter assays [42–44] by displaying CAR libraries, including different scFvs, hinge regions, and signaling domains directly in primary human immune cells. The CAR function can be directly linked to the NFAT promoter-driven iGOI expression or cytokine secretion and can be investigated after target antigen recognition. This allows the investigation of CAR specificity, functionality, efficacy, and dose-dependent cytokine secretion studies in one molecule.

The described vector system combines the advantage to exchange different modules of the construct to facilitate manufacturing for clinical application and reduce the risk of adverse events like insertional mutagenesis. Thus, the NFAT-promoter, as well as the inducible gene of interest, could be easily replaced by other promoters, reporters, and cytokines to adapt the "all-in-one" vector construct to other immune cell-based adoptive therapies. Adaptation to tumor therapies using checkpoint

inhibitors is also conceivable [47,48]. Alternatively, the "all-in-one" construct technology can be used for the application of anti-inflammatory therapies, for example, autoimmune diseases or transplantation studies. In this regard, this system could be applied to genetically manipulate regulatory T cells to express anti-inflammatory cytokines, like IL10 or TGF-β [49]. Hence, the "all-in-one" system offers a useful technology for various future therapeutic purposes, especially with regard to improved viral vector design and viral vector particle generation, and is transferable to other immune cells, vector systems, and cytokines.

4. Materials and Methods

4.1. Human Material

All experiments were performed with residual blood samples from platelet (PLT) apheresis disposables used for routine PLT collection and from regular anonymous healthy donors. As a requirement for donations, the respective donors had no signs of acute infection or a previous history of blood transfusion. The studies were approved by the local ethics committee (approval number 2519-2014).

4.2. Cell Lines

293T cells (#ACC 635; DSMZ, Brunswick, Germany) were used for lentiviral production and cultured in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate (all PAN Biotech, Aidenbach, Germany). In functionality and cytotoxicity experiments, the fibrosarcoma cell line HT1080 (#ACC 315; DSMZ), the HT1080 equipped with GD2 by retroviral transduction of a GD3S/GD2S transgene, and the established neuroblastoma cell line SH-SY5Y were used. All cell lines were cultivated similarly in DMEM. Primary glioblastoma samples were obtained from glioblastoma tumor resections after receiving the patient's informed consent (Nordstadt Cerebral Cancer Study (NoCCa-Study) Register- Nr. 6864). Cells were isolated and cultivated until further usage according to Hasselbach et al. [50] with modifications. In brief, the isolated spheroids were cultivated in DMEM/F12 (Gibco, Karlsruhe, Germany) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, N2 supplement (1×) (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany), 0.5 mg/mL bovine serum albumin (BSA) (PAN Biotech), 25 µg/mL gentamicin (Gibco), 20 ng/mL human basic fibroblast growth factor (bFGF), and human epidermal growth factor (EGF) (both PeproTech, Hamburg, Germany) in low-attachment flasks (Corning, Wiesbaden, Germany) until further usage. Primary T cell cultures and T cell-related assays were performed in Roswell Park Memorial Institute (RPMI) 1640 (PAN Biotech), supplemented with 10% FBS (PAN Biotech), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Darmstadt, Germany), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (PAN Biotech), 1% Minimal Essential Medium (MEM) non-essential amino acid solution (Gibco), 1% sodium pyruvate (PAN Biotech), and 100 IU/mL human IL-2 (ProLeukin S, Clinigen Healthcare B.V.). For some experiments (Figure 6, Figure 7 and Figure S4), T cell assays were performed in Tex magnetic activated cell sorting (TexMACS) medium (Miltenyi) supplemented with 3% human AB serum (c.c.pro, Oberdorla, Germany), and 12.5 ng/mL human IL-7 and human IL-15 (PeproTech, Rocky Hill, CT, USA) in humidified incubators at 37 °C and 5% CO_2.

4.3. Cloning of "All-in-One" Vector Constructs, Production, and Titration of Viral Supernatants

For the generation of NFATsyn.EGFP.GD2-CAR (pRRL.PPT.NFATenh.synTATA.EGFP. PGK. newMCS.GD2CAR.PRE), multiple cloning steps were performed. In brief, the synthesized NFATenh. synTATA.EGFP expression cassette (Thermo Fisher Scientific, Regensburg, Germany) was designed according to Merlet et al. [37], slightly modified, and individual modules (NFATenhancer, synTATA, and EGFP) were equipped with unique restriction sites (Figure 2A). After verification of the sequence by

Sanger sequencing, NFATenh.synTATA.EGFP was removed from the shuttle vector via XhoI and EcoRI and introduced into pRRL.PPT.PGK.newMCS.EBFP2.i2.Zeo.PRE via a three-fragment ligation using 529 bp *EcoRI*/*Age*I and 7871 bp *Age*I/*Xho*I fragments of pRRL.PPT.PGK.newMCS.EBFP2.i2.Zeo.pre. Subsequently, the EBFP2.i2.Zeo cassette of the resulting pRRL.PPT.NFATenh.synTATA [37]. EGFP.PGK.EBFP2.i2.Zeo.PRE plasmid was replaced by AgeI and SalI with the open reading frame (ORF) of the G_{D2}-CAR that was optimized for human codon-usage. For the generation of the NFATmIL2.EGFP.GD2-CAR, the 6xNFAT-mIL2 promoter plus EGFP cDNA was amplified from previously published construct SIN-$(NFAT)_6$-GFP [36] via PCR with 5′ flanking *Sal*I + *Afe*I restriction sites and 3′ flanking *EcoR*I + *Nhe*I restriction sites. The amplified and sequenced fragment was cloned via three-fragment ligation into vector pRRL.PPT.PGK.newMCS.EBFP2.i2.Zeo.PRE using the *Xho*I and *Nhe*I sites between the cPPT and the hPGK promoter sequences. The human codon-usage optimized ORF second generation CAR containing scFv (14.G2a), HCH_2CH_3 hinge, CD28, CD137 (4-1BB), and CD3ζ domains [25] was flanked by the restriction enzymes *Age*I and *Sal*I were cloned into the lentiviral "all-in-one" SIN vector driven by an hPGK promoter. The restriction sites *Mlu*I and *EcoR*I, or *Afe*I and *EcoR*I, respectively, were inserted to exchange the iGOI within the "all-in-one" vector construct (Figure 2A). To generate the lentiviral "all-in-one" SIN vector driven by 5′ CMV promoter, the fusion of the inducible element driven by NFAT, the constitutive CAR, and lentiviral backbone was obtained using *Not*I, *Age*I, and *Sca*I restriction sites. Constructs were validated by sequencing (Microsynth SeqLab, Germany). Cloning details and sequences are available on request.

Lentiviral particles were generated as described previously [51]. In brief, 5×10^6 293T cells were seeded. The transfection was performed using the calcium phosphate method in the presence of 25 μM chloroquine with the following plasmids: lentiviral vector plasmid (10 μg), pcDNA3.HIV-1.GP.4 × CTE (lentiviral gag/pol) (12 μg) [52], pRSV-Rev (5 μg; kindly provided by T. Hope, Northwestern University Chicago, IL), and VSVg-encoding pMD.G (1.5 μg) [53]. For better standardization, pcDNA3.HIV-1.GP.4×CTE, pRSV-Rev and pMD.G were produced and purified by PlasmidFactory (Bielefeld, Germany). Supernatants were harvested 36 h and 48 h after transfection and concentrated via ultracentrifugation at 4 °C and 13,238× *g* or 82,740× *g* for 16 h or 2 h, respectively.

Titration of viral supernatants was performed in HT1080 fibroblasts via spinoculation-mediated transduction (4.4.). Transduction efficiency was determined three days post transduction via flow cytometric staining of G_{D2}CAR expression. Functional viral vector titers were calculated from samples with G_{D2}CAR expression percentages of ≤30% to avoid cells with multiple integrations [54].

4.4. Transduction of Cell Lines and Primary T Cells

For spinoculation-mediated transduction of HT1080 fibroblasts, 1×10^5 cells were seeded in a 24-well plate. Supernatants containing viral particles and 4 μg/mL protamine sulfate (Sigma-Aldrich) were added and cells were centrifuged (1 h, 800× *g*, 37 °C). The NFAT-GFP hybridoma cell line was transduced identically. Primary human T cells were isolated by magnetic activated cell sorting (MACS) from human peripheral blood mononuclear cells (PBMCs) previously isolated via density centrifugation. Subsequently, isolated T cells were activated for 48 h prior to transduction using T Cell TransAct™ (αCD3/CD28 beads, Miltenyi) in complete RPMI1640 medium supplemented with 100 U/mL human IL-2. After activation, human T cells were transduced with the lentiviral constructs using Retronectin (Takara, Kusatsu, Japan). In brief, 48-well plates were coated with Retronectin (137 μL of 24 mg/mL in PBS) overnight, blocked with sterile-filtered PBS containing 2% BSA for 30 min, and washed with Hank's Balanced Salt Solution (HBSS)/HEPES. Wells were loaded with lentiviral supernatants at the desired multiplicity of infection (MOI 3 or 10) by centrifugation for 30 min, 400× *g*, 4 °C, followed by adding 2.5×10^5 T cells per well [55]. The transduction efficiency was analyzed 6–7 days post transduction by flow cytometry after staining the scFv with the Ganglidiomab-phycoerythrin (PE) mAb (kindly provided by H. Lode and N. Siebert and PE-conjugated) [56,57]. For some analyses, the transduced T cells were isolated via FACS-based cell sorting. This is indicated in the respective figure legends. For some experiments (Figure 6, Figure 7 and Figure S4), T cells were activated for 24 h

with αCD3/CD28 beads (Miltenyi) and transduced by centrifugation for 45 min, 800× *g*, 32 °C with lentiviral constructs using polybrene (5 μg/mL) (Merck Millipore, Burlington, VT, USA) in complete TexMACS medium.

4.5. Hybridoma Co-Culture Assay

The NFAT-GFP hybridoma cell line (kindly provided by Ludger Klein (TU Munich, city, Germany)) encodes an EGFP under the control of an NFAT-dependent IL-2 promoter [58]. All hybridoma cultures and hybridoma-related assays were performed in DMEM supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. The hybridoma cells were transduced with the lentiviral G_{D2}CAR constructs by spinoculation (800× *g*, 1 h, 37 °C) in the presence of protamine sulfate. Transduction efficiencies were analyzed via flow cytometric staining 48 h post transduction. For functional tests, HT1080 and HT1080 GD2 cells were used as target cells. The co-culture assay was performed for 20 h with G_{D2}CAR-transduced hybridoma cells with a 4:1 effector/target ratio (E/T) and analyzed via flow cytometric staining.

4.6. Flow Cytometric Analysis

Flow cytometry surface staining of T cell phenotype and activation markers were performed after co-culture experiments using the following anti-human antibodies CD3 (OKT3, eBioscience, San Diego, CA, USA), CD3 (OKT3, SK7), CD4 (RPA-T4), CD8 (SK1), CD69 (FN50), CD137 (4B4-1), CD25 (BC96), and CD154 (24-31) (all BioLegend, San Diego, CA, USA). GD2 expression on target cells was analyzed via an anti-human Ganglioside GD2 antibody (BioLegend).

4.7. Cytotoxicity Assay

Cell-mediated cytotoxicity of primary T cells against GD2-expressing target cells (HT1080 GD2, SH-SY5Y neuroblastoma cell line) was determined via measuring the release of lactate dehydrogenase (LDH) with the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific, Bremen, Germany) using co-culture supernatants according to the manufacturer's instructions. Briefly, the indicated E/T ratio and appropriate controls (volume controls and maximum controls according to manufacturer's protocol) were seeded in a 96-well plate. After 48 h of co-culture, 50 μL of the cell-free co-culture supernatants was transferred to another 96-well plate and incubated with 50 μL provided reaction mix for 30 min at room temperature, and then it was stopped by adding the provided stop solution. To determine LDH activity, absorbance at 490 nm and 680 nm was measured (SPECTRAmax 340PC, Molecular DEVICES, Software SOFTmax PRO 4.0) and subtracted (490 nm–680 nm), respectively. The % cytotoxicity was calculated according to the manufacturer's protocol.

4.8. Cytokine Analysis by ELISA and LEGENDplex

Cell culture supernatants from primary T cells cultured alone or after indicated time points of co-cultures with HT1080, HT1080 GD2, SH-SY5Y, or primary glioblastoma cells were collected and cytokine concentrations were determined using the Human IL-12 (p70) ELISA MAX™ Deluxe Set (BioLegend) and the Human Total IL-18 DuoSet® ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured with a SPECTRAmax 340PC plate reader (Molecular DEVICES, Biberach an der Riss, Germany, Software SOFTmax PRO 4.0). In addition, supernatants from in vitro co-cultures were analyzed using a customized LEGENDplex™ Multi-Analyte Flow Assay (BioLegend), which allowed for the simultaneous detection of human IL2, IL10, IL12 (p70), IL18, granzyme B, IFNγ, perforin, and TNFα. The assay was performed according to the manufacturer's instructions. Briefly, undiluted samples and standards were incubated with antibody-coated premixed beads overnight at 4 °C followed by incubation with detection antibody for 1 h at room temperature and streptavidin-PE for 30 min at room temperature. Samples were acquired on a BD FACSCanto™ Flow Cytometer (BD Biosciences, Heidelberg, Germany) and analyzed with the LEGENDplex v8.0 Software (BioLegend).

4.9. Cell Migration Assay

To test the chemoattractive potential of supernatants that were derived from co-culture experiments of primary human T cells transduced with the indicated lentiviral "all-in-one" constructs and $GD2^+$ target cells, the migration assay with MONO-MAC-6 monocytes (MM6 cells) [59] was performed using a Boyden chamber (NeuroProbe, Gaithersburg, MD, USA) [60]. In brief, a 96-well plate (Sarstedt, flat bottom) was first filled with 300 µL/Agar per well. Then, 110 µL supernatant of co-culture experiments (different concentrations of the CC-chemokine ligand CCL2 were used as positive controls) were added per well and the plate was placed in the Boyden chamber using a spacer. Afterward, a polycarbonate membrane (8 µm pore size, NeuroProbe) was placed over the plate and the chamber was closed. Subsequently, 200 µL MM6 monocytes (5×10^5 cells/well) were added to the upper chamber. The entire chamber was incubated for 4 h at 37 °C and 5% CO_2. The membrane was removed and cells attached to the top of the membrane were removed with a scraper. Migrated cells attached to the lower side and inside the membrane were analyzed by fixing and staining the membrane with methanol for 10 min (Roth, Karlsruhe, Germany) and Giemsa for 1 h, respectively. The cell number was calculated using an Olympus IX71 microscope.

4.10. Microscopy

Brightfield images and NFAT-induced EGFP expression in primary T cells after co-cultures with patient-derived glioblastoma spheroids were analyzed and performed using an AxioObserver microscope (ZEISS, Jena, Germany) and AxioVision Software Rel. 4.8. Scale bars are indicated in each figure.

4.11. Statistical Analysis

Statistical analyses were performed with the help of GraphPad Prism version 6.0 (GraphPad software, San Diego, CA, USA). One-way ANOVA and two-way ANOVA tests with Tukey's or Bonferroni's multiple comparison test and unpaired two-tailed *t*-test; *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns > 0.05 were used. Analyses and *p*-values are also specified in the figure legends.

5. Conclusions

In conclusion, the described "all-in-one" vector concept with its modular design and its combination of constitutive CAR expression and inducible NFAT-driven transgene expression provides a useful vector concept for targeted gene transfer for adoptive gene therapies, especially regarding easy vector transfer and clinical application. Thus, with the "all-in-one" vector design, improved vector titer capacities, and superior inducibility, our system will be useful for the scientific community as it is suitable for various manufacturing processes for therapeutic purposes. Moreover, the system will be applicable to any other settings in which expression of one constitutive and one inducible gene cassette is desirable.

Supplementary Materials: The following supplementary figures are available online at http://www.mdpi.com/2072-6694/12/2/375/s1, [Note to journal staff: Please add as correct], Figure S1. Co-culture of transduced T cells with $GD2^-$ target cells led to a moderate EGFP upregulation, Figure S2. Human IL12 and human IL18 secretion after stimulation with $GD2^-$ target cells, Figure S3. Inducible NFATmIL2-driven human IL12 and human IL18 secretion after CAR-specific stimulation, Figure S4. Cytokine analysis after G_{D2}CAR-specific activation in human T cells transduced with "all-in-one" vector constructs.

Author Contributions: Conceptualization, K.Z., J.K., H.A., and A.S.; methodology, K.Z., J.K., A.C.D., M.G., C.K., L.S.R., and B.E.-V.; formal analysis, K.Z., M.A.M., A.S., B.E.-V., H.A., T.M., and J.M.; resources, C.R., H.A., I.E.S., B.N., B.E.-V., B.A., M.G., M.A.M., and A.S.; writing-original draft preparation, K.Z. and A.S.; writing-review and editing, all authors; supervision, A.S. and H.A.; funding acquisition, A.S., T.M., B.E.-V., C.R., and H.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the following grants "From CARs to TRUCKs" (Krebshilfe/German Cancer Aid-Priority Program in Translational Oncology) and DFG-funded SFB738 (projects C4 & C9) and Cluster of Excellence REBIRTH (EXC62/2).

Acknowledgments: The authors thank Ludger Klein, Institute for Immunology, Biomedical Center Munich (BMC), Ludwig Maximilians Universität, Planegg-Martinsried, Germany for providing us the NFAT-GFP hybridoma reporter cell line. The authors also thank Holger Lode and Nikolai Siebert from the Department of Pediatric Oncology and Hematology, University Medicine Greifswald (Germany) as well as Ulrike Köhl and Wolfgang Glienke, Institute of Cellular Therapeutics, Integrated Research and Treatment Center Transplantation, Hannover Medical School (MHH), Germany for providing us the Ganglidiomab-PE antibody for G_{D2}CAR-specific staining as well as helpful suggestions and advice. Additionally, the authors thank Karina Müller-Wallat for excellent cooperation in providing us the patient-derived glioblastoma cells. The authors thank Markus Chmielewski for advice regarding cloning. Moreover, the authors thank Artur Zimmermann, Daniela Lenz, and Daniela Paasch for technical support and Matthias Ballmeier from the core facility cell sorting, MHH, Germany for FACS-based cell sorting of transduced T cells.

Conflicts of Interest: The authors declare no conflict of interest, except that authors H.A., A.S., J.K., and K.Z. have submitted a patent application describing the "all-in-one" TRUCK vector technology.

References

1. Dotti, G.; Gottschalk, S.; Savoldo, B.; Brenner, M.K. Design and development of therapies using chimeric antigen receptor-expressing T cells. *Immunol. Rev.* **2014**, *257*, 107–126. [CrossRef]
2. Maus, M.V.; June, C.H. Making Better Chimeric Antigen Receptors for Adoptive T-cell Therapy. *Clin. Cancer Res.: Off. J. Am. Assoc. Cancer Res.* **2016**, *22*, 1875–1884. [CrossRef]
3. Porter, D.L.; Levine, B.L.; Kalos, M.; Bagg, A.; June, C.H. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *New Engl. J. Med.* **2011**, *365*, 725–733. [CrossRef] [PubMed]
4. Axicabtagene ciloleucel (Yescarta) for B-cell lymphoma. *Med. Lett. Drugs Ther.* **2018**, *60*, e122–e123.
5. Bach, P.B.; Giralt, S.A.; Saltz, L.B. FDA Approval of Tisagenlecleucel: Promise and Complexities of a $475000 Cancer Drug. *Jama* **2017**, *318*, 1861–1862. [CrossRef] [PubMed]
6. Brentjens, R.J.; Davila, M.L.; Riviere, I.; Park, J.; Wang, X.; Cowell, L.G.; Bartido, S.; Stefanski, J.; Taylor, C.; Olszewska, M.; et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci. Transl. Med.* **2013**, *5*, 177ra38. [CrossRef] [PubMed]
7. Brudno, J.N.; Somerville, R.P.; Shi, V.; Rose, J.J.; Halverson, D.C.; Fowler, D.H.; Gea-Banacloche, J.C.; Pavletic, S.Z.; Hickstein, D.D.; Lu, T.L.; et al. Allogeneic T Cells That Express an Anti-CD19 Chimeric Antigen Receptor Induce Remissions of B-Cell Malignancies That Progress After Allogeneic Hematopoietic Stem-Cell Transplantation Without Causing Graft-Versus-Host Disease. *J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol.* **2016**, *34*, 1112–1121. [CrossRef]
8. Kochenderfer, J.N.; Somerville, R.P.T.; Lu, T.; Yang, J.C.; Sherry, R.M.; Feldman, S.A.; McIntyre, L.; Bot, A.; Rossi, J.; Lam, N.; et al. Long-Duration Complete Remissions of Diffuse Large B Cell Lymphoma after Anti-CD19 Chimeric Antigen Receptor T Cell Therapy. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2017**, *25*, 2245–2253. [CrossRef]
9. Abken, H. Driving CARs on the Highway to Solid Cancer: Some Considerations on the Adoptive Therapy with CAR T Cells. *Hum. Gene Ther.* **2017**, *28*, 1047–1060. [CrossRef]
10. Mata, M.; Gottschalk, S. Engineering for Success: Approaches to Improve Chimeric Antigen Receptor T Cell Therapy for Solid Tumors. *Drugs* **2019**, *79*, 401–415. [CrossRef]
11. Morgan, M.A.; Schambach, A. Engineering CAR-T Cells for Improved Function Against Solid Tumors. *Front. Immunol.* **2018**, *9*, e2493. [CrossRef]
12. Morgan, R.A.; Yang, J.C.; Kitano, M.; Dudley, M.E.; Laurencot, C.M.; Rosenberg, S.A. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2010**, *18*, 843–851. [CrossRef] [PubMed]
13. Chinnasamy, D.; Yu, Z.; Kerkar, S.P.; Zhang, L.; Morgan, R.A.; Restifo, N.P.; Rosenberg, S.A. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. *Clin. Cancer Res.: Off. J. Am. Assoc. Cancer Res.* **2012**, *18*, 1672–1683. [CrossRef] [PubMed]
14. Koneru, M.; Purdon, T.J.; Spriggs, D.; Koneru, S.; Brentjens, R.J. IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo. *Oncoimmunology* **2015**, *4*, e994446. [CrossRef] [PubMed]

15. Zhang, L.; Morgan, R.A.; Beane, J.D.; Zheng, Z.; Dudley, M.E.; Kassim, S.H.; Nahvi, A.V.; Ngo, L.T.; Sherry, R.M.; Phan, G.Q.; et al. Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. *Clin. Cancer Res.: Off. J. Am. Assoc. Cancer Res.* **2015**, *21*, 2278–2288. [CrossRef] [PubMed]
16. Lacy, M.Q.; Jacobus, S.; Blood, E.A.; Kay, N.E.; Rajkumar, S.V.; Greipp, P.R. Phase II study of interleukin-12 for treatment of plateau phase multiple myeloma (E1A96): A trial of the Eastern Cooperative Oncology Group. *Leuk. Res.* **2009**, *33*, 1485–1489. [CrossRef] [PubMed]
17. Chmielewski, M.; Abken, H. CAR T cells transform to trucks: Chimeric antigen receptor-redirected T cells engineered to deliver inducible IL-12 modulate the tumour stroma to combat cancer. *Cancer Immunol. Immunother.* **2012**, *61*, 1269–1277. [CrossRef]
18. Chmielewski, M.; Abken, H. CAR T Cells Releasing IL-18 Convert to T-Bet(high) FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell Rep.* **2017**, *21*, 3205–3219. [CrossRef]
19. Chmielewski, M.; Kopecky, C.; Hombach, A.A.; Abken, H. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Res.* **2011**, *71*, 5697–5706. [CrossRef]
20. Kunert, A.; Chmielewski, M.; Wijers, R.; Berrevoets, C.; Abken, H.; Debets, R. Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors. *Oncoimmunology* **2017**, *7*, e1378842. [CrossRef]
21. Liu, Y.; Di, S.; Shi, B.; Zhang, H.; Wang, Y.; Wu, X.; Luo, H.; Wang, H.; Li, Z.; Jiang, H. Armored Inducible Expression of IL-12 Enhances Antitumor Activity of Glypican-3–Targeted Chimeric Antigen Receptor–Engineered T Cells in Hepatocellular Carcinoma. *J. Immunol.* **2019**, *203*, 198–207. [CrossRef] [PubMed]
22. Unutmaz, D.; Carroll, R.G.; Carpenito, C.; Shan, X.; Danet-Desnoyers, G.; Liu, R.; Jiang, S.; Albelda, S.M.; Golovina, T.; Coukos, G.; et al. Distinct Effects of IL-18 on the Engraftment and Function of Human Effector CD8+ T Cells and Regulatory T Cells. *PLoS ONE* **2008**, *3*, e3289. [CrossRef]
23. Hu, B.; Ren, J.; Luo, Y.; Keith, B.; Young, R.M.; Scholler, J.; Zhao, Y.; June, C.H. Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18. *Cell Rep.* **2017**, *20*, 3025–3033. [CrossRef] [PubMed]
24. Avanzi, M.P.; Yeku, O.; Li, X.; Wijewarnasuriya, D.P.; van Leeuwen, D.G.; Cheung, K.; Park, H.; Purdon, T.J.; Daniyan, A.F.; Spitzer, M.H.; et al. Engineered Tumor-Targeted T Cells Mediate Enhanced Anti-Tumor Efficacy Both Directly and through Activation of the Endogenous Immune System. *Cell Rep.* **2018**, *23*, 2130–2141. [CrossRef] [PubMed]
25. Kailayangiri, S.; Altvater, B.; Lesch, S.; Balbach, S.; Gottlich, C.; Kuhnemundt, J.; Mikesch, J.H.; Schelhaas, S.; Jamitzky, S.; Meltzer, J.; et al. EZH2 Inhibition in Ewing Sarcoma Upregulates GD2 Expression for Targeting with Gene-Modified T Cells. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2019**, *27*, 933–946. [CrossRef] [PubMed]
26. Golinelli, G.; Grisendi, G.; Prapa, M.; Bestagno, M.; Spano, C.; Rossignoli, F.; Bambi, F.; Sardi, I.; Cellini, M.; Horwitz, E.M.; et al. Targeting GD2-positive glioblastoma by chimeric antigen receptor empowered mesenchymal progenitors. *Cancer Gene Ther.* **2018**. [CrossRef]
27. Johnsen, J.I.; Dyberg, C.; Wickstrom, M. Neuroblastoma-A Neural Crest Derived Embryonal Malignancy. *Front. Mol. Neurosci.* **2019**, *12*, e9. [CrossRef]
28. Kailayangiri, S.; Altvater, B.; Meltzer, J.; Pscherer, S.; Luecke, A.; Dierkes, C.; Titze, U.; Leuchte, K.; Landmeier, S.; Hotfilder, M.; et al. The ganglioside antigen GD2 is surface-expressed in Ewing sarcoma and allows for MHC-independent immune targeting. *Br. J. Cancer* **2012**, *106*, 1123–1133. [CrossRef]
29. Heczey, A.; Louis, C.U.; Savoldo, B.; Dakhova, O.; Durett, A.; Grilley, B.; Liu, H.; Wu, M.F.; Mei, Z.; Gee, A.; et al. CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2017**, *25*, 2214–2224. [CrossRef]
30. Louis, C.U.; Savoldo, B.; Dotti, G.; Pule, M.A.; Yvon, E.; Myers, G.D.; Rossig, C.; Russell, H.V.; Diouf, O.; Liu, E.; et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* **2011**, *118*, 6050–6056. [CrossRef]
31. Pule, M.A.; Savoldo, B.; Myers, G.D.; Rossig, C.; Russell, H.V.; Dotti, G.; Huls, M.H.; Liu, E.; Gee, A.P.; Mei, Z.; et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: Persistence and antitumor activity in individuals with neuroblastoma. *Nature Med.* **2008**, *14*, 1264–1270. [CrossRef] [PubMed]

32. Maude, S.L.; Frey, N.; Shaw, P.A.; Aplenc, R.; Barrett, D.M.; Bunin, N.J.; Chew, A.; Gonzalez, V.E.; Zheng, Z.; Lacey, S.F.; et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *New Engl. J. Med.* **2014**, *371*, 1507–1517. [CrossRef] [PubMed]
33. Ladenstein, R.; Pötschger, U.; Valteau-Couanet, D.; Luksch, R.; Castel, V.; Yaniv, I.; Laureys, G.; Brock, P.; Michon, J.M.; Owens, C.; et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): A multicentre, randomised, phase 3 trial. *Lancet Oncol.* **2018**, *19*, 1617–1629. [CrossRef]
34. Schambach, A.; Zychlinski, D.; Ehrnstroem, B.; Baum, C. Biosafety features of lentiviral vectors. *Hum. Gene Ther.* **2013**, *24*, 132–142. [CrossRef] [PubMed]
35. Noyan, F.; Zimmermann, K.; Hardtke-Wolenski, M.; Knoefel, A.; Schulde, E.; Geffers, R.; Hust, M.; Huehn, J.; Galla, M.; Morgan, M.; et al. Prevention of Allograft Rejection by Use of Regulatory T Cells With an MHC-Specific Chimeric Antigen Receptor. *Am. J. Transplant.: Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **2017**, *17*, 917–930. [CrossRef] [PubMed]
36. Hooijberg, E.; Bakker, A.Q.; Ruizendaal, J.J.; Spits, H. NFAT-controlled expression of GFP permits visualization and isolation of antigen-stimulated primary human T cells. *Blood* **2000**, *96*, 459–466. [CrossRef] [PubMed]
37. Merlet, E.; Lipskaia, L.; Marchand, A.; Hadri, L.; Mougenot, N.; Atassi, F.; Liang, L.; Hatem, S.N.; Hajjar, R.J.; Lompre, A.M. A calcium-sensitive promoter construct for gene therapy. *Gene Ther.* **2013**, *20*, 248–254. [CrossRef] [PubMed]
38. Yeku, O.O.; Brentjens, R.J. Armored CAR T-cells: Utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy. *Biochem. Soc. Trans.* **2016**, *44*, 412–418. [CrossRef]
39. David, R.M.; Doherty, A.T. Viral Vectors: The Road to Reducing Genotoxicity. *Toxicol. Sci.: Off. J. Soc. Toxicol.* **2017**, *155*, 315–325. [CrossRef]
40. Biasco, L.; Rothe, M.; Schott, J.W.; Schambach, A. Integrating Vectors for Gene Therapy and Clonal Tracking of Engineered Hematopoiesis. *Hematol. /Oncol. Clin. North Am.* **2017**, *31*, 737–752. [CrossRef]
41. Goswami, R.; Subramanian, G.; Silayeva, L.; Newkirk, I.; Doctor, D.; Chawla, K.; Chattopadhyay, S.; Chandra, D.; Chilukuri, N.; Betapudi, V. Gene Therapy Leaves a Vicious Cycle. *Front. Oncol.* **2019**, *9*, e297. [CrossRef] [PubMed]
42. Kulemzin, S.V.; Matvienko, D.A.; Sabirov, A.H.; Sokratyan, A.M.; Chernikova, D.S.; Belovezhets, T.N.; Chikaev, A.N.; Taranin, A.V.; Gorchakov, A.A. Design and analysis of stably integrated reporters for inducible transgene expression in human T cells and CAR NK-cell lines. *BMC Med Genom.* **2019**, *12*, e44. [CrossRef]
43. Jutz, S.; Leitner, J.; Schmetterer, K.; Doel-Perez, I.; Majdic, O.; Grabmeier-Pfistershammer, K.; Paster, W.; Huppa, J.B.; Steinberger, P. Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: Simultaneous measurement of NF-kappaB, NFAT and AP-1. *J. Immunol. Methods* **2016**, *430*, 10–20. [CrossRef] [PubMed]
44. Rydzek, J.; Nerreter, T.; Peng, H.; Jutz, S.; Leitner, J.; Steinberger, P.; Einsele, H.; Rader, C.; Hudecek, M. Chimeric Antigen Receptor Library Screening Using a Novel NF-kappaB/NFAT Reporter Cell Platform. *Mol. Ther.: J. Am. Soc. of Gene Ther.* **2019**, *27*, 287–299. [CrossRef] [PubMed]
45. Neelapu, S.S.; Tummala, S.; Kebriaei, P.; Wierda, W.; Gutierrez, C.; Locke, F.L.; Komanduri, K.V.; Lin, Y.; Jain, N.; Daver, N.; et al. Chimeric antigen receptor T-cell therapy-assessment and management of toxicities. *Nat. Rev. Clin. Oncol.* **2018**, *15*, 47–62. [CrossRef] [PubMed]
46. Zhang, L.; Kerkar, S.P.; Yu, Z.; Zheng, Z.; Yang, S.; Restifo, N.P.; Rosenberg, S.A.; Morgan, R.A. Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2011**, *19*, 751–759. [CrossRef]
47. Rafiq, S.; Yeku, O.O.; Jackson, H.J.; Purdon, T.J.; van Leeuwen, D.G. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. *Nat. Biotechnol.* **2018**, *36*, 847–856. [CrossRef]
48. Tray, N.; Weber, J.S.; Adams, S. Predictive Biomarkers for Checkpoint Immunotherapy: Current Status and Challenges for Clinical Application. *Cancer Immunol. Res.* **2018**, *6*, 1122–1128. [CrossRef]
49. Yi, L.; Weifan, Y.; Huan, Y. Chimeric antigen receptor-engineered regulatory T lymphocytes: Promise for immunotherapy of autoimmune disease. *Cytotherapy* **2019**, *21*, 925–934. [CrossRef]
50. Hasselbach, L.A.; Irtenkauf, S.M.; Lemke, N.W.; Nelson, K.K.; Berezovsky, A.D.; Carlton, E.T.; Transou, A.D.; Mikkelsen, T.; deCarvalho, A.C. Optimization of high grade glioma cell culture from surgical specimens for use in clinically relevant animal models and 3D immunochemistry. *J. Vis. Exp.: JoVE* **2014**, *83*, e51088. [CrossRef]

51. Klapdor, R.; Wang, S.; Hacker, U.; Buning, H.; Morgan, M.; Dork, T.; Hillemanns, P.; Schambach, A. Improved Killing of Ovarian Cancer Stem Cells by Combining a Novel Chimeric Antigen Receptor-Based Immunotherapy and Chemotherapy. *Hum. Gene Ther.* **2017**, *28*, 886–896. [CrossRef] [PubMed]
52. Schambach, A.; Bohne, J.; Chandra, S.; Will, E.; Margison, G.P.; Williams, D.A.; Baum, C. Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-methylguanine-DNA methyltransferase in hematopoietic cells. *Mol. Ther.: J. Am. Soc. Gene Ther.* **2006**, *13*, 391–400. [CrossRef] [PubMed]
53. Yang, Y.; Vanin, E.F.; Whitt, M.A.; Fornerod, M.; Zwart, R.; Schneiderman, R.D.; Grosveld, G.; Nienhuis, A.W. Inducible, High-Level production of Infectious Murine Leukemia Retroviral Vector Particles Pseudotyped with Vesicular Stomatitis Virus G Envelope Protein. *Hum. Gene Ther.* **1995**, *6*, 1203–1213. [CrossRef] [PubMed]
54. Fehse, B.; Kustikova, O.S.; Bubenheim, M.; Baum, C. Pois(s)on–it's a question of dose. *Gene Ther.* **2004**, *11*, 879–881. [CrossRef] [PubMed]
55. Suerth, J.D.; Morgan, M.A.; Kloess, S.; Heckl, D.; Neudorfl, C.; Falk, C.S.; Koehl, U.; Schambach, A. Efficient generation of gene-modified human natural killer cells via alpharetroviral vectors. *J. Mol. Med.* **2016**, *94*, 83–93. [CrossRef] [PubMed]
56. Lode, H.N.; Schmidt, M.; Seidel, D.; Huebener, N.; Brackrock, D.; Bleeke, M.; Reker, D.; Brandt, S.; Mueller, H.P.; Helm, C.; et al. Vaccination with anti-idiotype antibody ganglidiomab mediates a GD(2)-specific anti-neuroblastoma immune response. *Cancer Immunol. Immunother.* **2013**, *62*, 999–1010. [CrossRef]
57. Siebert, N.; Seidel, D.; Eger, C.; Brackrock, D.; Reker, D.; Schmidt, M.; Lode, H.N. Validated detection of anti-GD2 antibody ch14.18/CHO in serum of neuroblastoma patients using anti-idiotype antibody ganglidiomab. *J. Immunol. Methods* **2013**, *398-399*, 51–59. [CrossRef]
58. Aichinger, M.; Wu, C.; Nedjic, J.; Klein, L. Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. *J. Exp. Med.* **2013**, *210*, 287–300. [CrossRef]
59. Ziegler-Heitbrock, H.W.; Thiel, E.; Fütterer, A.; Herzog, V.; Wirtz, A.; Riethmüller, G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer* **1988**, *15*, 456–461. [CrossRef]
60. Humphries, J.; Gossage, J.A.; Modarai, B.; Burnand, K.G.; Sisson, T.H.; Murdoch, C.; Smith, A. Monocyte urokinase-type plasminogen activator up-regulation reduces thrombus size in a model of venous thrombosis. *J. Vasc. Surg.* **2009**, *50*, 1127–1134. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

3.3 GMP-compliant manufacturing of TRUCKS: CAR T cells targeting GD2 and releasing inducible IL-18

Manuscript in revision in Molecular Therapy – Methods & Clinical Development

Wolfgang Glienke[1,10,*], **Anna Christina Dragon**[2,10] , Katharina Zimmermann[3,10], Alexandra Martyniszyn-Eiben[1], Mira Mertens[1], Hinrich Abken[4], Claudia Rossig[5], Bianca Altvater[5], Krasimira Aleksandrova[6] , Lubomir Arseniev[6], Christina Kloth[3], Thomas Moritz[3], Holger N. Lode[7], Nikolai Siebert[7], Rainer Blasczyk[2], Lilia Goudeva[2], Axel Schambach[3,8], Ulrike Köhl[1,7,9,11], Britta Eiz-Vesper[2,11], and Ruth Esser[1,11]

[1]ATMP-GMP Development Unit, Institute of Cellular Therapeutics, Integrated Research and Treatment Center for Transplantation, Hannover Medical School, 30625 Hannover, Germany

[2]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, 30625 Hannover, Germany

[3]Institute of Experimental Hematology, Hannover Medical School, 30625 Hannover, Germany; Division of Hematology/Oncology,

[4]Regensburg Centre for Interventional Immunology (RCI), Department of Genetic Immunotherapy, and University Hospital Regensburg, 93053 Regensburg, Germany

[5]Department of Pediatric Hematology and Oncology, University Children's Hospital Muenster, 48149 Muenster, Germany

[6]Cellular Therapy Center, Institute of Cellular Therapeutics, Hannover Medical School, 30625 Hannover, Germany

[7]University Medicine Greifswald, Department of Pediatric Hematology and Oncology 17489 Greifswald, Germany

[8]Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

[9]Fraunhofer Institute for Cell Therapy and Immunology (IZI), Leipzig as well as Clinical Immunology, University of Leipzig 04103 Leipzig, Germany

[10]These authors contributed equally to this work.

[11]These authors share senior authorship.

* Correspondence Glienke.Wolfgang@mh-hannover.de; Tel.: +49-511-532-8211

Author contribution statement

Anna Christina Dragon generated the TRUCKs via primary T-cell transduction of the lentiviral "all-in-one" TRUCK vector construct under laboratory conditions (laboratory scale) and characterized their pre-activation and transduction efficiency in comparison to clinical-scale TRUCKs (Fig. 3, S2). She planned and performed the *in vitro* functionality testing for both, the laboratory- and clinical-scale TRUCKs. This included performance and analysis of co-culture

experiments of both TRUCKs with target cells for the assessment of activation marker expression, cytokine release and cytotoxicity (Fig. 4, 5, 6A, S3, S4, Table 1, S2), as well as analysis of the *in vitro* migration assays (Fig. 6B-D). She participated in development of the study concept, wrote parts of the manuscript, and reviewed and edited the whole manuscript.

All author contributions: W.G. performed and evaluated the clinical-scale manufacturing (Fig. 1, 2, S1, S5, S6), participated at conceptualization of the study and wrote parts of the manuscript. K.Z. generated the lentiviral vector and participated in development of the concept. A.M.-E. helped with performing and evaluating the clinical-scale manufacturing (Fig. 1, 2, S1, S5, S6) and contributed to writing parts of the manuscript. M.M. helped in the clinical-scale manufacturing. H.A., C.R., T.M. and R.B. contributed to funding acquisition. B.A. developed the G_{D2}-specific CAR. K.A. helped to analyze parameters from the clinical-scale manufacturing (Fig. 1, 2, S1, S5, S6). L.A., H.N.L. and N.S. contributed to the development of the clinical-scale manufacturing protocol. C.K. performed the migration assays (Fig. 6B-D). L.G. contributed the leukapheresis. A.S., U.K. and B.E.V. contributed to the conceptualization of the study, funding acquisition and data interpretation. R.E. performed and evaluated the clinical-scale manufacturing (Fig. 1, 2, S1, S5, S6), participated at conceptualization of the study and wrote parts of the manuscript. All authors reviewed and edited the manuscript.

The article was submitted for publication to Molecular Therapy – Methods & Clinical Development in March 2021 and is in revision since April 2021.

Glienke, W., Dragon, A.C., Zimmermann, K., Martyniszyn-Eiben, A., Mertens, M., Abken, H., Rossig, C., Altvater, B., Aleksandrova, K., Arseniev, L., Kloth, C., Moritz, T., Lode, H.N., Siebert, N., Blasczyk, R., Goudeva, L., Schambach, A., Köhl, U., Eiz-Vesper, B., and Esser, R., *GMP-compliant manufacturing of TRUCKS: CAR T cells targeting GD2 and releasing inducible IL-18*. 2021: Manuscript in revision.

Molecular Therapy Methods & Clinical Development

GMP-COMPLIANT MANUFACTURING OF TRUCKS: CAR T CELLS TARGETING GD2 AND RELEASING INDUCIBLE IL-18.

Wolfgang Glienke[1,10], Anna Christina Dragon[2,10], Katharina Zimmermann[3,10], Alexandra Martyniszyn-Eiben[1], Mira Mertens[1], Hinrich Abken[4], Claudia Rossig[5], Bianca Altvater[5], Krasimira Aleksandrova[6], Lubomir Arseniev[6], Christina Kloth[3], Thomas Moritz[3], Holger N. Lode[7], Nikolai Siebert[7], Rainer Blasczyk[2], Lilia Goudeva[2], Axel Schambach[3,8*], Ulrike Köhl[1,6,9,11], Britta Eiz-Vesper[2,11], and Ruth Esser [1,11]

1 ATMP-GMP Development Unit, Institute of Cellular Therapeutics, Integrated Research and Treatment Center for Transplantation, Hannover Medical School, 30625 Hannover, Germany glienke.wolfgang@mh-hannover.de (W.G.); esser.ruth@mh-hanover.de (R.E.); a.martyniszyn@gmx.net (A.M.E.); mertens.mira@mh-hannover.de (M.M.); 2 Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, 30625 Hannover, Germany; dragon.anna@mh-hannover.de (A.C.D.); eiz-vesper.britta@mh-hannover.de (B.E.-V.); blasczyk.rainer@mh-hannover.de (R.B.); goudeva.lilia@mh-hannover.de (L.G.)3 Institute of Experimental Hematology, Hannover Medical School, 30625 Hannover, Germany; Division of Hematology/Oncology, schambach.axel@mh-hannover.de (A.S.); zimmermann.katharina@mh-hannover.de (K.Z.); Sirus22@gmx.de (C.K.); moritz.thomas@mh-hannover.de (T.M.); 4 Regensburg Centre for Interventional Immunology (RCI), Department of Genetic Immunotherapy, and University Hospital Regensburg, 93053 Regensburg, Germany; hinrich.abken@klinik.uni-regensburg.de (H.A.); 5 Department of Pediatric Hematology and Oncology, University Children's Hospital Muenster, 48149 Muenster, Germany; rossig@ukmuenster.de (C.R.); bianca.altvater@ukmuenster.de 6 Cellular Therapy Center, Institute of Cellular Therapeutics, Hannover Medical School, 30625 Hannover, Germany; aleksandrova.krasimira@mh-hannover.de (K.A.); arseniev.lubomir@mh-hannover.de (L.A.); 7 University Medicine Greifswald, Department of Pediatric Hematology and Oncology 17475 Greifswald, Germany; lode@med.uni-greifswald.de (H.N.L.); nikolai.siebert@med.uni-greifswald.de (N.S.); 8 Boston Children's Hospital, Harvard Medical School, Boston, MA, USA; 9 Fraunhofer Institute for Cell Therapy and Immunology (IZI), Leipzig as well as Clinical Immunology, University of Leipzig 04103 Leipzig, Germany ulrike.koehl@izi.fraunhofer.de (U.K)

[10] These authors contributed equally to this work.

[11] These authors share senior authorship.

Correspondence should be addressed to W.G. (Glienke.Wolfgang@mh-hannover.de; Tel.: +49-511-532-8211)

***Correspondence**[subject IL-18 TRUCK "all-in-one" Lentiviral vector]**:** Axel Schambach

E-mail: Schambach.Axel@mh-hannover.de; Tel.: +49-511-532-5170

Keywords: TRUCK, IL-18, GD2-CAR, Prodigy, GMP, 4th generation CAR

Molecular Therapy Methods & Clinical Development

ABSTRACT

Chimeric antigen receptor (CAR)-engineered T cells) can be highly effective in the treatment of hematological malignancies. Approaches using 4th generation CAR T cells secreting immunomodulatory cytokines upon CAR signaling, known as TRUCKs ("*T cells redirected for universal cytokine-mediated killing*") are currently under investigation. The feasibility of a GMP-compliant manufacturing of IL-18 TRUCKs targeting GD2 using the automated and closed CliniMACS Prodigy® system is shown herewith. The recently described all-in-one lentiviral vector system and previous extensive experience regarding CAR-T-cell-manufacturing allowed for a rapid translation to the GMP-compliant generation of TRUCKs. High expansion and transduction efficiencies resulted in a yield of 4.7 x 10^9 engineered cells after 12 days. Preclinical characterization revealed a specific GD2-CAR-mediated activation after co-cultivation with GD2$^+$ target cells, which was shown by increased activation marker expression as well as release of pro-inflammatory cytokines and cytolytic mediators. In the same target-specific manner, the release of IL-18 was induced. Moreover, the manufactured TRUCKs exhibited cytotoxicity towards target cells, which was even improved when compared to TRUCKs generated in a laboratory scale, and further not impeded by cryopreservation. Our results demonstrate that GMP-compliant manufacturing of TRUCKs is feasible and can yields a high quality T cell product.

Molecular Therapy Methods & Clinical Development

INTRODUCTION

One of the most significant recent developments in cancer therapy is the CAR T cell technology. To enable and improve CAR T cell proliferation, activity, resistance to apoptosis and *in vivo* persistence, five generations of CARs have been developed meanwhile[1]. A promising strategy to target solid tumors with phenotypic heterogeneity has led to the fourth generation of CARs also known as TRUCKs or armored CARs[2 3]. TRUCKs are CAR T cells that release a transgenic protein upon CAR engagement of cognate antigen and signaling. TRUCKs are thereby used as "living factories" to produce and deposit substances with anti-tumor activity in the targeted tissue. These factors include but are not limited to cytokines, such as IL-(interleukin), IL-12 and IL-18 or enzymes and costimulatory ligands augmenting T cell activation. Innate immune cells are attracted and activated by IL-12 to eliminate antigen-low expressing or antigen-negative cancer cells within the tumor[2]. CAR T cells engineered with inducible IL-18 release modulate the T cell effector functions and exhibited superior activity against pancreatic and lung tumors in mice that were refractory to CAR T cells without cytokines[4 5]. In this study we focused on the manufacturing of TRUCKs targeting disialoganglioside GD2. Physiological expression of GD2 is restricted to low densities on neurons, skin melanocytes and peripheral pain fibers[6]. GD2 is highly and consistently expressed in the childhood cancer neuroblastoma and can be found on the cell surface also of other solid cancer entities including breast cancer[7], osteosarcoma[8], melanoma[9], glioblastoma[10] and Ewing sarcoma[11 12]. GD2 therefore is a promising target for redirected immunotherapy. Initial GD2-CAR T cell clinical studies targeting neuroblastoma by first to third generation CAR T cells showed only moderate or temporary anti-tumor responses but failed to produce sustained remissions, emphasizing the need to modulate the T cell response [13 14 15] With the increasing expectation of GD2 as a broad target for CAR T cell therapy and the expected benefit in applying TRUCKs with transgenic IL-18 release, there is a need to manufacture such cellular medicinal products in a safe, validated and reproducible fashion. CAR T cell manufacturing for clinical use is a complex process and places high standard demands on safety, quality and efficacy. Chemistry, Manufacturing and Controls (CMC) even in the preclinical phase of drug development includes significant quality attributes and critical process parameters, including cell composition and transduction efficiency, assessment of potency, product sterility, process validation,

stability and production at multiple manufacturing sites[16]. The CliniMACS Prodigy® (Miltenyi Biotec) allows *ex vivo* magnetic bead-based cell separation followed by activation, transduction, expansion, final formulation and sampling of T cells in a single device leading to robust, reproducible and automated, supervised cost-effective manufacturing processes (Process of CAR T cell Therapy in Europe EHA Guidance Document, 2019). The feasibility of the T cell transduction (TCT) process for use in automated and closed GMP compliant manufacturing of CAR T cells on the CliniMACS Prodigy® platform for was shown by us and others [17] [18] [19] [20]. Here we demonstrate the automated processing of TRUCKs using the CliniMACS Prodigy® with TCT software with the IL-18 TRUCKs targeting GD2 as an example. Preclinical characterization of the final product showed equivalent quality and function of the final clinical-scale product compared to manually produced IL-18 TRUCKs in small laboratory-scale.

Molecular Therapy Methods & Clinical Development

RESULTS

GMP-compliant manufacturing process of IL-18 TRUCKs targeting GD2 using automated cell processing in a closed system

To assess feasibility, we performed a complete GMP-compliant process to manufacture IL-18 TRUCKs targeting GD2 using an automated TCT protocol for CliniMACS Prodigy® as shown in **Figure 1**.

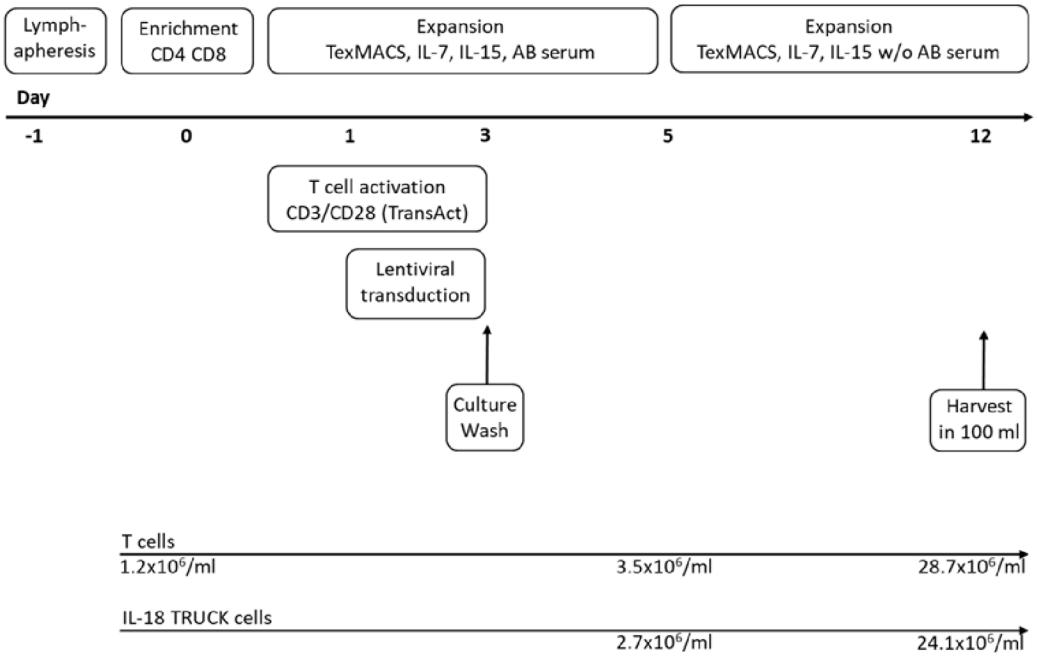


Figure 1. Schematic diagram of producing IL-18 TRUCKs targeting GD2 utilizing the CliniMACS Prodigy® automated cell processor (clinical-scale process): The entire 12-day process from lymphapheresis material to formulated final product was performed using the CliniMACS Prodigy®. Day -1: Overnight storage of the lymphapheresis at 4°C. Day 0: Immunomagnetic enrichment of $CD4^+$ and $CD8^+$ cells. Start of T cell activation with CD3/CD28 TransAct Beads (day 0-3) and cultivation in TexMACS™ basal medium supplemented with IL-7, IL-15 and human AB serum until day 5. Day 1-3: Lentiviral transduction. Day 3: Culture wash and start of culture agitation. Day 5: Switch to TexMACS™ basal medium supplemented with IL-7, IL-15 without human AB serum for following media exchanges. Day 12: Wash of cells and harvest in 100 ml formulation buffer for following cryopreservation. During T cell expansion several feeding steps by medium exchange was included in the activity matrix (not shown in this figure).

Molecular Therapy Methods & Clinical Development

Recovery and purity of $CD4^+$/$CD8^+$ cells enriched in an automated process

Starting with the lymphapheresis, 1.1 x 10^9 $CD4^+$ and $CD8^+$ cells (20.6% of lymphapheresis) were applied for enrichment. an amount of 0.67 x 10^9 $CD4^+$ and $CD8^+$ cells were obtained representing a recovery of 58.5%,. Thereby, the isolation of $CD4^+$ cells with a recovery of 63.9% was more effective than enrichment of $CD8^+$ cells with 46.8% recovery, respectively (**Figure 2A**). The CD4/CD8 ratio pre-enrichment was 2.2 and post-enrichment 2.9 reflecting a superior recovery of $CD4^+$ cells (data not shown). The achieved T cell purity was 79.1% with contaminating $CD56^+CD3^+$ NKT cells (12.0%), $CD56^+CD3^-$ NK cells (6.4%), $CD14^+$ monocytes (1.9%) and $CD20^+$ B cells (0.2%) (**Figure 2B**). Due to their expression of CD8, NK as well as NKT cells were not completely depleted.

Expanded IL-18 TRUCKs targeting GD2 display a high purity and favorable phenotype

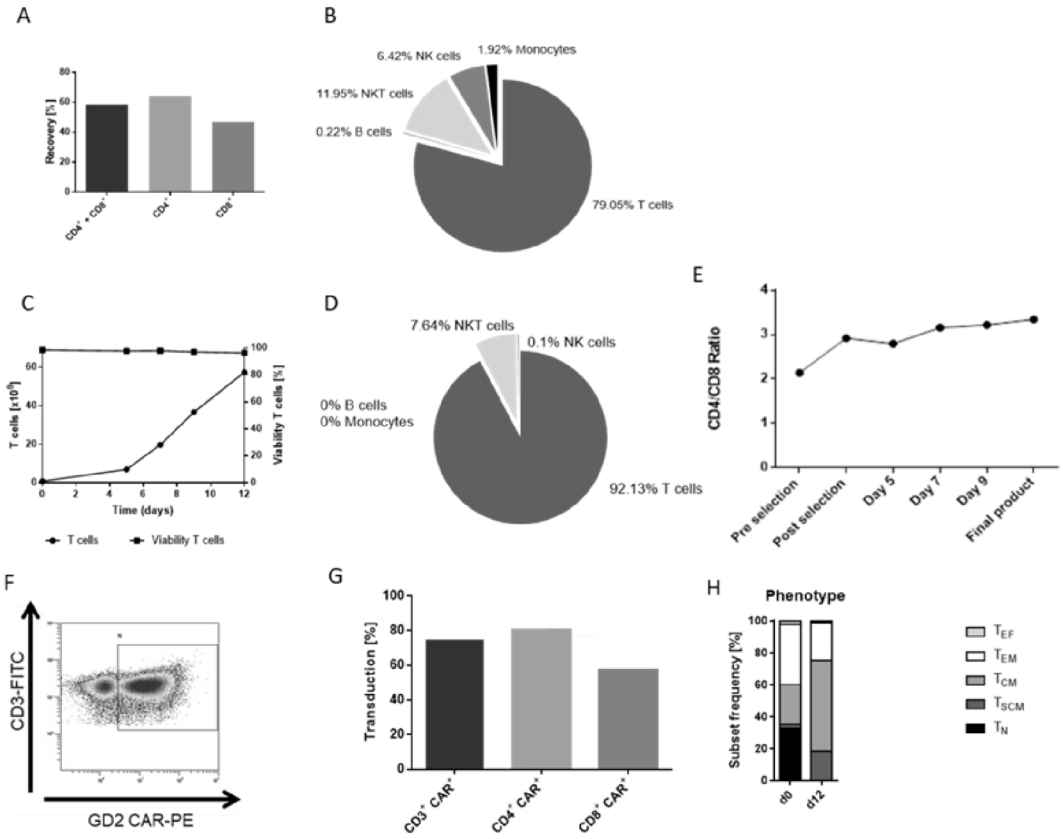
In accordance with the recommendation of the TCT protocol, the cell expansion started with 0.85 x 10^8 viable T cells (corresponding 1.08 x 10^8 viable WBC) after enrichment of $CD4^+$ and $CD8^+$ cells. The expansion rate during the 12-day manufacturing process was 67.5-fold for T cells. Viability of the cells was >96% during the whole process until final harvest of IL-18 TRUCKs targeting GD2 (**Figure 2C**). Cell concentration increased in a constant culture volume from 3.75 x 10^6/ml on day 5 to 31.16 x 10^6/ml in the final product (**Figure S1A**). Monitoring of cell culture condition revealed a constant pH-level of 7.1 and a glucose level above the critical level of 100 mg/dl. **(Figure S1B)**. Cell composition of the final product is shown in **Figure 2D** with a purity of 92.1% T cells and impurities of NKT cells (7.6%) as well as residual 0.1% NK cells. Neither B cells nor monocytes were detectable in the final product. During expansion, the CD4/CD8 ratio increased from 2.8 to 3.3 reflecting the higher expansion rate of $CD4^+$ cells (69.5-fold) compared to $CD8^+$ cells (60.6-fold; data not shown). The proportion of T cell phenotypes during *ex vivo* cell expansion was analyzed by comparing T cell phenotypes in the initial product (post $CD4^+$ and $CD8^+$ enrichment, process day 0) and in the final product (IL-18 TRUCKs targeting GD2, process day 12). The analyses were performed by flow cytometry based on the expression of CD45RO, CCR7 and CD95 among viable $CD3^+$ cells to define naïve (T_N: $CD45RO^-$, $CCR7^+$, $CD95^-$), stem-cell memory (T_{SCM}: $CD45RO^-$, $CCR7^+$, $CD95^+$), central memory (T_{CM}: $CD45RO^+$, $CCR7^+$), effector memory (T_{EM}: $CD45RO^+$, $CCR7^-$) and effector (T_{EF}:

Molecular Therapy Methods & Clinical Development

CD45RO$^-$, CCR7$^-$) T cell subsets. The gating strategy is shown in **Figure S6**. T_N (33.1 %), T_{CM} (25.1 %) and T_{EM} (37.6 %) cells were represented predominantly levels in the initial product with a wide variety of the T cell subsets (T_{SCM}: 2.2%, T_{EF}: 2.1%). In contrast, analysis of the final TRUCK cell product revealed less differentiated memory phenotypes with a strong increase in the portion of T_{CM} (56.9%) and T_{SCM} (18.3%) cells, a decrease in the portion of T_{EM} (23.6%) and T_{EF} (1.2%) cells and the disappearance of T_N (<0.1%) cells (**Figure 2H**).

Transduction of T cells with an "all-in-one" lentiviral vector is highly efficient

A recently described "all-in-one" lentiviral vector [21] encoding constitutive GD2 CAR and inducible IL-18 expression was used for cell transduction on day 1 of the manufacturing processing with a multiplicity of infection (MOI) of 29. The percentage of transduced CD3$^+$ cells during the process was 73.1%, 74.1% and 73.8% on days 5, 7 and 9, respectively (data not shown). In the final product, CAR$^+$ T cells was 74.9%, whereby CD4$^+$ cells exhibited a higher efficiency (80.6%) compared to CD8$^+$ cells (57.2%) (**Figure 2G**). The VCN determined by qPCR of genomic DNA was below 2.6 copies per cell (data not shown).



152

153 **Figure 2. Automated GMP-compliant manufacturing of IL-18 TRUCKs targeting GD2 using the**
154 **CliniMACS Prodigy® (clinical-scale process): (A)** Recovery (cell population in enriched fraction in
155 percent of cell population pre-enrichment) of $CD4^+$ and $CD8^+$ cells after enrichment. **(B)** Cell
156 composition after enrichment of $CD4^+$ and $CD8^+$ cells. **(C)** Expansion and viability of T cells ($CD3^+$
157 $CD56^-$) during 12-day manufacturing. **(D)** Cell composition of the final product after 12 day-
158 expansion. **(E)** CD4/CD8 ratio during cultivation. The CD4/CD8 ratio was slightly increasing during
159 the manufacturing process (post enrichment) from 2.9 to 3.3 in the final product reflecting superior
160 recovery of $CD4^+$ cells. **(F)** Flow cytometric presentation of $CD3^+$ IL-18 TRUCK targeting GD2 using
161 the anti-Ganglidiomab-PE antibody. **(G)** Transduction rate of $CD3^+$ cells and $CD4^+$ $CD8^+$ subtypes in
162 the final product. **(H)** T cell phenotypic analysis of the starting material on day 0 (lymphapheresis) and
163 in the final product on day 12. T cell phenotypes were defined based on their expression of the
164 markers CD45RO, CCR7 and CD95 among viable $CD3^+$ cells: naïve (T_N: $CD45RO^-$ $CCR7^+$ $CD95^-$),
165 stem-cell memory (T_{SCM}: $CD45RO^-$ $CCR7^+$ $CD95^+$), central memory (T_{CM}: $CD45RO^+$ $CCR7^+$),
166 effector memory (T_{EM}: $CD45RO^+$ $CCR7^-$) and effector (T_{EF}: $CD45RO^-$ $CCR7^-$) T cells. For cell
167 composition, cells (**B**, **D**) were gated as viable $CD45^+$ cells using lineage-specific markers: T cells
168 ($CD3^+$ $CD56^-$), monocytes ($CD14^+$), NK cells ($CD56^+$ $CD16^+$), NKT cells ($CD56^+$ $CD3^+$), B cells
169 (CD20). n = 1.

Preclinical *in vitro* characterization using clinical- and laboratory-scale manufactured TRUCKs: Clinical-scale- and laboratory-scale-manufactured IL-18 TRUCKs targeting GD2 are transduced and pre-activated at similar efficacies

To characterize IL-18 TRUCKs targeting GD2 manufactured using the CliniMACS Prodigy® (referred to as clinical-scale TRUCKs), they were compared to the respective TRUCKs generated under laboratory conditions (referred to as laboratory-scale TRUCKs). These were manufactured by using the same isolated $CD4^+$ and $CD8^+$ T cell starting population, lentiviral vector, expansion media, cytokines, and experimental timing, but the cells were processed manually as previously described[22]. Untransduced T cells were generated accordingly in laboratory scale. From a starting fraction of 6.3 x 10^5 cells, laboratory-scale TRUCKs expanded to a cell number of 5.4 x 10^7 cells, which was slightly lower when compared to untransduced T cells with a final cell number of 7.0 x 10^7 cells (**Figure S2A**). Both, laboratory- and clinical-scale IL-18 TRUCKs, showed an enhanced frequency of $CD4^+$ T cells after expansion with CD4/CD8 ratios of 2.5 for clinical- and 1.6 for laboratory-scale TRUCKs, whereas it was 1.0 for untransduced T cells (**Figures 3A, B**). Utilizing the recently described all-in-one vector[21], we obtained a high percentage of transduced cells with 75% and 80% under clinical scale as well as under laboratory conditions, respectively, with a higher frequency of transduced $CD4^+$ T cells than $CD8^+$ T cells (**Figures 3C, D**). Activation marker expression on clinical-scale TRUCKs, assessed after expansion of T cells with anti-CD3/CD28 stimulation and IL-7 and IL-15 supplementation, revealed CD25 and CD69 expression on $CD3^+$ T cells (63 and 35%, respectively), moderate CD154 expression on $CD4^+$ cells (21%) and absent CD137 expression on $CD8^+$ cells (**Figures 3 E-H**). For all markers except for CD137, this was slightly enhanced compared to the expression on untransduced T cells, but similar or even less than on laboratory-scale TRUCKs. Clinical use of modified cells often requires cryopreservation to allow for centralized manufacturing and administration flexibility [23]. To assess the feasibility of cryopreservation of the obtained T cell product, the manufactured T cells were also characterized after freezing and thawing. Cryopreservation of clinical-scale and laboratory-scale IL-18 TRUCKs led to a reduction of the activation state to the level of untransduced T cells (Figures 3E-H). CD4/CD8 ratios and the frequency of transduced cells did not change (**Figures 3A, B, D**).

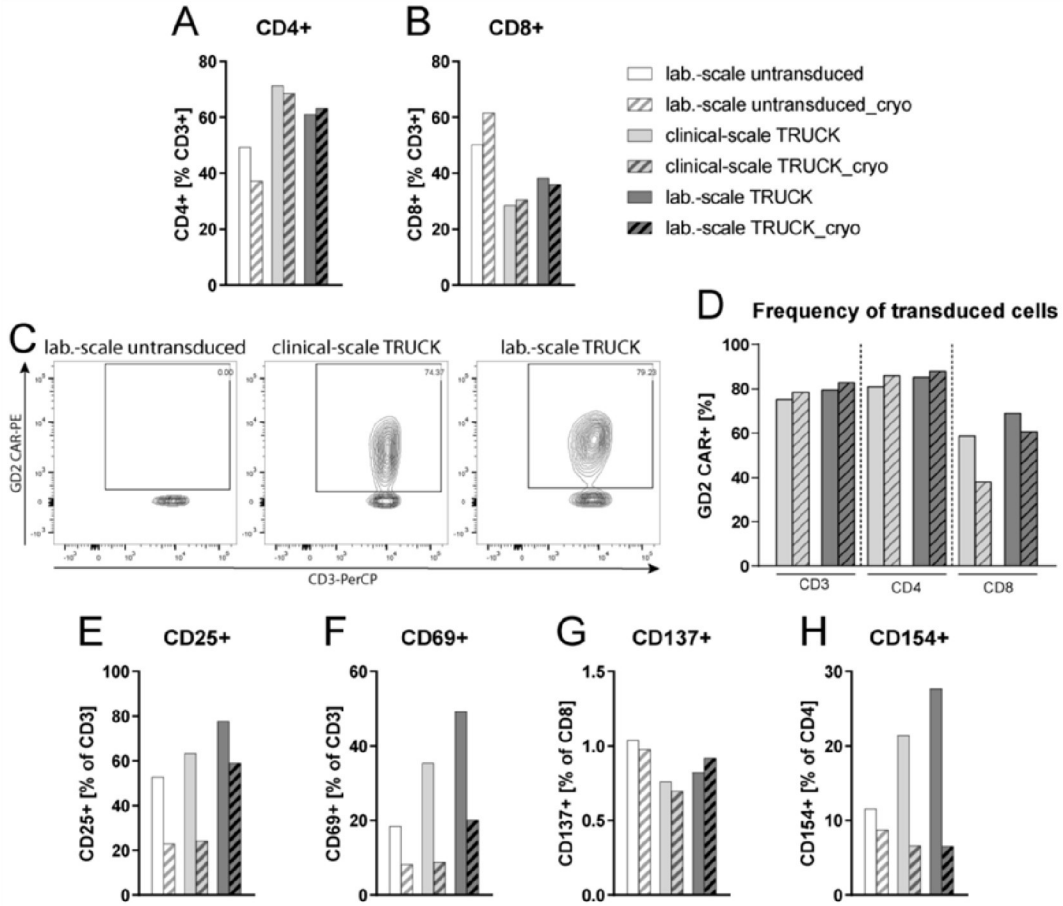


Figure 3. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 are transduced and pre-activated at similar efficacies compared to laboratory-scale-manufactured TRUCKs: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were either characterized directly after the generation process (d12) or after cryopreservation and thawing (cryo). n=1. **(A, B)** Frequency of **(A)** $CD4^+$ and **(B)** $CD8^+$ T cells in the final cell product. **(C, D)** Percentage of CAR^+ cells of **(C)** $CD3^+$ cells shown as representative plots and **(D)** $CD3^+$, $CD4^+$ and $CD8^+$ cells as assessed by staining of the scFv-domain of TRUCKs with a Ganglidiomab antibody after expansion. **(E-H)** Expression of the activation markers **(E)** CD25 on $CD3^+$, **(F)** CD69 on $CD3^+$, **(G)** CD137 on $CD8^+$ and **(H)** CD154 on $CD4^+$ T cells.

Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically respond to $GD2^+$ target cells with an increase of activation marker expression

To test the ability of the generated TRUCKs to recognize and react towards $GD2^+$ target cells, they were co-cultivated either with HT1080, HT1080 cells expressing GD2 or with the neuroblastoma cell line SH-SY5Y as target cells and examined for activation marker expression. IL-18 TRUCKs targeting GD2 responded with increased expression of CD25 and CD69 by 56 – 61% and 61 - 79%,

respectively, on all $CD3^+$ T cells to both GD2-expressing target cells, whereas they did not exhibit an antigen-independent response towards unmodified HT1080 cells (**Figures 4B, C; S3A-D**). The slightly lower activation response towards SH-SY5Y in comparison to HT1080-GD2 coincides with the described GD2 expression levels [21] . Clinical-scale compared to laboratory-scale TRUCKs even reacted with an enhanced activation marker increase for most of the markers and target cells. Regarding expression of CD137 on $CD8^+$ and CD154 on $CD4^+$ T cells, the same effects were observed, indicating cells of the $CD4^+$ and $CD8^+$ populations in manufactured IL-18 TRUCKs were specifically and effectively activated in response to $GD2^+$ target cells (**Figures 4A, D, E; S3E-H**). Assessment of respective activation marker expression levels as mean fluorescence intensity (MFI) or in further effector-to-target (E:T) ratios confirmed the observed results (**Figures S3A-H**).

Molecular Therapy Methods & Clinical Development

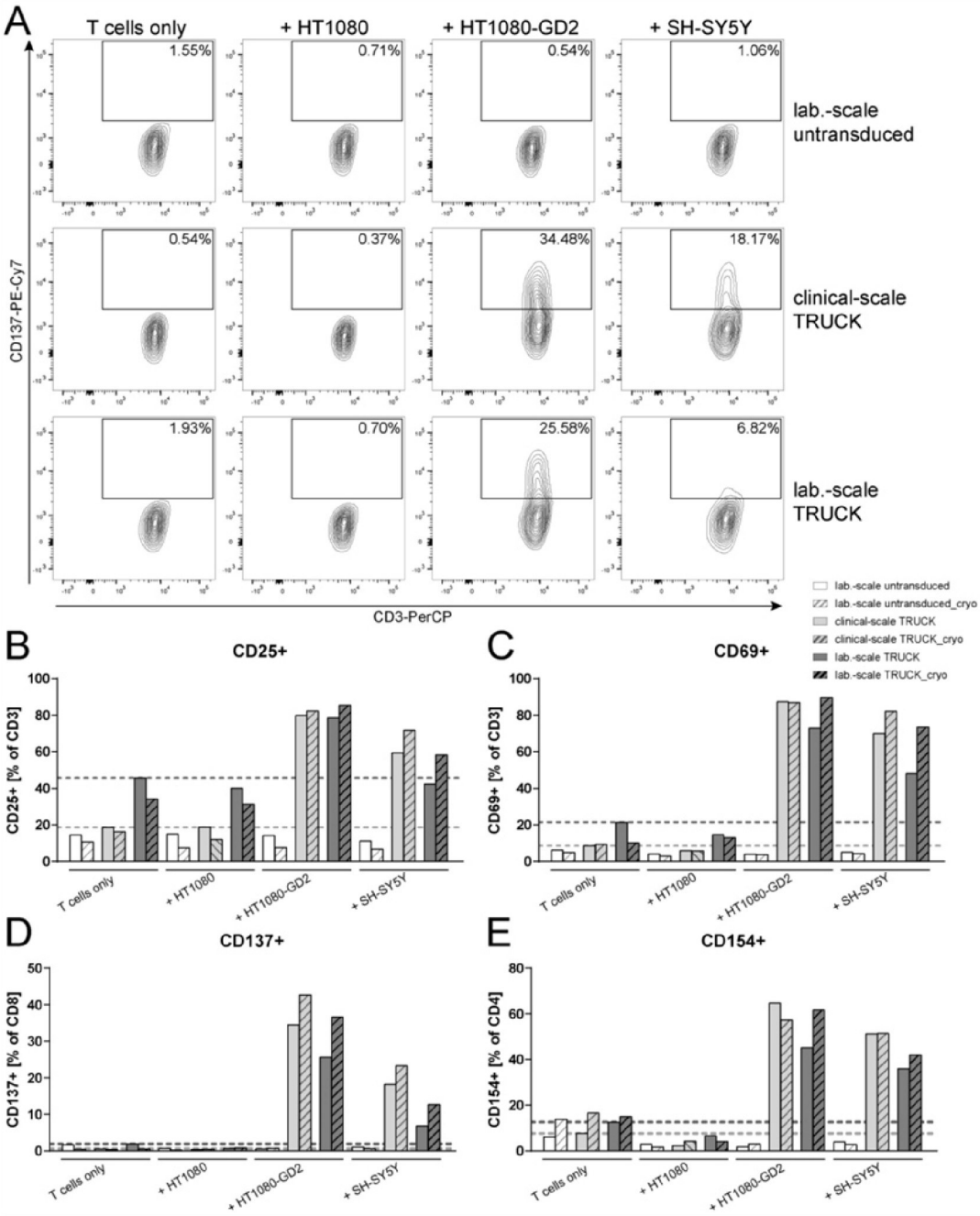


Figure 4. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically respond to GD2$^+$ target cells with an increase of activation marker expression. IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were tested for GD2-CAR-mediated activation either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells for 48h in an effector-to-target (E:T) ratio of 4:1 or cultivation alone (T cells only). n=1. **(A-E)** Frequency of **(A)** CD137$^+$ of CD8$^+$ as representative plots, **(B)** CD25$^+$ of CD3$^+$, **(C)** CD69$^+$ of CD3$^+$, **(D)** CD137$^+$ of CD8$^+$ and **(E)** CD154$^+$ of CD4$^+$ T cells as determined by flow cytometry. A dashed line

236 indicates background levels of the respective expression by clinical-scale (light grey) and laboratory-
237 scale (dark grey) TRUCKs cultured alone.
238
239

240 **Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically react to target**
241 **recognition with increased release of activation-specific cytokines**

242 To further test the functionality and specificity of the engineered T cells, the release of cytokines into
243 the cell co-culture supernatants upon target contact was assessed. In co-cultures with HT1080-GD2
244 cells and, to a lower extent also with SH-SY5Y cells, the release of several cytokines was upregulated
245 in clinical-scale IL-18 TRUCKs compared to background secretion: IL-2 (37 and 13-fold), IL-4 (33
246 and 7.9-fold), IL-10 (11 and 2.2-fold), interferon (IFN)-γ (380 and 39-fold), granzyme B (>120 and
247 30-fold), perforin (7.2 and 2.6-fold) and tumor necrosis factor (TNF)-α (160 and 39-fold; all in an E:T
248 ratio of 16:1) (**Table 1**). Thereby, antigen-induced cytokine release was similar or even enhanced
249 compared to laboratory-scale TRUCKs. Cryopreservation of cells led to an even increased target cell-
250 induced release of IL-2, IL-4 and TNF-α, but lower release of granzyme B, perforin and IFN-γ
251 (**Supplementary Table 1**). Regarding TNF-α, a higher expression of the cytokine by IL-18 TRUCKs
252 upon specific target contact was confirmed by intracellular staining (**Figure S3I**).

Effector cells	E:T ratio	Target cells	IL-2 [pg/mL]	IL-4 [pg/mL]	IL-10 [pg/mL]	Granzyme B [pg/mL]	IFN-γ [pg/mL]	Perforin [pg/mL]	TNF-α [pg/mL]
untransduced	4:1	T cells only	2.57	0.78	0.66	246.64	7.86	222.19	1.05
clinical-scale	4:1	T cells only	2.04	1.03	0.82	64.10	18.23	231.48	1.23
lab.-scale	4:1	T cells only	2.04	0.85	0.68	671.21	6.83	136.75	1.05
untransduced	4:1	HT1080	< 1.54	0.71	0.68	82.78	5.09	182.33	< 0.98
clinical-scale	4:1	HT1080	2.04	0.71	< 0.61	32.50	8.31	166.85	< 0.98
lab.-scale	4:1	HT1080	< 1.54	0.92	< 0.61	764.11	12.49	252.12	1.21
untransduced	4:1	HT1080-GD2	< 1.54	0.61	0.68	124.85	4.66	147.00	1.05
clinical-scale	4:1	HT1080-GD2	1689.94	71.16	4.78	28368.61	14789.44	803.09	272.12
lab.-scale	4:1	HT1080-GD2	11.42	3.77	0.97	1273.69	25.51	264.99	5.15
untransduced	4:1	SH-SY5Y	< 1.54	0.68	< 0.61	209.84	6.10	190.89	1.13
clinical-scale	4:1	SH-SY5Y	216.91	26.34	1.77	5526.97	1969.48	583.55	178.80
lab.-scale	4:1	SH-SY5Y	2.04	0.58	< 0.61	9.14	4.38	3.84	1.05
untransduced	8:1	T cells only	4.66	1.13	0.68	389.95	9.50	401.39	1.05
clinical-scale	8:1	T cells only	2.57	0.88	< 0.61	174.28	28.77	465.70	1.13
lab.-scale	8:1	T cells only	3.07	1.17	0.62	1345.82	12.64	324.93	1.24
untransduced	8:1	HT1080	< 1.54	0.58	< 0.61	112.96	4.09	208.26	< 0.98
clinical-scale	8:1	HT1080	4.16	0.82	< 0.61	134.91	26.23	486.36	1.33
lab.-scale	8:1	HT1080	3.63	0.85	0.66	937.59	14.43	365.16	1.03
untransduced	8:1	HT1080-GD2	5.72	0.78	0.91	318.66	10.26	402.99	1.12
clinical-scale	8:1	HT1080-GD2	472.18	67.65	8.15	> 32296.04	> 21943.96	2761.78	439.96
lab.-scale	8:1	HT1080-GD2	30.80	16.44	6.24	27773.20	454.12	1257.24	35.79
untransduced	8:1	SH-SY5Y	3.63	1.00	0.97	418.80	10.40	343.97	1.82
clinical-scale	8:1	SH-SY5Y	54.09	15.82	1.94	10173.94	3367.96	1869.57	158.34
lab.-scale	8:1	SH-SY5Y	5.18	3.11	0.88	2478.90	56.51	592.36	4.65
untransduced	16:1	T cells only	< 1.54	0.82	0.66	499.08	7.57	408.00	1.01
clinical-scale	16:1	T cells only	3.07	1.10	0.65	266.76	58.02	877.97	1.74
lab.-scale	16:1	T cells only	3.60	1.13	0.65	2450.29	24.31	675.36	1.27
untransduced	16:1	HT1080	< 1.54	0.78	0.82	380.46	7.57	490.41	1.01
clinical-scale	16:1	HT1080	10.27	1.48	0.70	383.53	76.12	1083.58	2.49
lab.-scale	16:1	HT1080	7.28	2.11	1.14	4264.08	54.08	1197.29	3.25
untransduced	16:1	HT1080-GD2	3.07	0.78	0.99	455.00	9.20	439.54	< 0.98
clinical-scale	16:1	HT1080-GD2	115.10	36.00	7.38	> 32296.04	> 21943.96	6334.52	282.16
lab.-scale	16:1	HT1080-GD2	13.50	7.35	4.68	> 32296.04	555.66	2839.83	12.61
untransduced	16:1	SH-SY5Y	2.04	0.71	1.02	506.85	9.06	465.92	1.13
clinical-scale	16:1	SH-SY5Y	39.55	8.66	1.46	8020.51	2244.12	2273.24	67.24
lab.-scale	16:1	SH-SY5Y	< 1.54	2.31	1.17	5659.88	70.38	1287.11	3.05

Table 1. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically react to target recognition with increased release of soluble mediators: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale) or under laboratory conditions (lab.-scale). Untransduced T cells (untransduced) served as control. The manufactured cells were tested for functionality by co-cultivation with the indicated target cells in the indicated effector-to-target (E:T) ratios or cultivation alone (T cells only) directly after their generation. The concentration of released cytokines in the cell culture supernatants after 48h was assessed by LEGENDPlex™.

Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically eliminate GD2$^+$ target cells

Finally, the killing capacity of manufactured IL-18 TRUCKs targeting GD2 was determined by flow cytometric analysis of target cells in the co-cultures. Relative to co-cultures of untransduced T cells with HT1080-GD2 and SH-SY5Y, clinical-scale TRUCKs eliminated 43 - 81% of HT1080-GD2 and

SH-SY5Y cells at different E:T ratios, whereas the frequency of HT1080 in co-cultures with the clinical-scale TRUCKs was only very slightly reduced at the highest E:T ratio of 16:1 (**Figure 5A**). Compared to TRUCKs generated in the laboratory scale, clinical-scale-manufactured TRUCKs were even more cytotoxic shown by the ability to eliminate $GD2^+$ target cells at a lower E:T ratio of 4:1. Cryopreservation of TRUCKs did not impede cytotoxicity. The release of lactate dehydrogenase (LDH) into the supernatant as parameter for cytolysis confirmed this result as it was enhanced in co-cultures of both IL-18 TRUCKs with HT1080-GD2 cells (20 – 36% and 23 - 40% for large- and laboratory-scale TRUCKs, respectively) compared to the respective co-cultures with unmodified HT1080 cells, in which LDH was not released above the background of cells cultured alone (**Figure S4A**). Target cell death by LDH release in co-cultures of clinical-scale TRUCKs with SH-SY5Y was also enhanced at all E:T ratios (9 - 22%) and more than compared to laboratory-scale TRUCKs (3 - 4%), which were moreover not able to induce LDH release by SH-SY5Y cells at the lowest E:T ratio of 4:1. LDH measurements in the co-cultures with thawed T cells showed a background induced by cells that died in the process of freezing and thawing. These cells were not incorporated for the cell number determination but were present in the co-cultures. However, a negligible increase of LDH release over background could be observed for thawed TRUCK co-cultured with HT1080-GD2 and SH-SY5Y. For thawed IL-18 TRUCKs, real-time measurement of target cell viability confirmed their cytotoxic capability. After addition of both TRUCKs to adherent SH-SY5Y cells, the cell index was rapidly reduced, resulting in almost complete absence of adherent target cells after co-cultivation for 100 h (**Figure 5B**). Clinical-scale TRUCKs eliminated the target cells more effectively compared to the laboratory-scale TRUCKs, demonstrated by an earlier onset of cell index reduction after T cell addition. Transmitted-light microscopy visualized the process of target cell elimination. In co-cultures of HT1080 with all T cell products, cells are distributed equally, and the target cells stayed viable in all E:T ratios (**Figure 5C**). In co-cultures of both TRUCKs with HT1080-GD2 or SH-SY5Y, the target cells were diminished or even absent after 72 h and T cells formed large clusters around the target cells. For thawed TRUCKs, the clusters were even larger and already appeared at low E:T ratios in co-cultures with SH-SY5Y cells (**Figure S4B**).

Molecular Therapy Methods & Clinical Development

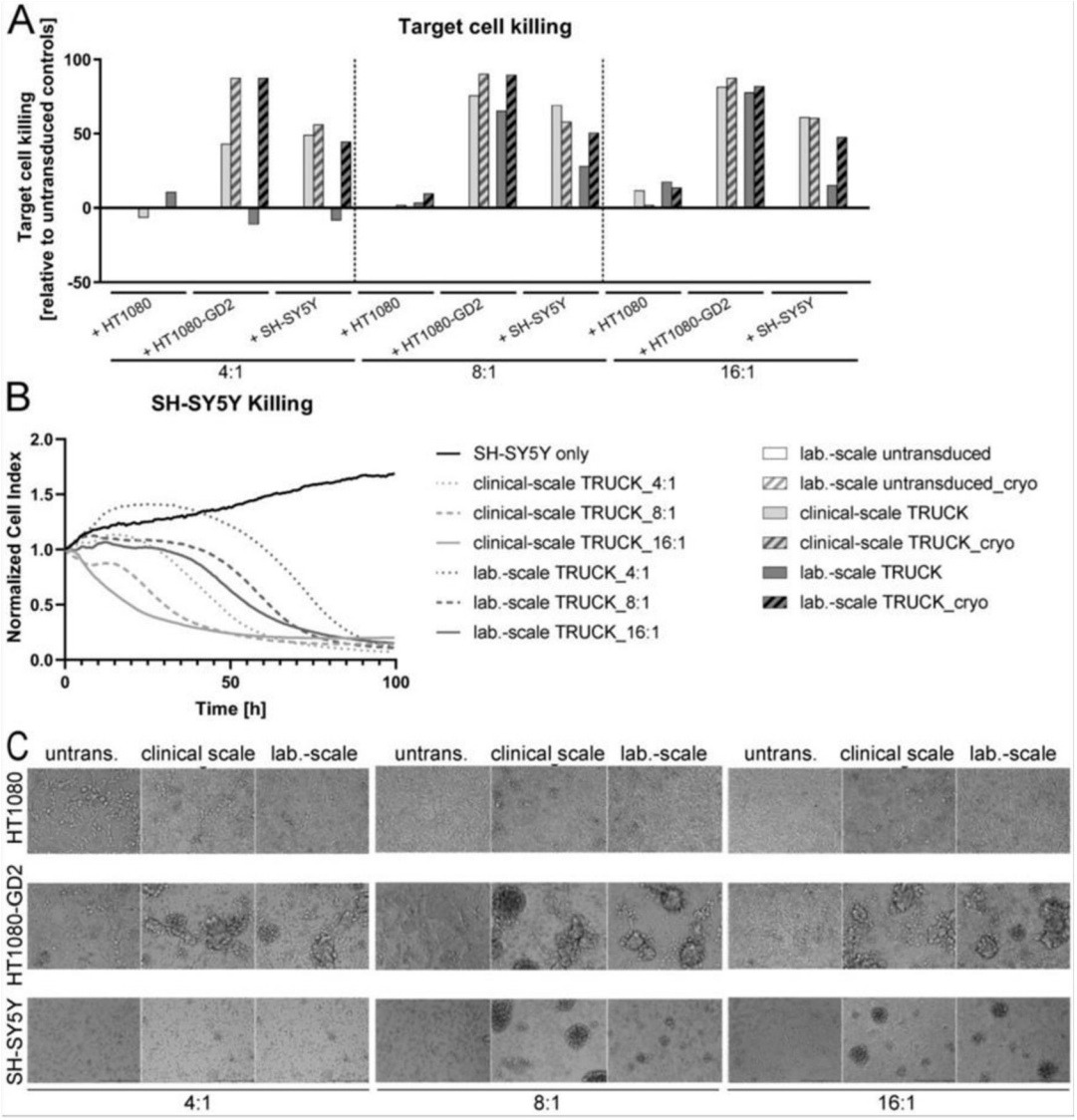


Figure 5. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically eliminate GD2$^+$ target cells: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were tested for cytotoxicity either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells and in the indicated effector-to-target (E:T) ratios. n=1. **(A)** Target cell killing after 48h was measured by flow cytometry as percentage of killed CD3$^-$ cells relative to those eliminated in co-cultures with untransduced T cells. **(B)** Killing of SH-SY5Y cells determined with the XCelligence Real-Time Cell Analyzer. Cell indices were normalized to the respective indices after T cell addition. **(C)** Transmitted-light microscope images of co-cultures of fresh effector cells with target cells taken after 48h by an Olympus IX81 microscope combined with a digital B/W camera using 10x objective lenses.

Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 released IL-18 in a target-specific manner leading to innate immune cell attraction

To address the ability of the generated TRUCKs to selectively release IL-18 following CAR engagement of GD2 target antigen, the cytokine was measured in the co-culture supernatants. Importantly, the release of IL-18 into the cell culture supernatant by clinical-scale TRUCKs was induced in a target-specific manner up to 63 pg/ml upon HT1080-GD2 and 21 pg/ml upon SH-SY5Y encounter (**Figure 6A**). The IL-18 release was reduced by the freeze-and-thaw cycle. To assess the effect of anti-GD2 IL-18 TRUCK-induced cytokines with respect to the recruitment of innate immune cells, we used a modified Boyden chamber assay to compare the migration potential of cell supernatants collected from co-culture experiments of IL-18 TRUCKs vs. untransduced T cells and $GD2^+$ target cells (HT1080-GD2 and SH-SY5Y) to promote the recruitment of monocytes (THP-1 cells) and NK cells (NK-92 cells). The capacity of IL-18 containing supernatants to recruit innate immune cells was shown by positive Giemsa staining of the transwell membrane through which migrating cells were recruited (**Figure 6B**). Giemsa staining of migrated cells revealed that greater numbers of the monocytic cell line THP-1 and NK-92 cells were recruited by supernatants collected from IL-18 TRUCKs co-cultured with HT1080-GD2 or SH-SY5Y target cells compared to untransduced T cells or upon co-culture with $GD2^-$ target cells, likely as a result of induced IL-18 cytokine secretion (**Figure 6C, D**). Taken together, supernatants from TRUCKs releasing IL-18 in an inducible manner by CAR activation upon antigen recognition exhibit an innate immune cell recruitment potential *in vitro* indicating clinical-scale manufacturing maintains antigen-induced IL-18 release and its biological activity.

Molecular Therapy Methods & Clinical Development

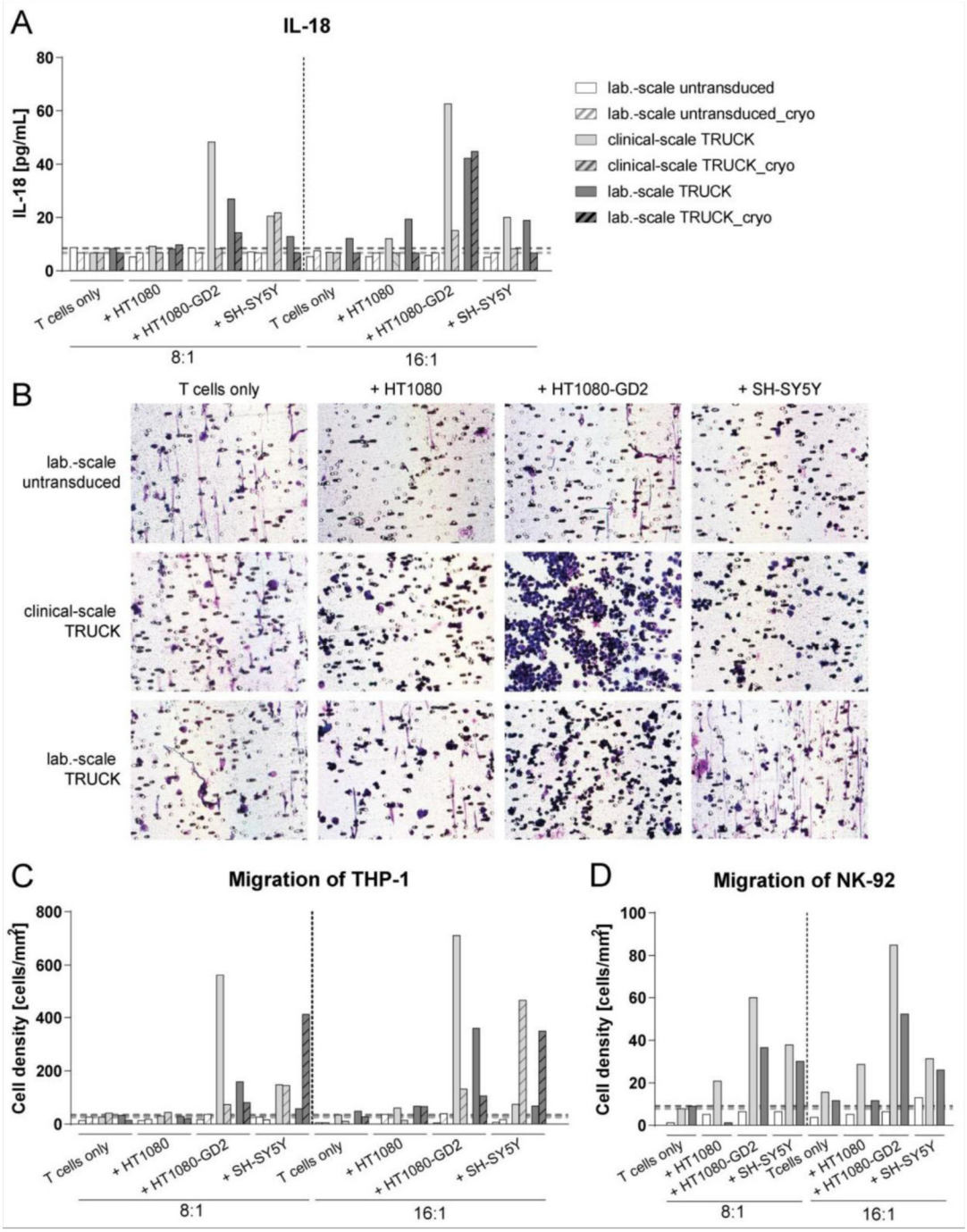


Figure 6. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 released IL-18 in a target-specific manner leading to innate immune cell attraction: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were tested for functionality either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells and in the indicated effector-to-target (E:T) ratios. n=1. **(A)** The concentration of released cytokines in the cell culture supernatants after 48h was assessed by LEGENDPlex™. The chemoattractive potential of IL-18 released by TRUCKs upon target recognition in terms of the migration of **(B, C)** THP-1 and **(D)**

NK-92 cells was assessed. Supernatant of engineered T cells cultured alone (T cells only) or together with the indicated target cells for 48h was placed in a Boyden chamber, covered with an 8 µm polycarbonate membrane and incubated for another 4h. Medium served as the control supernatant. Cells migrated through the membranes were Giemsa stained. **(B)** Representative pictures of Giemsa-stained THP-1 cells (violet) on the bottom of the membrane. **(C, D)** The number of cells that migrated through the membrane was determined.

Molecular Therapy Methods & Clinical Development

DISCUSSION

TRUCKs combine the redirected CAR T cell attack with the on-site release of a biologically active protein while avoiding its systemic toxicity and thereby hold promise to be suitable to modulate the environment of the targeted solid tumor. Based on design and broad *in vitro* characterization of the lentiviral vector combining constitutive anti-GD2 CAR expression and inducible IL-18[21] as well as our previous development and validation of an automated and closed processing for GMP compliant manufacturing of CAR T cells,[19 20] we present the proof of feasibility for translation of this method to activate and expand IL-18 TRUCKs targeting GD2 for clinical application.

Our protocol is optimized to produce CAR-engineered T cells in clinically sufficient numbers under GMP-compliance using the CliniMACS Prodigy® platform that integrates different steps of manufacturing including cell isolation, activation, transduction, cell washing, cultivation and formulation of the final product in a single process and thus minimizes variability emanating from various manual work steps. This fully integrated yet modular system allows flexibility and standardized procedure at the same time which is the key for the production of personalized cell products of various kinds. Multiple steps are required to produce gene modified effector cells starting with enrichment of $CD4^+/CD8^+$ T cell subset followed by activation, transduction and expansion of effector cells. $CD4^+/CD8^+$ enrichment is regarded as a safety procedure to decrease blast counts in the culture[24]. $CD4^+/CD8^+$ enrichment also decreases contaminating cells such as monocytes, which were shown to inhibit CAR T cell expansion[25]. The primary objective was the feasibility of cell production for 3 dose levels (e. g. 5×10^5, 1×10^6, and 3×10^6 anti-GD2 IL-18 TRUCKs/kg). Dosage protocols vary across different clinical studies from $1 \times 10^4 - 1 \times 10^{10}$ cells/kg[26]. After $CD4^+/CD8^+$ enrichment we found a high T cell purity with low contaminating cell populations of $CD8^+$ NK and NKT cells which are not removed during the $CD4^+/CD8^+$ enrichment step also described by other groups[18 24]. With an expansion rate of 67.5-fold and a transduction rate of 74.9% we reached a sufficient cell dose of 4.7×10^9 transduced WBCs. This cell dose would allow the application of 58.8×10^6/kg IL-18 TRUCKs targeting GD2 to a recipient weighing 80 kg even in a multi-dose base. A risk adapted dosing for use of the more potent TRUCKs may be adjusted.

We and others could show the eligibility of the T cell transduction (TCT) process developed by Miltenyi Biotec with fixed process parts (enrichment, activation and transduction) as well as the possibility of variable culture set up by modular programming of the activity matrix[17 18 19 20 24 27 28 29 30].

Safety aspects in the clinical use of ATMPs have high priority. Lentiviral vectors are known to have a lower risk for mutational oncogenesis than γ-retroviral vectors[24]. Moreover, by using an "all-in-one" vector, ideally only a single vector integration event is needed, which also reduces the risk of insertional mutagenesis. A European Medicines Agency (EMA) reflection paper (EMA/CAT/190186/2012) on the management of clinical risks deriving from insertional mutagenesis highlighted the VCN as a risk factor for oncogenesis and recommended risk assessment and management of the integration copy numbers, integration profile and sites in cellular products. The IL-18 TRUCK final product contained 2.6 copies/cell, which is below the limit of < 5 copies/cell that is considered safe[31]. The transduction rate of 75% in the final product is higher than reported by other groups using CliniMACS Prodigy® for production of CAR T cells[17 18 20 24 27 28 29 30 32]. Transduction efficiency was higher for $CD4^+$ compared to $CD8^+$ T cells, respectively as also shown by previous reports[20 30].

Regarding prolonging the *in vivo* persistence of CAR T cells in patients, one promising strategy is the enrichment of cell products with less differentiated T cell subsets such as central memory (T_{CM}) or stem cell memory T (T_{SCM}) T cells. These subpopulations have gained substantial attention, as the adoptive transfer of even low numbers of T cells from these subsets can reconstitute robust and long-term maintained immune responses[33 34]. Most T cells in our IL-18 TRUCK final product showed a T_{cm}/T_{scm} phenotype and thus promise functional advantages for adoptive T cell transfer. The use of IL-7 and IL-15 on naïve T (T_N) cells during culture conditions promotes the acquisition of T_{CM} or T_{SCM} phenotypes[17 18 24 35]. This is in line with previous reports [18 30]. In addition, whereas T cells expressing CARs with CD28 domains predominantly differentiate into effector memory T (T_{EM}) cells, *in vitro*

400 expansion of 4-1BB-containing CAR T cells as the TRUCKs used here produces a higher proportion
401 of T_{CM} cells [36].
402
403 *In vitro* characterization of the obtained engineered T cell product revealed an upregulation of
404 activation markers (CD25, CD69, CD137 and CD154) on both $CD4^+$ and $CD8^+$ T cells and a variety
405 of proinflammatory cytokines and cytotoxic mediators (IL-2, IL-4, granzyme B, perforin, IFN-γ, TNF-
406 α) upon specific recognition of GD2-expressing target cells, but not after co-cultivation with a control
407 cell line lacking the target. In addition, TRUCKs induced release of the engineered cytokine IL-18 in
408 an antigen-dependent manner and mediated only a very low background secretion upon co-cultivation
409 with target-negative cells or cultivation alone. However, potential toxicities associated to systemic
410 presence of the cytokine need to be evaluated in further studies, since another study revealed severe
411 toxicity of T cells engineered with inducible IL-12 in a melanoma mouse model due to non-specific
412 cytokine secretion [37]. However, other studies with IL-12 TRUCKs reported a safe administration into
413 mice and such toxicities were not observed for IL-18 TRUCKs [2 3 4 38]. GMP-compliant manufactured
414 IL-18 TRUCKs targeting GD2 showed a high cytotoxic capability and were able to eliminate tumor
415 cells while forming clusters around target cells. After cryopreservation of the T cell products, the
416 specificity and cytotoxicity of TRUCKs was maintained. Attempts to treat large solid tumors with
417 redirected T cells have largely failed so far, with very few patients responding and with only transient
418 and partial tumor regression[39 40 41 14 13 42 43]. The poor clinical outcome is thought to be due at least in
419 part to an unfavorable environment in the tumor tissue that suppresses CAR T cell responses. We
420 confirmed that TRUCK-secreted cytokine IL-18 led to increased recruitment of monocytes and NK
421 cells in an *in vitro* cell migration assay. This could contribute to reprogramming the tumor stroma
422 towards a more favorable environment for CAR T function and thereby enhance their efficacy in the
423 treatment of solid tumors. Furthermore, IL-18 was shown to polarize TRUCKs themselves towards
424 more potent pro-inflammatory effector cells that do not drive into functional exhaustion in the long
425 term[4]. CAR T cells can be engineered to release any protein with therapeutic potential. Candidates for
426 therapeutic proteins are immune checkpoint inhibitors, in particular monoclonal antibodies against

427 CTLA-4 and against PD-1. Such antibody fragments can also be produced by engineered T cells and
428 released after CAR signaling[44 34] as an alternative to cytokines.
429
430 In conclusion, the feasibility of GMP-compliant manufacturing of IL-18 TRUCKs targeting GD2
431 using the automated closed CliniMACS Prodigy® system was shown, enabling the manufacturing of a
432 sufficient number of cells for clinical application. The automatic mode of operation improves
433 standardization and robustness of the manufacturing process. This benefits the manufacturing at
434 different sites for an academia-initiated multicenter trial. The smooth adaption of the process
435 established and validated for the manufacturing of CAR T cells to generate IL-18 TRUCKs gives hope
436 to the possibility for further translation to other cells and targets. This work is the basis to validate the
437 manufacturing process as well as the quality control methods including microbiological examination
438 carried out in accordance with Ph. Eur. chapter 2.6.27, the endotoxin testing according to Ph. Eur.
439 Chapter 2.6.14 and the mycoplasma testing according to Ph. Eur. chapter 2.6.7, respectively. As a next
440 step, the concept can enter early clinical development to benefit patients with GD2-expressing cancers.

Molecular Therapy Methods & Clinical Development

441 MATERIALS AND METHODS

442 **Human sample materials**

443 For the manufacturing of TRUCK cells in a clinical-scale process (n = 1) on the CliniMACS Prodigy®
444 (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany) and for the manufacturing of
445 laboratory-scale TRUCK cells (n = 1) a lymphapheresis product from a healthy donor was obtained
446 from the Institute for Transfusion Medicine of Hannover Medical School (MHH) after donors' written
447 informed consent. According to standard donation requirements, the donor had no signs of acute
448 infection and no previous history of blood transfusion.

449

450 **IL-18 TRUCK construct and production of lentiviral supernatants**

451 The generation of the lentiviral IL-18 TRUCK SIN vector was described previously[21]. In brief, the
452 lentiviral 3rd generation SIN vector pCCL.PPT.NFATsyn.hIL18.PGK.GD2CAR.PRE was used. The
453 human codon-usage optimized ORF second-generation CAR containing scFv (14.G2a), HCH2CH3
454 hinge, CD28, CD137 (4-1BB), and CD3ζ domains was flanked by restriction enzymes AgeI and SalI
455 and cloned into the lentiviral "all-in-one" SIN vector driven by an hPGK promoter[45]. The construct
456 was validated by sequencing (Microsynth SeqLab, Germany). Additional information is available on
457 request.
458 Lentiviral vector particles were generated as described previously [46] [21]. Briefly, 5×10^6 293T cells
459 were used for calcium phosphate transfection in the presence of 25 µM chloroquine. For transfection,
460 the following plasmids were used: lentiviral vector plasmid (10 µg), pcDNA3.HIV-1.GP.4 × CTE
461 (lentiviral gag/pol) (12 µg)[47], pRSV-Rev (5 µg; kindly provided by T. Hope, Northwestern University
462 Chicago, IL), and VSVg-encoding pMD.G (1.5 µg[48]. For better standardization, pcDNA3.HIV-
463 1.GP.4×CTE, pRSV-Rev and pMD.G were produced and purified by PlasmidFactory (Bielefeld,
464 Germany). 36h and 48h post transfection, supernatants were harvested and concentrated via
465 ultracentrifugation at 4°C and 13,238× g or 82,740× g for 16h or 2h, respectively. The particles were
466 resuspended in TexMACS™ GMP medium. Lentiviral supernatant was titrated in HT1080 fibroblasts
467 via spinoculation-mediated transduction, i. e. 1×10^5 cells were seeded, the supernatant containing
468 viral particles and 4 µg/ml protamine sulfate (Sigma-Aldrich, St. Louis, USA) were added and cells

were centrifuged (1h, 800× g, 37°C). Three days post transduction, transduction efficiency was determined via flow cytometric staining of GD2-CAR expression and functional viral vector titers were calculated from samples with GD2-CAR expression percentages of ≤30% to avoid cells with multiple integrations [49 21].

GMP-compliant manufacturing of IL-18 TRUCKs targeting GD2 with CliniMACS Prodigy® (clinical-scale process)

Instrumentation and tubing set

GMP-compliant manufacturing of IL-18 TRUCKs was performed using the CliniMACS Prodigy® platform, which allows automated cell processing in a closed system controlled by operating software version V1.3 and process software for T cell transduction (TCT) version V2.0 (released). For overview of clinical-scale process see **Figure 1**. Within the scope of the automatically running process, the input of different variable process parameter like time points of transduction, media exchange, culture wash, harvesting and volume of media exchange is possible (activity matrix). Installation of CliniMACS Prodigy® Tubing Set TS520 as well as operational interventions were carried out via the graphical user interface (GUI) of the Prodigy. Components of the Prodigy include bag hangers for cell products and reagents as well as pinch valves, a peristaltic pump and liquid sensors to operate fluids. The gas mix unit controls the atmosphere in the culture chamber, which is integral part of the TS520 tubing set used for the entire process. The centrifugation- and culture chamber (CentriCult Chamber) is a central component of the TS520 and allows the important automated process steps like washing of cells, media exchange and sampling. Integrated sample pouches allow sampling without opening the system. Prepared buffer, media, starting cell material and vector were connected directly to TS520 via sterile tubing welder device (TSCDII Terumo BCT).

Buffer, media, reagents, culture conditions, and operational sequence

If available, clinical grade materials for IL-18 TRUCK cell production were used. Detailed information regarding the materials was recorded, including the supplier, lot number, and expiration date. GMP-compliant reagents obtained from Miltenyi Biotec were used if not otherwise stated. Starting material for manufacturing of IL-18 TRUCKs were $CD3^+$ T cells derived from a non-mobilized lymphapheresis. Cell processing started within 24 h after product collection with

immunomagnetic enrichment of 1 x 10^9 $CD4^+$ and $CD8^+$ T cells using CliniMACS® CD4 Reagent, CliniMACS® CD8 Reagent and CliniMACS® PBS/EDTA buffer supplemented with human serum albumin (HSA, Human albumin 200 g/L Baxalta, Shire Deutschland GmbH, Berlin, Germany) to a final concentration of 0.5%. For cultivation, the basal medium TexMACS™ GMP medium was supplemented with 12.5 ng/ml MACS GMP Recombinant Human IL-7, 12.5 ng/ml MACS GMP Recombinant Human IL-15 and 3% heat-inactivated human AB serum (pool-human serum P-HS/Tü, Centre for Clinical Transfusion Medicine Tübingen/ZKT, Germany) until day 5. T cells were activated for 72h (day 0 to day 3) with CD3/CD28 MACS GMP T Cell TransAct Beads. On day 1 of culture transduction took place by adding 10 ml lentiviral vector with a multiplicity of infection (MOI) of 29 in total volume of 100 ml. At day 3 CD3/CD28 T cell TransAct beads and non-bound vector were washed out (culture wash) and culture volume increased to 200 ml. The culture was fed every 12 – 24h after day 5 of culture. Hereby the concentration of AB serum in the culture was reduced continuously using supplemented TexMACS™ GMP medium without AB serum for further medium exchange. On day of harvest (day 12), cells were formulated in Composol PS (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) with 2.86% (w/v) HSA for later cryopreservation. During cultivation, the temperature and atmosphere was maintained at 37°C with 5% CO_2. After 3 days of static culture shaking modus was activated (culture agitation) enabling high cell concentrations in the limited volume of the CentriCult Chamber.

Monitoring of culture and in process controls

Total cell number and viability was analyzed by flow cytometric analysis as described below. The glucose concentration of the cell-free culture supernatant was determined with the blood glucose meter Accu-Chek® Aviva (Roche, Mannheim, Germany). For analysis of the pH-value during cell cultivation pH-indicator strips MColorpHast™ pH 6.5 - 10.0 (Merck Millipore, Darmstadt, Germany) were used.

Flow cytometric characterization of IL-18 TRUCK manufactured in clinical-scale process

Flow cytometric analysis was performed by using anti-human monoclonal antibodies. Cellular composition antibody table is shown in **Supplement Table S1**. Transduction efficiency was determined by GD2-CAR detection with the antibody Ganglidiomab, which was conjugated with PE

524 by Miltenyi Biotec, herein after referred to as Ganglidiomab-PE. The antigen-specific single-chain
525 variable fragment (scFv) of the GD2-CAR is derived from the anti-GD2 antibody 14G2a [50 51].
526 Ganglidiomab is a monoclonal anti-idiotype antibody to 14G2a and therefore allows a direct detection
527 of the GD2-CAR on cells. Subsequently after antibody staining (10 min at RT) cells were incubated
528 (10 min at RT) with freshly prepared red blood cell lysis solution (Miltenyi Biotec). T cell phenotype
529 antibody panel is shown in **Supplement Table S1**. After antibody staining (10 min at RT in PBS
530 supplemented with 4% FCS) cells were washed and resuspended with PBS supplemented with 4%
531 FCS (both Merck, Darmstadt, Germany). Prior to flow cytometric analysis 7-AAD and Flow-Count
532 Fluorospheres (both Beckman Coulter) were added to the samples for dead cell discrimination and
533 single platform cell quantification, respectively. Flow cytometric analysis was performed with the
534 Navios flow cytometer (Navios 3L 10C, Software 1.3, Beckman Coulter). For gating strategies see
535 **Supplement Figures S5, 6**.

536 **Cell lines and cell culture**
537 293T cells (#ACC 635; DSMZ, Brunswick, Germany) were cultured in Dulbecco's modified Eagle's
538 medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine
539 serum (FBS, Sigma-Aldrich, St. Louis, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1
540 mM sodium pyruvate (all PAN Biotech, Aidenbach, Germany). The fibrosarcoma cell line HT1080
541 (#ACC 315; DSMZ, Brunswick, Germany), HT1080 GD2 equipped with GD2 by retroviral
542 transduction of a GD3S/GD2S transgene, and the neuroblastoma cell line SH-SY5Y (#ACC209;
543 DSMZ, Brunswick, Germany) were used for functionality analysis. All target cells were cultivated in
544 Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FBS. SH-SY5Y
545 additionally received 1 mM sodium pyruvate (c.c.pro, Oberdorla, Germany). NK-92 (#ACC 488;
546 DSMZ, Brunswick, Germany) were cultivated in RPMI 1640 medium (Lonza, Basel, Switzerland)
547 supplemented with 10% FBS and 2 mM L-glutamine (c.c.pro, Oberdorla, Germany). THP-1 (human
548 acute monocytic leukemia, #ACC 16; DSMZ, Brunswick, Germany) cells were cultured in RPMI-
549 1640 medium with 2mM L-glutamine, 2-mercaptoethanol to a final concentration of 0.05 mM, 10%
550 (v/v) heat-inactivated fetal bovine serum (HI-FBS), and 50 IU/ml penicillin and 50 µg/ml
551 streptomycin. All cells were tested for mycoplasma contamination on a regular basis using the

552 MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland) according to the manufacturer's
553 protocol.

554 **Laboratory manufacturing of IL-18 TRUCKs targeting GD2 (laboratory-scale process)**

555 $CD4^+$ and $CD8^+$ T cells isolated by the CliniMACS Prodigy® (see clinical-scale manufacturing) were
556 transduced and expanded as previously described[22]. Briefly, they were activated with anti-CD3/CD28
557 beads (Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1:1 in TexMACS™ (Miltenyi
558 Biotec) with 3% human serum (c.c.pro, Oberdorla, Germany) supplemented with 12.5 ng/ml IL-7 and
559 IL-15 (PeproTech, Rocky Hill, NJ, USA). On the following day, T cells were either left untransduced
560 or transduced with lentiviral particles by spinoculation using an MOI of 7 and addition of 5 µg/ml
561 Polybrene Infection/Transfection Reagent (Merck Millipore, Burlington, MA, USA). The anti-
562 CD3/CD28 beads were removed on the following day and cells were further cultivated in
563 TexMACS™ medium supplemented with 3% human serum, 12.5 ng/ml IL-7 and IL-15 and splitting
564 1:2 every 2-3 days for a total expansion time of 12 days.

565 **Cryopreservation and thawing of IL-18 TRUCKs targeting GD2**

566 Untransduced and transduced T cells were cryopreserved in Composol® PS/2.86% HSA after
567 manufacturing by adjusting the cell counts and addition of DMSO (CryoSure-DMSO, USP grade;
568 WAK Chemie, Steinbach, Germany) to final concentration of 10% (v/v). After cryopreservation in a <
569 -80°C freezer overnight the cells were stored in the vapor phase above liquid nitrogen at < -140°C. For
570 T cell phenotype analysis cells were thawed in RPMI-1640 medium with 20% FCS (both Merck,
571 Darmstadt, Germany) and rested for 1h in RPMI-1640 with 10% FCS (37°C, 5% CO_2) before flow
572 cytometric analysis. For functional analysis of the cryopreserved cells, they were thawed and seeded in
573 TexMACS™ medium in a cell density of 2.5 x 10^6 cells/ml and resting them overnight.

574 **Co-culture of laboratory- and clinical-scale IL-18 TRUCKs with target cells**

575 Directly after expansion or after cryopreservation and thawing (cryo), functionality of the laboratory-
576 and clinical-scale IL-18 TRUCKs in comparison to untransduced T cells was assessed by co-culturing
577 them with target cells. For flow cytometry and soluble mediator measurements, 5 x 10^4 target cells
578 were seeded in 800 µL of their respective culture medium, which was removed after 24h followed by

Molecular Therapy Methods & Clinical Development

addition of effector cells according to the specified effector-to-target (E:T) ratio in 800 µL CTL medium. For cytotoxicity, microscopy and intracellular cytokine assessment, 2 x 10^4 target cells were seeded in 200µL and co-cultured with effector cells accordingly.

Flow cytometry of laboratory-scale experiments

The antibodies used for flow cytometric analysis are listed in **Supplement Table S1**. The transduction efficiency was analyzed with the Ganglidiomab-PE mAb. Intracellular staining of TNF-α was performed by using the IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Samples were read on a BD FACSCanto™ Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). To determine cytotoxicity of engineered T cells, target cells were gated as $CD3^-$ cells. Percentage of killed cells was normalized to untransduced cell co-cultures using the following formula:

$$CD3^- \text{ Killing} = 100\% - \frac{CD3^- \text{ frequency (co-culture)}}{CD3^- \text{ frequency (respoective co-culture with untransduced T cells)}}$$

Multiplex cytokine analysis

Cytokine concentrations in the supernatant were determined using a customized LEGENDplex™ Multi-Analyte Flow Assay (BioLegend, San Diego, CA, USA), which allowed for the detection of human IL-2, IL-4, IL-10, IL-18, granzyme B, perforin, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. Samples were analyzed with LEGENDplex v8.0 software (BioLegend, San Diego, CA, USA).

Determination of cytotoxicity by LDH Assay

The release of lactate dehydrogenase (LDH) into the cell culture supernatant was assessed by using the Cytotoxicity Detection Kit (Roche, Basel, Switzerland). Cells lysed by adding Triton X-100 (Merck, Darmstadt, Germany) to a final concentration of 1% to all control wells served as maximum controls. Absorbance was assessed at a wavelength of 490 nm with a reference wavelength of 690 nm on a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). LDH release (%) was calculated according to the manufacturer's protocol.

Microscopy

Transmitted-light microscope images of co-cultures of target and effector cells were taken with an Olympus IX81 microscope combined with a digital B/W camera using 10x objective lenses and analyzed with Xcellence Pro image software (all from Olympus, Hamburg, Germany). Representative pictures are shown.

XCelligence

Target cell killing by cryopreserved and thawed TRUCKs was furthermore determined with the XCelligence RTCA S16 Real Time Cell Analyzer and using E-Plates 16 PET (both ACEA Biosciences, San Diego, CA, USA). Background impedance of all wells was assessed with cell culture medium measurements. Afterwards, target cells were seeded in an amount of 1 x 10^5 cells (SH-SY5Y) or 2 x 10^4 cells (HT1080, HT1080-GD2) in 200µL of their respective culture medium and adhesion was checked by measurements every 30 min. T cells were added after target cell adhesion shown by a constant impedance after 19 - 25h. For this, 150 µL medium were carefully removed and replaced by T cells in the specified E:T-ratios in 150 µL CTL medium. Impedance was measured every 30 min. Cell indices were normalized to the respective indices after T cell addition.

Cell Migration Assay

To test the chemoattractive potential of supernatants that were derived from co-culture experiments of primary human T cells transduced with the IL-18 TRUCK vector and $GD2^+$ target cells, a migration assay with THP-1 or NK-92 cells was performed using a Boyden chamber (NeuroProbe, Gaithersburg, MD, USA). In brief, a 96-well plate (Sarstedt, flat bottom) was first filled with 300 µL Agar per well. Then, 110 µL supernatant of co-culture experiments was added per well and the plate was placed in the Boyden chamber using a spacer. Afterward, a polycarbonate membrane (8 µm pore size, NeuroProbe, Gaithersburg, USA) was placed over the plate and the chamber was closed. Subsequently, 200 µL THP-1 or NK-92 cells (5 × 10^5 cells/well) were added to the upper chamber. The entire chamber was incubated for 4h at 37°C and 5% CO_2. The membrane was removed, and cells attached to the top of the membrane were removed with a scraper. Migrated cells attached to the lower side and inside the membrane were analyzed by fixing and staining the membrane with methanol for

631 10 min (Roth, Karlsruhe, Germany) and Giemsa for 1h, respectively. The cell number was calculated
632 using an Olympus IX71 microscope.

633 **Isolation of genomic DNA and determination of the VCN by qPCR**

634 Genomic DNA was isolated from 1 x 10^6 transduced or untransduced cells (-20°C frozen cell pellets)
635 with the QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's
636 protocol. Standard curve quantification was performed with five serial dilutions (1 x 10^6 -1 x 10^2
637 copies) of the PTBP2-WPRE copy gene containing vector "pQPCR-Stdx4". Specific primers for
638 WPRE forward GAGGAGTTGTGGCCCGTTGT; reverse: TGACAGGTGGTGGCAATGCC; probe
639 CTGTGTTTGCTGACGCAAC (5'-FAM-3'-BHQ1) PTBP2 forward
640 TCTCCATTCCCTATGTTCATGC; reverse GTTCCCGCAGAATGGTGAGGTG; probe
641 ATGTTCCTCGGACCAACTTG (5'-JOE-3'-BHQ1) (BioSpring, Frankfurt am Main, Germany) [52]
642 were used with the TaqMan® Fast Advanced Mastermix (Thermo Fisher Scientific, Darmstadt,
643 Germany) and the StepOnePlus™ Real-Time PCR Detection System (Applied Biosystems, Darmstadt,
644 Germany). Cycling conditions were 95°C 2 min (initial step); 95°C 1 sec; 58°C 5 sec (data
645 accumulation) with 40 cycles. VCN was calculated as the ratio of WPRE copy number (multiplied
646 with factor 2) to PTBP2 copy number multiplied with the transduction efficiency (factor 0.75). All
647 samples were tested in triplicate, and negative controls were included to monitor sample cross-
648 contamination. The qPCR was performed according to the MIQE guidelines to facilitate
649 standardization [53].

650 SUPPLEMENTAL INFORMATION

651

652 AUTHOR CONTRIBUTIONS

653 Conceptualization: RE, WG, BEV, ACD, UK, KZ, AS; methodology: ACD, WG, RE, MM, AME,
654 CK, KZ, AS; formal analysis: WG, ACD, RE, AME, MM, CK, KZ, KA; resources: HNL, NS, CR;
655 writing original draft application: WG, ACD, RE, AME; writing review and editing: All authors;
656 supervision: HA, LA, TM, RB, LG, AS, BEV, UK; funding acquisition: BEV, HA, TM, RB, AS, UK,
657 CR. All authors have read and agreed to the published version of the manuscript.

658

659 CONFLICTS OF INTEREST

660 The authors declare no conflict of interest, except that authors HA, AS, and KZ have submitted a
661 patent application describing the "all-in-one" TRUCK vector technology. HA serves on the Scientific
662 Advisory Board of Miltenyi Biotec. The University Medicine of Greifswald licensed Ganglidiomab to
663 AnYxis Immuno-oncology for commercialization.

664

665 ACKNOWLEDGEMENTS

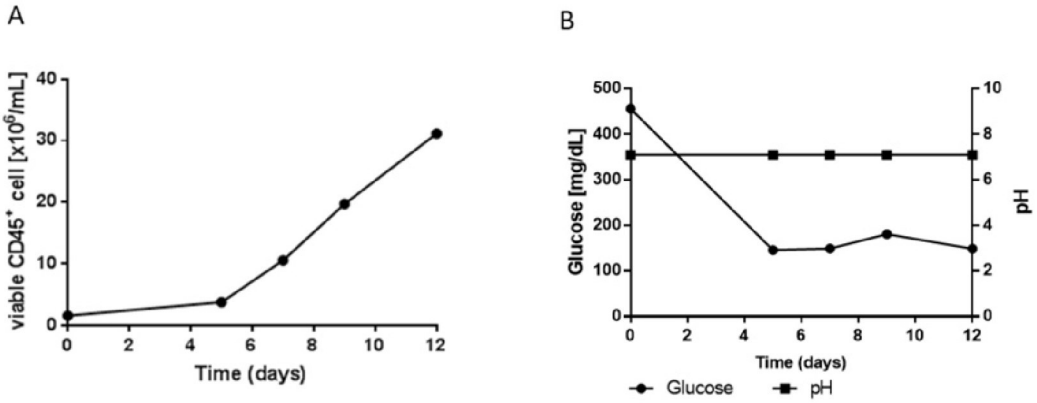
666 This work was supported by the following grants: "From CARs to TRUCKs: Induction of a concerted
667 antitumor immune response by engineered T cells" (Deutsche Krebshilfe/German Cancer Aid-Priority
668 Program in Translational Oncology #111975) and DFG-funded SFB738 (projects C4 & C9) and
669 Cluster of Excellence REBIRTH (EXC62/2). The pQPCR-Stdx4"-plasmid was kindly provided by
670 Michael Rothe. We thank Anna-Magdalena Bartels, Andrea Quaiser, Sarina Lukis and Elvira Schulde
671 for excellent technical assistance. Furthermore, we would like to thank Dr. Bastian Schirmer and Prof.
672 Dr. Detlef Neumann (Institute of Pharmacology, Hannover Medical School) for providing the
673 XCelligence RTCA S16 Real Time Cell Analyzer. We thank Thea Boehm and Caren Clark (Miltenyi
674 Biotec) for helpful discussions and valuable technical advice for the development of process protocol
675 for automated cell-manufacturing.

676 STATEMENT OF ETHICS

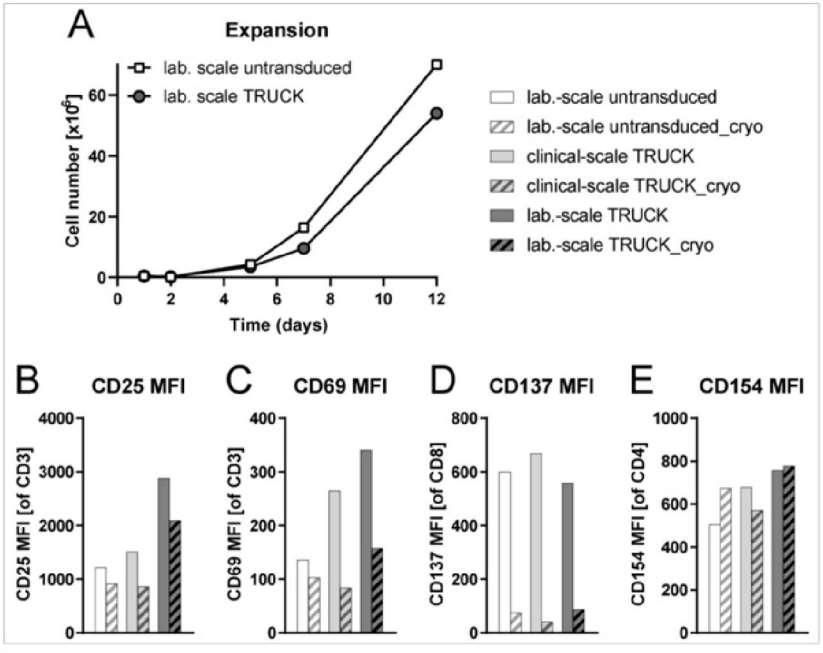
677 For this study donors gave their written informed consent and approval was granted by the
678 corresponding ethics committee (No. 2829-2015). The study was approved by the ethics committee of
679 Hannover Medical School (2519-2014, 2830-2015, 3639-2017).

Molecular Therapy Methods & Clinical Development

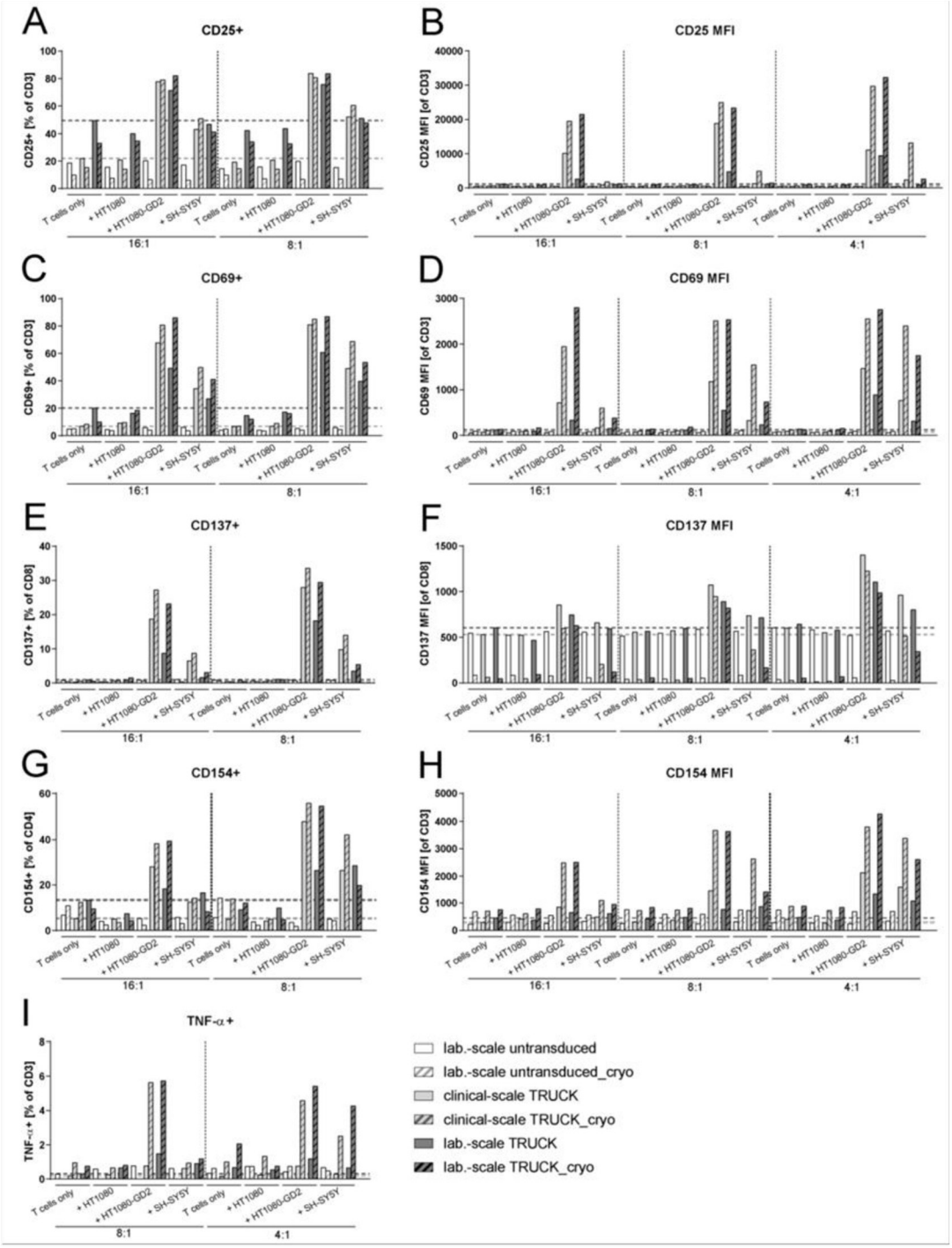
SUPPLEMENTARY FIGURES



Supplementary Figure S1. Monitoring of viable CD45$^+$ cell concentration, pH-value and glucose. **(A)** Concentration of viable CD45$^+$ cells**.** The starting cell concentration was 1.5 x 10^6 viable CD45$^+$ cells/ml. During expansion and media exchange cell concentration achieved 31.16 x 10^6 CD45$^+$ cells/ml. After increase of culture volume on day 1 (transduction) to 100 ml and on day 3 (culture wash) the volume kept constant at 200 ml. From day 3 after culture wash for removal of vector and activation reagent the culture was shaken automatically. **(B)** Monitoring of culture conditions with measurement of pH and concentration of glucose during 12-day cultivation.

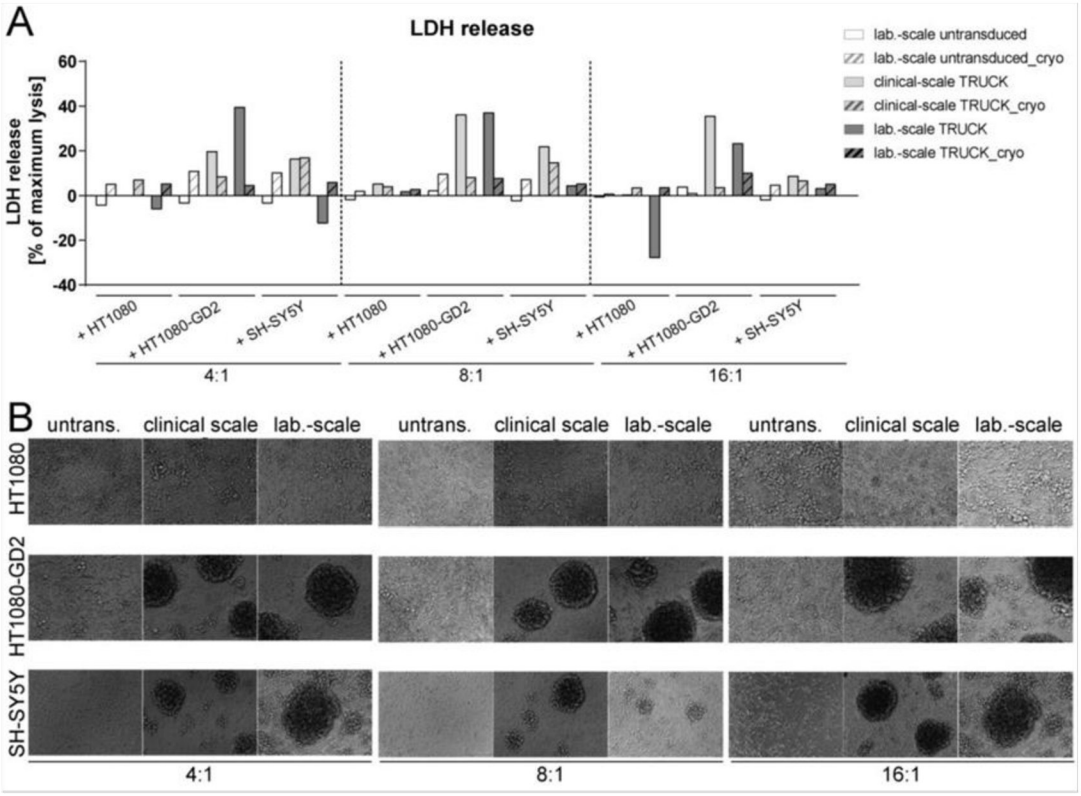


Supplementary Figure S2. Expansion of laboratory-scale TRUCKs and pre-activation of clinical- and laboratory-scale TRUCKs: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were either characterized directly after generation or after cryopreservation and thawing (cryo). n=1. **(A)** The expansion of untransduced TC and TRUCKs generated in the laboratory scale with a starting population of 6.3 x 10^5 cells was determined by cell counting. **(B-E)** Expression of the activation markers **(B)** CD25 on CD3$^+$, **(C)** CD69 on CD3$^+$, **(D)** CD137 on CD8$^+$ and **(E)** CD154 on CD4$^+$ T cells as median fluorescence intensity (MFI).

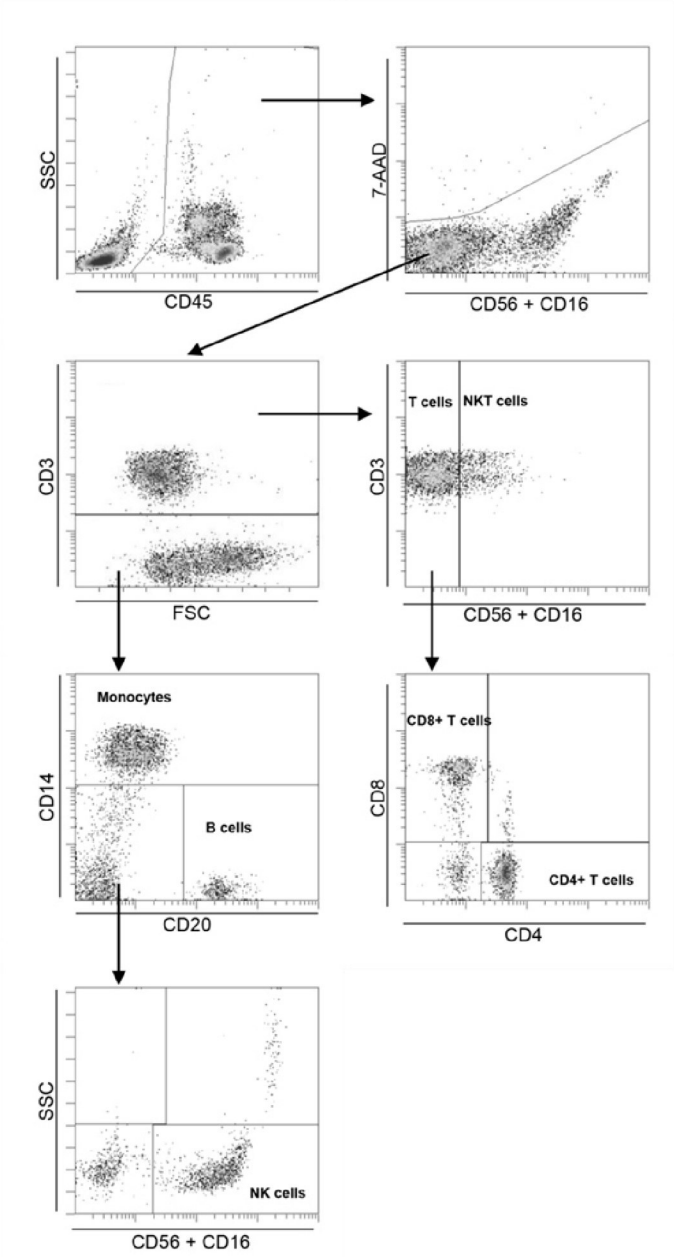


Supplementary Figure S3. Clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically respond to GD2$^+$ target cells with an increase of activation marker expression and release of TNF-α: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were tested for GD2-CAR-mediated activation either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells for 48h in the indicated effector-to-

target (E:T) ratios or cultivation alone (T cells only). n=1. **(A-E)** Expression of the activation markers **(A, B)** $CD25^+$ of $CD3^+$, **(C, D)** $CD69^+$ of $CD3^+$, **(E, F)** $CD137^+$ of $CD8^+$ and **(G, H)** $CD154^+$ of $CD4^+$ T cells as **(A, C, E, G)** frequency or **(B, D, F, H)** median fluorescence intensity (MFI). **(I)** The concentration of released TNF-α in the cell culture supernatants after 48h was assessed by LEGENDPlex™. **(A-I)** A dashed line indicates background levels of the respective expression by clinical-scale-manufactured (light grey) and laboratory-scale-manufactured (dark grey) TRUCKs cultured alone.

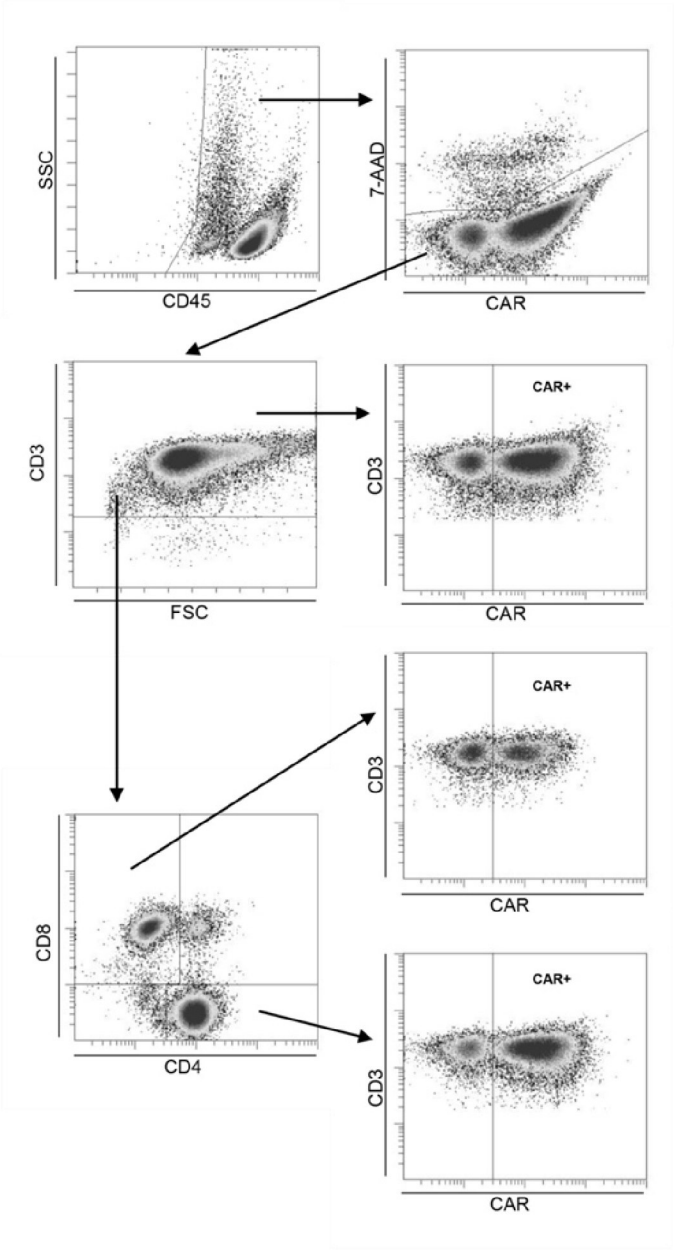


Supplementary Figure S4. Clinical-scale-manufactured GD2-specific IL-18 TRUCKs specifically eliminate $GD2^+$ target cells: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK) or under laboratory conditions (lab.-scale TRUCK). Untransduced T cells (lab.-scale untransduced) served as control. The manufactured cells were tested for cytotoxicity either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells and in the indicated effector-to-target (E:T) ratios for 48 h. n=1. **(A)** The release of lactate dehydrogenase (LDH) into the cell culture supernatant as an indicator of the killing of target cells by T cells. LDH levels are expressed as a percentage of the maximum lysis level obtained using controls lysed with 1% Triton X-100. **(B)** Transmitted-light microscope images of co-cultures of cryopreserved effector cells with target cells taken by an Olympus IX81 microscope combined with a digital B/W camera using 10x objective lenses.



730

731 **Supplementary Figure S5. Gating strategy to determine cellular composition during clinical-**
732 **scale process manufactured IL-18 TRUCKs by flow cytometry exemplarily shown for**
733 **lymphapheresis on process day -1:** Cells were stained for surface antigens and analyzed based on
734 their expression of different markers: T cells (7-AAD$^-$ CD45$^+$ CD3$^+$ CD56$^-$/CD16$^-$), CD4$^+$ T cells (7-
735 AAD$^-$ CD45$^+$ CD3$^+$ CD56$^-$/CD16$^-$ CD4$^+$), CD8$^+$ T cells (7-AAD$^-$ CD45$^+$ CD3$^+$ CD56$^-$/CD16$^-$ CD8$^+$),
736 NKT cells (7-AAD$^-$ CD45$^+$ CD3$^+$ CD56$^+$/CD16$^+$), NK cells (7-AAD$^-$ CD45$^+$ CD3$^-$ CD56$^+$/CD16$^+$), B
737 cells (7-AAD$^-$ CD45$^+$ CD3$^-$ CD20$^+$), monocytes (7-AAD$^-$ CD45$^+$ CD3$^-$ CD14$^+$).



738

739 **Supplementary Figure S6. Gating strategy to determine transduction rate during clinical-scale**
740 **process manufactured IL-18 TRUCKs by flow cytometry exemplarily shown for the final**
741 **product on process day 12:** Cells were stained for surface antigens and analyzed based on their
742 expression of different markers: $CD3^+CAR^+$ (7-AAD^- $CD45^+$ $CD3^+$ CAR^+), $CD4^+CAR^+$ (7-AAD^-
743 $CD45^+$ CD3+ $CD4^+$ CAR^+), $CD8^+CAR^+$ (7-AAD^- $CD45^+$ $CD3^+$ $CD8^+$ CAR^+).

744 **A**

Antibody	Clone	Company
CD3-FITC	REA613	Miltenyi Biotec
CD4-VioBlue	VIT4	Miltenyi Biotec
CD8-APC-Vio770	BW135/80	Miltenyi Biotec
CD14-APC	TÜK4	Miltenyi Biotec
CD16-PE	REA423	Miltenyi Biotec
CD20-PE-Vio770	REA780	Miltenyi Biotec
CD45-VioGreen	REA747	Miltenyi Biotec
CD56-PE	REA196	Miltenyi Biotec

745 **B**

Antibody	Clone	Company
CD3-PacificBlue	UCHT1	Beckman Coulter
CD4-Krome Orange	13B8.2	Beckman Coulter
CD8-APC-Vio770	BW135/80	Miltenyi Biotec
CD45RO-FITC	UCHL1	Becton Dickinson
CD95-APC	DX2	Becton Dickinson
CCR7-PE-Vio615	REA546	Miltenyi Biotec

746 **C**

Antibody	Clone	Company
CD3-PerCP	SK7	BioLegend
CD4-BV510	RPA-T4	BioLegend
CD4-PE-Cy7	SK3	BioLegend
CD8-AF700	SK1	BioLegend
CD8-APC	SK1	BioLegend
CD25-BV421	BC96	BioLegend
CD69-BV605	FN50	BioLegend
CD137-PE-Cy7	4B4-1	BioLegend
CD154-APC-Cy7	24-31	BioLegend
TNF-α-APC	Mab 11	BioLegend

747

748 **Supplementary Table S1.** List of antibodies except Ganglidiomab-PE Ab used for **(A)** clinical-scale
749 cell composition, **(B)** clinical-scale T cell phenotype and (C) laboratory-scale analyses.

Effector cells	E:T ratio	Target cells	IL-2 [pg/mL]	IL-4 [pg/mL]	IL-10 [pg/mL]	Granzyme B [pg/mL]	IFN-γ [pg/mL]	Perforin [pg/mL]	TNF-α [pg/mL]
untransduced	4:1	T cells only	< 2.53	< 2.71	1.55	< 38.40	< 4.05	< 1.31	78.60
clinical-scale	4:1	T cells only	< 2.53	< 2.71	1.55	< 38.40	7.40	2.07	43.71
lab.-scale	4:1	T cells only	< 2.53	< 2.71	1.88	101.12	8.54	< 1.31	75.19
untransduced	4:1	HT1080	< 2.53	< 2.71	1.55	< 38.40	< 4.05	< 1.31	95.32
clinical-scale	4:1	HT1080	< 2.53	2.83	< 1.51	< 38.40	9.71	2.69	< 5.80
lab.-scale	4:1	HT1080	< 2.53	< 2.71	1.63	< 38.40	7.40	1.48	111.55
untransduced	4:1	HT1080-GD2	< 2.53	< 2.71	< 1.51	< 38.40	7.40	< 1.31	64.87
clinical-scale	4:1	HT1080-GD2	3077.45	54.40	2.39	6404.04	3072.59	697.17	309.32
lab.-scale	4:1	HT1080-GD2	512.46	233.90	25.40	34451.98	2424.20	867.29	517.27
untransduced	4:1	SH-SY5Y	< 2.53	< 2.71	< 1.51	< 38.40	< 4.05	< 1.31	68.33
clinical-scale	4:1	SH-SY5Y	1151.79	44.12	2.48	5914.62	1772.37	425.38	218.86
lab.-scale	4:1	SH-SY5Y	94.35	59.44	6.23	7491.83	523.98	120.49	346.84
untransduced	8:1	T cells only	< 2.53	< 2.71	1.53	43.57	5.18	< 1.31	141.15
clinical-scale	8:1	T cells only	< 2.53	2.77	1.61	55.88	6.30	1.97	43.12
lab.-scale	8:1	T cells only	< 2.53	< 2.71	1.88	73.36	13.38	1.48	172.11
untransduced	8:1	HT1080	< 2.53	< 2.71	1.70	43.57	< 4.05	< 1.31	134.88
clinical-scale	8:1	HT1080	4.31	3.36	1.70	77.21	12.18	1.58	42.20
lab.-scale	8:1	HT1080	2.93	2.83	1.88	164.13	15.94	1.87	183.46
untransduced	8:1	HT1080-GD2	< 2.53	< 2.71	1.57	< 38.40	< 4.05	< 1.31	122.64
clinical-scale	8:1	HT1080-GD2	1705.64	37.69	2.98	6667.23	1888.73	513.02	284.13
lab.-scale	8:1	HT1080-GD2	186.60	204.50	47.34	27992.35	2829.72	767.30	980.73
untransduced	8:1	SH-SY5Y	< 2.53	< 2.71	1.57	43.57	4.65	< 1.31	65.01
clinical-scale	8:1	SH-SY5Y	525.44	38.08	2.76	7298.61	1990.83	418.72	414.67
lab.-scale	8:1	SH-SY5Y	23.87	13.94	4.08	5637.68	273.66	43.69	433.30
untransduced	16:1	T cells only	< 2.53	2.77	< 1.51	43.57	6.30	1.79	227.96
clinical-scale	16:1	T cells only	< 2.53	< 2.71	< 1.51	49.51	9.44	< 1.31	97.32
lab.-scale	16:1	T cells only	< 2.53	< 2.71	< 1.51	54.68	5.18	< 1.31	246.31
untransduced	16:1	HT1080	< 2.53	< 2.71	< 1.51	55.88	5.18	< 1.31	217.66
clinical-scale	16:1	HT1080	< 2.53	< 2.71	< 1.51	< 38.40	59.36	1.88	266.40
lab.-scale	16:1	HT1080	2.73	< 2.71	1.96	519.56	38.08	2.70	473.17
untransduced	16:1	HT1080-GD2	< 2.53	< 2.71	1.53	43.57	< 4.05	< 1.31	389.95
clinical-scale	16:1	HT1080-GD2	3581.84	82.39	6.24	27909.83	5222.66	1329.03	1190.55
lab.-scale	16:1	HT1080-GD2	148.36	160.80	55.08	35262.32	3784.14	553.18	1631.56
untransduced	16:1	SH-SY5Y	< 2.53	< 2.71	< 1.51	< 38.40	5.18	< 1.31	287.03
clinical-scale	16:1	SH-SY5Y	116.79	16.85	2.62	6874.00	2329.63	274.26	635.41
lab.-scale	16:1	SH-SY5Y	< 2.53	< 2.71	1.86	1698.36	141.23	4.80	353.22

Supplementary Table S2. Cryopreserved clinical-scale-manufactured IL-18 TRUCKs targeting GD2 specifically react to target recognition with an increase of soluble mediator release: IL-18 TRUCKs targeting GD2 were generated using the CliniMACS Prodigy® (clinical-scale) or under laboratory conditions (lab.-scale). Untransduced T cells (untransduced) served as control. The manufactured cells were tested for functionality by co-cultivation with the indicated target cells in the indicated effector-to-target (E:T) ratios or cultivation alone (T cells only) after cryopreservation and thawing. The concentration of released cytokines in the cell culture supernatants after 48h was assessed by LEGENDPlex™.

759 REFERENCES

760

761 1. Kagoya, Y, Tanaka, S, Guo, T, Anczurowski, M, Wang, CH, Saso, K, *et al.* (2018). A novel
762 chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior
763 antitumor effects. *Nat Med* **24**: 352-359.
764 2. Chmielewski, M, Kopecky, C, Hombach, AA, and Abken, H (2011). IL-12 release by
765 engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-
766 independent macrophage response on tumor cells that have shut down tumor antigen
767 expression. *Cancer Res* **71**: 5697-5706.
768 3. Chinnasamy, D, Yu, Z, Kerkar, SP, Zhang, L, Morgan, RA, Restifo, NP, *et al.* (2012). Local
769 delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple
770 vascularized tumors in mice. *Clin Cancer Res* **18**: 1672-1683.
771 4. Chmielewski, M, and Abken, H (2017). CAR T Cells Releasing IL-18 Convert to T-Bet(high)
772 FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell*
773 *Rep* **21**: 3205-3219.
774 5. Tokarew, N, Ogonek, J, Endres, S, von Bergwelt-Baildon, M, and Kobold, S (2019). Teaching
775 an old dog new tricks: next-generation CAR T cells. *Br J Cancer* **120**: 26-37.
776 6. Svennerholm, L, Bostrom, K, Fredman, P, Jungbjer, B, Lekman, A, Mansson, JE, *et al.*
777 (1994). Gangliosides and allied glycosphingolipids in human peripheral nerve and spinal cord.
778 *Biochim Biophys Acta* **1214**: 115-123.
779 7. Seitz, CM, Schroeder, S, Knopf, P, Krahl, AC, Hau, J, Schleicher, S, *et al.* (2020). GD2-
780 targeted chimeric antigen receptor T cells prevent metastasis formation by elimination of
781 breast cancer stem-like cells. *Oncoimmunology* **9**: 1683345.
782 8. Chulanetra, M, Morchang, A, Sayour, E, Eldjerou, L, Milner, R, Lagmay, J, *et al.* (2020).
783 GD2 chimeric antigen receptor modified T cells in synergy with sub-toxic level of
784 doxorubicin targeting osteosarcomas. *Am J Cancer Res* **10**: 674-687.
785 9. Navid, F, Santana, VM, and Barfield, RC (2010). Anti-GD2 antibody therapy for GD2-
786 expressing tumors. *Curr Cancer Drug Targets* **10**: 200-209.
787 10. Golinelli, G, Grisendi, G, Prapa, M, Bestagno, M, Spano, C, Rossignoli, F, *et al.* (2020).
788 Targeting GD2-positive glioblastoma by chimeric antigen receptor empowered mesenchymal
789 progenitors. *Cancer Gene Ther* **27**: 558-570.
790 11. Kailayangiri, S, Altvater, B, Spurny, C, Jamitzky, S, Schelhaas, S, Jacobs, AH, *et al.* (2017).
791 Targeting Ewing sarcoma with activated and GD2-specific chimeric antigen receptor-
792 engineered human NK cells induces upregulation of immune-inhibitory HLA-G.
793 *Oncoimmunology* **6**: e1250050.
794 12. Casey, DL, Lin, TY, and Cheung, NV (2019). Exploiting Signaling Pathways and Immune
795 Targets Beyond the Standard of Care for Ewing Sarcoma. *Front Oncol* **9**: 537.
796 13. Heczey, A, Louis, CU, Savoldo, B, Dakhova, O, Durett, A, Grilley, B, *et al.* (2017). CAR T
797 Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients
798 with Neuroblastoma. *Mol Ther* **25**: 2214-2224.
799 14. Louis, CU, Savoldo, B, Dotti, G, Pule, M, Yvon, E, Myers, GD, *et al.* (2011). Antitumor
800 activity and long-term fate of chimeric antigen receptor-positive T cells in patients with
801 neuroblastoma. *Blood* **118**: 6050-6056.
802 15. Straathof, K, Flutter, B, Wallace, R, Jain, N, Loka, T, Depani, S, *et al.* (2020). Antitumor
803 activity without on-target off-tumor toxicity of GD2-chimeric antigen receptor T cells in
804 patients with neuroblastoma. *Sci Transl Med* **12**.
805 16. Cauchon, NS, Oghamian, S, Hassanpour, S, and Abernathy, M (2019). Innovation in
806 Chemistry, Manufacturing, and Controls-A Regulatory Perspective From Industry. *J Pharm*
807 *Sci* **108**: 2207-2237.
808 17. Mock, U, Nickolay, L, Philip, B, Cheung, GW, Zhan, H, Johnston, ICD, *et al.* (2016).
809 Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy
810 using CliniMACS prodigy. *Cytotherapy* **18**: 1002-1011.
811 18. Lock, D, Mockel-Tenbrinck, N, Drechsel, K, Barth, C, Mauer, D, Schaser, T, *et al.* (2017).
812 Automated Manufacturing of Potent CD20-Directed Chimeric Antigen Receptor T Cells for
813 Clinical Use. *Hum Gene Ther* **28**: 914-925.

19. Priesner, C, Aleksandrova, K, Esser, R, Mockel-Tenbrinck, N, Leise, J, Drechsel, K, *et al.* (2016). Automated Enrichment, Transduction, and Expansion of Clinical-Scale CD62L(+) T Cells for Manufacturing of Gene Therapy Medicinal Products. *Hum Gene Ther* **27**: 860-869.
20. Aleksandrova, K, Leise, J, Priesner, C, Melk, A, Kubaink, F, Abken, H, *et al.* (2019). Functionality and Cell Senescence of CD4/ CD8-Selected CD20 CAR T Cells Manufactured Using the Automated CliniMACS Prodigy(R) Platform. *Transfus Med Hemother* **46**: 47-54.
21. Zimmermann, K, Kuehle, J, Dragon, AC, Galla, M, Kloth, C, Rudek, LS, *et al.* (2020). Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-GD2 CAR Expression and Inducible Cytokines. *Cancers (Basel)* **12**.
22. Dragon, AC, Zimmermann, K, Nerreter, T, Sandfort, D, Lahrberg, J, Kloss, S, *et al.* (2020). CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation. *J Immunother Cancer* **8**.
23. Panch, SR, Srivastava, SK, Elavia, N, McManus, A, Liu, S, Jin, P, *et al.* (2019). Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics. *Mol Ther* **27**: 1275-1285.
24. Blaeschke, F, Stenger, D, Kaeuferle, T, Willier, S, Lotfi, R, Kaiser, AD, *et al.* (2018). Induction of a central memory and stem cell memory phenotype in functionally active CD4(+) and CD8(+) CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19(+) acute lymphoblastic leukemia. *Cancer Immunol Immunother* **67**: 1053-1066.
25. Stroncek, DF, Lee, DW, Ren, J, Sabatino, M, Highfill, S, Khuu, H, *et al.* (2017). Elutriated lymphocytes for manufacturing chimeric antigen receptor T cells. *J Transl Med* **15**: 59.
26. Pettitt, D, Arshad, Z, Smith, J, Stanic, T, Hollander, G, and Brindley, D (2018). CAR-T Cells: A Systematic Review and Mixed Methods Analysis of the Clinical Trial Landscape. *Mol Ther* **26**: 342-353.
27. Zhu, F, Shah, N, Xu, H, Schneider, D, Orentas, R, Dropulic, B, *et al.* (2018). Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS Prodigy device at an academic medical center. *Cytotherapy* **20**: 394-406.
28. Zhang, W, Jordan, KR, Schulte, B, and Purev, E (2018). Characterization of clinical grade CD19 chimeric antigen receptor T cells produced using automated CliniMACS Prodigy system. *Drug Des Devel Ther* **12**: 3343-3356.
29. Castella, M, Boronat, A, Martin-Ibanez, R, Rodriguez, V, Sune, G, Caballero, M, *et al.* (2019). Development of a Novel Anti-CD19 Chimeric Antigen Receptor: A Paradigm for an Affordable CAR T Cell Production at Academic Institutions. *Mol Ther Methods Clin Dev* **12**: 134-144.
30. Castella, M, Caballero-Banos, M, Ortiz-Maldonado, V, Gonzalez-Navarro, EA, Sune, G, Antonana-Vidosola, A, *et al.* (2020). Point-Of-Care CAR T-Cell Production (ARI-0001) Using a Closed Semi-automatic Bioreactor: Experience From an Academic Phase I Clinical Trial. *Front Immunol* **11**: 482.
31. Zhao, Y, Stepto, H, and Schneider, CK (2017). Development of the First World Health Organization Lentiviral Vector Standard: Toward the Production Control and Standardization of Lentivirus-Based Gene Therapy Products. *Hum Gene Ther Methods* **28**: 205-214.
32. Vedvyas, Y, McCloskey, JE, Yang, Y, Min, IM, Fahey, TJ, Zarnegar, R, *et al.* (2019). Manufacturing and preclinical validation of CAR T cells targeting ICAM-1 for advanced thyroid cancer therapy. *Sci Rep* **9**: 10634.
33. Busch, DH, Frassle, SP, Sommermeyer, D, Buchholz, VR, and Riddell, SR (2016). Role of memory T cell subsets for adoptive immunotherapy. *Semin Immunol* **28**: 28-34.
34. Rafiq, S, Hackett, CS, and Brentjens, RJ (2020). Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat Rev Clin Oncol* **17**: 147-167.
35. Martinez, M, and Moon, EK (2019). CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol* **10**: 128.
36. Kawalekar, OU, RS, OC, Fraietta, JA, Guo, L, McGettigan, SE, Posey, AD, Jr., *et al.* (2016). Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity* **44**: 712.

868 37. Kunert, A, Chmielewski, M, Wijers, R, Berrevoets, C, Abken, H, and Debets, R (2017). Intra-
869 tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and
870 counteracts immune evasion of solid tumors. *Oncoimmunology* **7**: e1378842.
871 38. Zhang, L, Kerkar, SP, Yu, Z, Zheng, Z, Yang, S, Restifo, NP, *et al.* (2011). Improving
872 adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor
873 environment. *Mol Ther* **19**: 751-759.
874 39. Ahmed, N, Brawley, VS, Hegde, M, Robertson, C, Ghazi, A, Gerken, C, *et al.* (2015). Human
875 Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified
876 T Cells for the Immunotherapy of HER2-Positive Sarcoma. *J Clin Oncol* **33**: 1688-1696.
877 40. Feng, K, Guo, Y, Dai, H, Wang, Y, Li, X, Jia, H, *et al.* (2016). Chimeric antigen receptor-
878 modified T cells for the immunotherapy of patients with EGFR-expressing advanced
879 relapsed/refractory non-small cell lung cancer. *Sci China Life Sci* **59**: 468-479.
880 41. Beatty, GL, Haas, AR, Maus, MV, Torigian, DA, Soulen, MC, Plesa, G, *et al.* (2014).
881 Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor
882 activity in solid malignancies. *Cancer Immunol Res* **2**: 112-120.
883 42. Keu, KV, Witney, TH, Yaghoubi, S, Rosenberg, J, Kurien, A, Magnusson, R, *et al.* (2017).
884 Reporter gene imaging of targeted T cell immunotherapy in recurrent glioma. *Sci Transl Med*
885 **9**.
886 43. O'Rourke, DM, Nasrallah, MP, Desai, A, Melenhorst, JJ, Mansfield, K, Morrissette, JJD, *et al.*
887 (2017). A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates
888 antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl*
889 *Med* **9**.
890 44. Li, S, Siriwon, N, Zhang, X, Yang, S, Jin, T, He, F, *et al.* (2017). Enhanced Cancer
891 Immunotherapy by Chimeric Antigen Receptor-Modified T Cells Engineered to Secrete
892 Checkpoint Inhibitors. *Clin Cancer Res* **23**: 6982-6992.
893 45. Kailayangiri, S, Altvater, B, Lesch, S, Balbach, S, Gottlich, C, Kuhnemundt, J, *et al.* (2019).
894 EZH2 Inhibition in Ewing Sarcoma Upregulates GD2 Expression for Targeting with Gene-
895 Modified T Cells. *Mol Ther* **27**: 933-946.
896 46. Klapdor, R, Wang, S, Hacker, U, Buning, H, Morgan, M, Dork, T, *et al.* (2017). Improved
897 Killing of Ovarian Cancer Stem Cells by Combining a Novel Chimeric Antigen Receptor-
898 Based Immunotherapy and Chemotherapy. *Hum Gene Ther* **28**: 886-896.
899 47. Schambach, A, Bohne, J, Chandra, S, Will, E, Margison, GP, Williams, DA, *et al.* (2006).
900 Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-
901 methylguanine-DNA methyltransferase in hematopoietic cells. *Mol Ther* **13**: 391-400.
902 48. Yang, Y, Vanin, EF, Whitt, MA, Fornerod, M, Zwart, R, Schneiderman, RD, *et al.* (1995).
903 Inducible, high-level production of infectious murine leukemia retroviral vector particles
904 pseudotyped with vesicular stomatitis virus G envelope protein. *Hum Gene Ther* **6**: 1203-
905 1213.
906 49. Fehse, B, Kustikova, OS, Bubenheim, M, and Baum, C (2004). Pois(s)on--it's a question of
907 dose. *Gene Ther* **11**: 879-881.
908 50. Lode, HN, Schmidt, M, Seidel, D, Huebener, N, Brackrock, D, Bleeke, M, *et al.* (2013).
909 Vaccination with anti-idiotype antibody ganglidiomab mediates a GD(2)-specific anti-
910 neuroblastoma immune response. *Cancer Immunol Immunother* **62**: 999-1010.
911 51. Siebert, N, Seidel, D, Eger, C, Brackrock, D, Reker, D, Schmidt, M, *et al.* (2013). Validated
912 detection of anti-GD2 antibody ch14.18/CHO in serum of neuroblastoma patients using anti-
913 idiotype antibody ganglidiomab. *J Immunol Methods* **398-399**: 51-59.
914 52. Maetzig, T, Galla, M, Brugman, MH, Loew, R, Baum, C, and Schambach, A (2010).
915 Mechanisms controlling titer and expression of bidirectional lentiviral and gammaretroviral
916 vectors. *Gene Ther* **17**: 400-411.
917 53. Bustin, SA, Benes, V, Garson, JA, Hellemans, J, Huggett, J, Kubista, M, *et al.* (2009). The
918 MIQE guidelines: minimum information for publication of quantitative real-time PCR
919 experiments. *Clin Chem* **55**: 611-622.

920

4 Results and Discussion

An adoptive transfer of T cells into patients suffering from virus infections or cancer has emerged as therapy option [3], whereby introduction of a CAR, an artificial molecule comprising antigen binding and signaling domains, enables redirection towards defined target specificity [12]. Although CAR-T cells exhibited impressive anti-tumor activity in patients with hematological malignancies [19-22], treatment of solid tumors mostly failed due to heterogeneous antigen expression and formation of an immunosuppressive TME by tumor cells [32]. Within the TME, soluble mediators and redirected immune cells suppress an anti-tumor immune response [33]. To approach this, TRUCKs, the fourth generation of CAR-T cells, were developed, which contain a CAR that is constitutively expressed and an NFAT-sensitive, inducible cassette for a soluble mediator [36, 37]. Thereby, CAR activation upon target recognition at the tumor site induces the release of the molecule in a locally-restricted manner, which enables re-modulation of the TME, recruitment and activation of other immune cells as well as improvement of the CAR-T cell function and phenotype [32].

4.1 A virus peptide as CAR target provides high specificity and coverage

One of the key determinants for the success of a CAR-T cell product is the choice of antigen targeted by the CAR, for which several requirements are posed. The target antigen should ideally have a high coverage being expressed on the surface of all tumor cells, high specificity, meaning not being expressed by healthy cells, and high expression stability to prevent antigen escape [44]. Since only surface molecules, which cover a small proportion of all cellular components, can be recognized by a CAR, and most known tumor-associated antigens overexpressed by tumor cells can also be found on healthy cells, ideal target antigens are difficult to find. The usage of a TCR-like mAb to confer antigen specificity of the CAR widens the spectrum of potential target antigens, as they specifically bind to a distinct peptide of extra- or intracellular origin when presented in context of an HLA molecule, and such CARs were shown to efficiently target tumor cells in *in vivo* models for melanoma [68, 69], leukemia and ovarian cancer [70].

In this study [83], the mAb TÜ165 recognizing the EBNA-3C-derived peptide LPPHDITPY in context of HLA-B*35 was used to confer antigen specificity of CAR-T cells towards EBV-

associated PTLD cells. PTLD is a severe complication in patients that underwent HSCT or SOT [45]. The majority of PTLD cases results from an uncontrolled expansion of EBV-infected B cells upon primary infection or virus reactivation after transplantation [46]. Although EBV enters a latency program with several strategies to evade immune destruction enabling livelong persistence of the virus in the host, immune cells of healthy individuals are able to restrict reactivation of viral lytic cycles and subsequent spreading of EBV, a finely tuned balanced that is interfered upon delayed immune reconstitution or immunosuppressive regimen applied to prevent organ rejection after transplantation [51]. Considering 1% to 20% of transplant patients are affected displaying a three-year overall survival of 49% to 80% [45, 47-49], and current treatment options are unspecific resulting in varying response rates and severe adverse effects [52-54], the need of novel treatment options for PTLD is high. For this, the mAb TÜ165 provides a great specificity for PTLD cells based on their origination from EBV-infected B cells of the latency type III gene expression program [51] and thus spares healthy B cells.

Binding of the mAb TÜ165 to K562 cells expressing HLA-B*35:01 (K562-B*35) loaded with the LPPHDITPY peptide and equally loaded HLA-B*35-positive PBMCs was shown in this study [83], whereas respective cells that were unloaded or loaded with other peptides were not, or only at very high loading concentrations unlikely to be reached under physiological conditions, recognized. Moreover, TÜ165 bound to B-lymphoblastoid cell lines (B-LCLs) of HLA-B*35$^+$ donors, which were generated by infection of B cells with the EBV strain B95.8 resulting in an EBV latency type III expression profile similar to malignant EBV-associated PTLD cells [54]. Not only the here utilized laboratory EBV strain, but also the natural strains HKNPC1 (GenBank no. JQ009376.2), C666-1 (GenBank no. AB828190.1), and M81 (GenBank no. KF373730.1) incorporate the genetic information of the LPPHDITPY peptide indicating conservation of this motif and consequent stability of expression, as well as an almost complete coverage of the TÜ165 target on all malignant PTLD cells. Thereby, the target antigen addressed by the mAb TÜ165 is mostly ideal. One drawback, however, is HLA-dependency of the TCR-like mAb restricting the proportion of potentially treatable patients to HLA-B*35$^+$ individuals accounting 12.4% to 30.5% of the population depending on the geographic regions when analyzing all available data with a sample size of more than 1000 [84]. From these, TÜ165, until now, was only described to bind the HLA-B*35:01 subtype [72], which could be confirmed here. However, further recognition of B-LCLs with the HLA-B*35:03 subtype was detected in our study and prediction results from the NetMHCpan server revealed that all occurring HLA-B*35 alleles should bind the EBNA-3C-

derived peptide with 31/39 analyzed subtypes being classified as even strong binders [85]. The ability of TÜ165 to bind to these complexes of EBV peptide with other HLA-B*35 subtypes needs to be clarified in further studies. Furthermore, extension of this proof-of-concept to target additional HLA types would be desirable, for which other described TCR-like mAbs recognizing EBV-derived peptides in context of HLA-A*02:01 [65, 66] could be used. The HLA frequency analysis revealed a prevalence of 31.3 % to 58.1% for HLA-A*02 in different regions [86] increasing the proportion of potentially treatable patients and more TCR-like mAbs with various specificities for the usage in CARs can be generated applying hybridoma and phage display technologies [58, 59].

Currently, two main strategies dominate the field of engineered T cells: modification of effector T cells with either a recombinant TCR or a CAR. One major concern in the generation of TCR-T cells is the occurrence of unexpected specificities upon mispairing of the introduced TCR chains with either the endogenous TCR α or β chain potentially resulting in the formation of dangerous self-reactive TCR-T cells [8-10]. Further, competition with the endogenous TCR may result in low expression of the introduced TCR leading to a decreased T-cell functionality, since it correlates with TCR expression levels on the cell surface. This provoked the development of several strategies to enhance expression of the introduced TCR including disruption or silencing of the endogenous TCR or introducing the TCR into alternative cells (reviewed in [11]). Using a TCR-like mAb to confer antigen specificity for a CAR combines advantages of both strategies, since intracellular peptides can be targeted, while formation of dimers with the endogenous TCR is prevented. Furthermore, in this study [83], no naturally occurring TCRs targeting LPPHDITPY in HLA-B*35 context could be found, which can indicate missing immunodominance of the epitope, undetectably low respective T-cell frequencies or deletion of specific T cells by tolerance mechanisms and, importantly, prevents the generation of TCR-T cells for this epitope.

The generated TÜ165-based CAR-T cells showed a high proportion of central memory T cells (T_{CM}), the T-cell subset that was shown to exhibit superior functionality *in vivo* accompanied by long-term persistence and occupancy of memory T-cell niches [28, 87]. CARs with a 4-1BB co-stimulation domain as utilized in TÜ165 CAR-T cells also elsewhere predominantly drove a T_{CM} phenotype with an enhanced respiratory capacity in *in vitro* cultures as opposed to CAR-T cells with a CD28-based co-stimulatory domain, which mainly differentiated into T_{EM} [88]. Moreover, confirming the observed phenotypes in this study, the utilized cytokines IL-7 and IL-15 were also shown to promote a T_{SCM} and T_{CM} differentiation from naïve T cells during cultivation in other

studies [89-92]. Further, the manufactured T cells exhibited a favorable phenotype with low expression of programmed cell death protein 1 (PD-1) indicating that they were not exhausted by the amplification process.

Such generated TÜ165 CAR-T cells exhibited high specificity for the EBNA-3C-derived peptide/HLA-B*35 complex in a peptide-concentration-dependent manner in a reporter assay with EBV-peptide-loaded K562-B*35 as target cells. K562-B*35 that were unloaded or loaded with different EBV- or cytomegalovirus (CMV)-derived peptides were not recognized by TÜ165 CAR-T cells. Other researchers developing TCR-like CAR-T cells faced the problem that a high-affinity TCR-like mAb targeting Wilms tumor suppressor gene 1 (WT1) peptide/HLA-A*02 complexes lost specificity when used to provide antigen specificity of CAR-T cells resulting in the unspecific elimination of HLA-A*02-positive, but WT1 peptide-negative cells [93]. However, low-affinity TCR-like CAR-Ts with an affinity in the range of a TCR did not exhibit loss of specificity and elicited potent cytotoxicity, which is thought to be facilitated by sequential engagement of peptide/HLA complexes needed for complete T-cell activation [69, 93]. The mAb TÜ165 is an immunoglobulin M (IgM) antibody with ten sites for antigen binding, suggesting that the single site present in the TÜ165 CAR may feature a markedly reduced avidity resulting in a weak binding, which, equally to the aforementioned study, maintains specificity and functionality. This was confirmed by upregulation of the activation markers CD137, CD25 and CD69, release of IFN-γ and the cytotoxic mediators perforin and granzyme B, as well as cluster-formation of primary TÜ165 CAR-T cells upon encounter with LPPHDITPY-loaded K562-B*35 or autologous B-LCLs, whereas the respective control target cells, unloaded K562-B*35 and uninfected PBMCs, did not induce any response in this study [83]. However, these effector functions were rather mild, especially when compared to CARs targeting CD19, which can be explained by the dependency of CAR-T-cell functionality on target expression levels [94], and the comparably lower expression levels of distinct peptide/HLA complexes when compared to surface molecules such as CD19. In the next step, *in vivo* functionality of TÜ165 CAR-Ts should be evaluated, exemplary by using immune-competent mice engrafted with B-LCLs, which were previously described for testing of a TCR-like mAb with EBV specificity [65].

4.2 Peptide-specific TRUCKs exhibit highly improved functionality

To further improve the effector functions of TÜ165 CAR-T cells and potentially enable recruitment and activation of other immune cells that are otherwise suppressed and in low numbers in immunocompromised PTLD patients, TÜ165-based TRUCKs with inducible enhanced green fluorescent protein (EGFP) reporter or IL-12 expression were generated in this study [83]. Equally to TÜ165 CAR-T cells, they displayed a phenotype with low expression of PD-1 and a high frequency of the T_{CM} subtype after expansion. Assessment of these markers after co-cultivation with target cells would be interesting, since TRUCKs with inducible IL-12, in contrast to TRUCKs with IL-18, showed an exhausted phenotype upon antigen encounter in another study that was characterized by the enhanced expression levels of T-box expressed in T cells (T-bet) and Forkhead box protein O1 (FoxO1) [42].

Opposed to that, the here generated TÜ165 TRUCKs with inducible IL-12 exhibited potent functionality towards specific target cells *in vitro* that was markedly improved when compared to TÜ165 CAR-Ts including a higher extent of activation marker CD25 upregulation, as well as release of IL-2, IFN-γ and TNF-α. This confirms previous observations that various T cells with IL-12 expression including virus-specific T cells, CAR-T cells or TCR-T cells modified to express IL-12 in an inducible or constitutive manner secreted more IFN-γ and/or TNF-α, which is also one of the major physiological functions of IL-12 [38], compared to their counterparts without IL-12 [36, 37, 42, 95-97]. TNF-α was critically important for IL-12-TRUCK-mediated elimination of solid tumors with heterogeneous antigen expression by involvement of macrophages in an immune-competent mouse model [36], and both, IFN-γ and TNF-α, were shown to mediate resistance to immunosuppressive factors often released by EBV^+ Hodgkin's disease [95]. Further, TÜ165 TRUCKs with inducible IL-12 not only released elevated amounts of the cytolytic mediators perforin and granzyme B, but further had the tendency of increased cytotoxicity and potently eliminated autologous B-LCLs as PTLD model. This is contradictory to IL-12 TRUCKs with other specificities exhibiting comparable cytotoxicity [36, 96], as well as CD107a and granzyme B expression [96] to their respective T-cell counterparts without IL-12 expression. However, one major difference is that CARs targeting carcinoembryonic antigen (CEA) as well as TCRs specific for a melanoma antigen used for the generation of the aforementioned T cells with inducible IL-12 already showed high effector functions in CAR- or TCR-T cells without IL-12. Thus, the explanation might be that the mild cytotoxicity of weakly-binding CAR-T cells can be improved by incorporation of an inducible IL-12 cassette, whereas more potent CAR-T cells do

not profit from the addition in such a large extent. Considering the fact that TCR-like CAR-T cells with a low affinity were shown to maintain target specificity but react rather mildly [69, 93], an addition of inducible IL-12 may serve as new strategy to ensure safety as well as enable effective functionality of these cells at the same time. If this strategy is applicable for more weakly-binding TCR-like CAR-T cells would be of major interest for further studies.

TÜ165 CAR-T cells specifically enhanced the expression of NFAT upon target encounter as shown in a reporter assay in this study [83]. Thus, the NFAT-sensitive promoter in TÜ165 TRUCKs allowed for the target-specific expression of a GOI, which was demonstrated by an increased EGFP reporter expression and elevated IL-12 secretion after co-cultivation of TÜ165 TRUCKs incorporating inducible EGFP and IL-12, respectively, with LPPHDITPY/HLA-B*35^{+} target cells. The released IL-12 induced after target-recognition by TÜ165 IL-12-TRUCKs was capable to effectively recruit a monocyte and NK cell line and, moreover, improved the functionality of addressed NK-92 cells, since they displayed an increased killing capacity and augmented IFN-γ expression. This confirms the physiologic function of IL-12, which is, amongst other effects, acting on NK cells and promoting their effector function [38] and suggests that the release of cytokines may also lead to a beneficial modulation of the TME by attraction of supportive immune cells including macrophages as it was shown for other TRUCKs [36, 37, 42]. This should be confirmed in clinically relevant *in vivo* models with immune-competent mice. The attraction and activation of further immune cells including NK, NKT and endogenous T cells is of major interest in this context, since IL-12 is described to promote their proliferation, cytotoxic capacity, as well as production of cytotoxic mediators and cytokines, and further drives differentiation of T_H1 and CTLs [38]. Especially the beneficial effect of TÜ165 TRUCKs on NK-92 cells should be evaluated for endogenous NK cells in the mouse model, because importance of these cells in the control of latently EBV-infected cells was shown, since NK cell immunodeficiencies predispose for EBV-associated diseases [98], a distinct NK cell population was found that was able to restrict EBV-mediated B-cell transformation [99], and one NK cell subset could even mediate cytotoxicity against B-LCLs accompanied by IFN-γ production and CD107a mobilization [100]. In PTLD, T cells and especially NK cells show an exhausted phenotype with enhanced PD-1 expression [101], indicating the potential TME re-modulation could restore the endogenous immune response towards PTLD cells.

Furthermore, in immune-competent mouse models, release of immunosuppressive mediators by tumor cells and their impact on the transferred cells can be studied, as TCR-T cells with constitutive

IL-12 expression were able to resist TGF-β released by EBV$^+$ Hodgkin's disease [95]. One additional focus of future *in vivo* testing of TÜ165 TRUCKs should be IL-12 driven toxicities, since TRUCKs with inducible IL-12 were reported as being safe [36, 37, 42, 96], but one study observed severe IL-12-driven toxicity due to non-specific release of the cytokine and resulting highly increased systemic IFN-γ and TNF-α levels [97], although the here measured IL-12-background levels were markedly lower compared to the aforementioned study.

4.3 Functionality of G_{D2}-targeting TRUCKs for the treatment of solid tumors

An additional tumor entity, for which CAR-T cells were developed [79], is neuroblastoma, one of the most common solid tumors for children showing a long-term survival below 50% for high-risk patients despite availability of aggressive therapy options [76]. These include immunotherapeutic approaches applying antibodies targeting the disialoganglioside G_{D2}. This antigen is expressed at low levels on neuronal cells of the CNS, peripheral nerves, mesenchymal stroma cells, and skin melanocytes, but abundantly presented on tumor cells of neuroblastoma, melanoma and several more childhood and adult cancer entities (reviewed in [73]). Administration of ch14.18, a chimeric antibody based on the mAb 14G2a targeting G_{D2}, was shown to increase the event-free survival from a median of 46% for neuroblastoma patients treated with standard therapy to 66% when applied in combination with IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [102] leading to its approval by the European Medicines Agency (EMA; Qarziba; EUSA Pharma B.V., Amsterdam, Netherlands) [103]. However, repetitive administration with extended infusion times is required for this antibody [76]. Although another antibody with shorter infusion times is currently being investigated for approval [104, 105], an immunotherapeutic approach using CAR-T cells would circumvent the need for sequential administration and potentially provide lifelong protection. However, CARs based on the mAb 14.G2a, although exhibiting impressive short-term tumor lysing capabilities, largely failed to sustain long-term remission in clinical trials [80].

To approach this, TRUCKs based on the G_{D2}-targeting CAR with inducible cytokine expression were developed in this study [106]. In initial studies, TRUCKs were generated by co-transduction of two viral vectors into the same cell conferring the genetic information for the CAR and the inducible cytokine, respectively [36, 37]. However, compared to the manufacturing of conventional

CARs, which was shown to be feasible and safe leading to the approval of three CAR-T-cell products [19-22], this bears an increased risk for insertional mutagenesis since two instead of one integration events are required [26]. Further, generation of viral vectors is laborious and cost-intensive – especially when it is performed under GMP-grade conditions that are needed for clinical manufacturing. To increase safety and simplify the manufacturing process, an "all-in-one"-vector was designed [106]. The vector combined expression of a CAR targeting G_{D2} driven by a constitutive, human phosphoglycerate kinase (hPGK) promoter and an inducible expression for human IL-12 or IL-18 in one lentiviral self-inactivating (SIN) vector and was further optimized for a high induction capacity and yielding clinically applicable viral titers. Besides self-inactivating properties, an enhanced safety is achieved by the usage of a lentiviral vector since the most common alternative, γ-retroviral vectors, were shown to have a higher risk for mutational oncogenesis due to their preferential insertion in cancer-associated genes [107]. Moreover, there was no evidence of vector-mediated adverse events in lentiviral therapies as shown by a long-term follow-up study evaluating more than 200 infusion events [108].

The G_{D2} CAR specifically induced the expression of NFAT after encounter with G_{D2}-expressing target cells (HT1080-G_{D2} and SH-SY5Y) in a reporter assay in this study [106]. Exploiting this and combining the G_{D2} CAR with an NFAT-sensitive expression cassette enabled specific expression of a GOI, such as the reporter gene EGFP or the cytokines IL-12 or IL-18, upon co-culture with G_{D2}^+ target cells, whereas the expression was on a marginal baseline level in T cells cultivated alone or in a co-culture with a cell line lacking G_{D2}.

Primary T cells transduced with these TRUCK vectors exhibited cytotoxicity towards G_{D2}^+ target cells in a range that was similar to G_{D2} CAR-T cells without inducible cytokine expression, which was also observed for other IL-12-engineered T cells [36, 42]. Further, a strong induction of the expression of the activation markers CD25 and CD69 on $CD3^+$ and CD137 on $CD8^+$ T cells, as well as a slight induction of CD154 on $CD4^+$ T cells upon specific target contact was even more pronounced than for G_{D2} CAR-T cells. In line, the pro-inflammatory cytokines TNF-α, IFN-γ, IL-2 and the cytotoxic mediators perforin and granzyme B were released in a target-dependent manner by TRUCKs, whereby G_{D2} TRUCKs with inducible IL-12 secreted markedly higher amounts than TRUCKs with inducible IL-18 and G_{D2} CAR-T cells. Only perforin was released to a similar extent by both TRUCKs, but also more than by CAR-T cells. Thereby, the designed TRUCKs were shown to be functional, maintain target specificity of the G_{D2} CAR, and even display a slightly improved activation response, and, in case of IL-12 TRUCKs, a markedly augmented cytokine release

towards G_{D2}^+ target cells. This confirms observations made for the TÜ165 TRUCK with inducible IL-12 [83] and other IL-12-engineered T cells showing an elevated IFN-γ and TNF-α release [36, 37, 42, 95-97]. In contrast to here, a study with TCR-T cells targeting a melanoma-associated peptide combined with inducible IL-12 expression did not reveal an increased production of the cytotoxic mediators CD107a and granzyme B by the IL-12-modified cells [96], and TÜ165 TRUCKs with inducible IL-12 exhibited an increased release of both markers and even an improved cytotoxicity [83] compared to their counterpart without IL-12 release. This indicates that these effects are, most likely, dependent on the examined combination of tumor target and specificity as well as affinity of the effector cells. TCR-T cells and CAR-T cells with different specificities and constitutive or inducible IL-18 expression, analogously to those with IL-12 expression, were shown to exhibit an increased release of IFN-γ, IL-2 and/or improved proliferation upon specific target recognition [42, 43, 97, 109], but did not exhibit an enhanced cytotoxicity [42, 109], confirming the results of this study.

In this study [106], specifically after recognition of G_{D2}^+ target cells, the respective TRUCKs induced the release of IL-12 or IL-18 leading to an increased recruitment of a monocyte cell line in an *in vitro* migration assay, whereby IL-12 TRUCKs exhibited a higher recruitment potential when compared to IL-18 TRUCKs. Equally to TÜ165 TRUCKs with inducible IL-12 [83], modulatory effects on the TME should be evaluated in further *in vivo* models using immune-competent mice (see chapter 4.2). Since another TRUCK with inducible IL-12 expression even mediated eradication of antigen-negative tumor cells in a mouse model by this recruitment *in vivo* [36], efficacy of the G_{D2} TRUCKs to target heterogeneous tumor lesions as they are exemplary described for Ewing sarcoma expressing variable amounts of G_{D2} among the cells in individual tumors [81], would be interesting to study.

Chmielewski and Abken [42] directly compared TRUCKs targeting CEA in context of pancreatic cancer with either inducible IL-12 or IL-18 release. Both TRUCKs revealed an improved target-dependent IFN-γ release and proliferation in comparison to conventional CEA CAR-T cells, whereby IL-12 TRUCKs were more potent. However, antigen encounter induced the expression of both T-bet and FoxO1 revealing an exhausted phenotype in IL-12 TRUCKs, whereas IL-18 TRUCKs upregulated T-bet but downregulated FoxO1 expression, which indicates an activated and pro-inflammatory phenotype. With this, both TRUCKs were able to control pancreatic tumor cells in an immune-competent mouse model, enhanced the presence of protective NK cells and reduced the numbers of immunosuppressive cells at the tumor site, whereby IL-18 TRUCKs were

more effective. In another study comparing TCR-engineered T cells with inducible IL-12 and IL-18, both cells enhanced the release of IFN-γ compared to the TCR-T-cell counterpart, whereby T cells with inducible IL-12 reacted stronger [97]. However, transfer of TCR-T cells with inducible IL-12 caused severe IL-12-driven toxicity and reduced persistence of the therapeutic T cells in melanoma-bearing mice. Thus, since lacking persistence and exhaustion of CAR-T cells targeting G_{D2} is thought to be a major reasons for their failure in the treatment of neuroblastoma patients [80], combination of the CAR with an inducible IL-18 expression driving the T cells towards a more inflammatory and less exhausted phenotype seems to be a promising approach to overcome reappearance of the tumor.

4.4 Feasibility of GMP-compliant manufacturing of TRUCKs

Once developed in an academic center, manufacturing of promising CAR-T-cell candidates needs to be transferred, scaled up, optimized and validated to perform large global clinical trials for the approval as clinical therapy. As an example, tisagenlecleucel (Kymriah; Novartis Pharma, Basel, Switzerland), the first approved CAR-T-cell product in the United States and the European Union, underwent accurate optimization of the manufacturing process upon transfer from university to large-scale trials and the commercial use [27]. Thereby, the process was improved regarding compliance and robustness to ensure reproducibility and allow for global distribution. This included the GMP-compliant production of the lentiviral CAR vector that was refined to reduce variability, as well as to maximize quantity and transduction efficiency [110].

Since the pre-clinical evaluation of the tested TRUCKs was promising [83, 106], the feasibility of up-scaling manufacturing of TRUCKs under GMP-compliant conditions was tested in this study [111], whereby the developed “all-in-one” vector meeting several requirements including an enhanced safety, yield and transfer efficiency was applied [106]. Here, previous extensive *in vitro* characterization of the lentiviral vector [106] and experience in the development and validation of CAR-T-cell manufacturing for clinical trials [112] were used to implement generation of TRUCKs into an automated and closed process. In order to yield high cell numbers for the use in patients, different systems of bioreactors providing an improved gas exchange and culture mixing properties were used for the generation of CAR-T cells [110]. In contrast to other utilized bioreactors, such as the WAVE Bioreactor (GE Healthcare Life Sciences) or the G-Rex (Wilson Wolf), the

CliniMACS Prodigy® (Miltenyi Biotec) not only allows for an improved expansion of T cells, but accomplishes all steps of T-cell preparation, enrichment, activation, transduction, washing steps, cultivation, media exchanges, final formulation and sampling in a single device [89]. Further, the system is closed and fully automated, thereby minimizing the risk for contamination, cell loss and errors in hands-on steps, and providing an opportunity for reproducible and robust cell processing independent of the manufacturing site.

By using the CliniMACS Prodigy® and a protocol for T-cell transduction (TCT) that was established for the automated generation of CAR-T cells [90], and whose feasibility to yield an end product sufficient for the use in clinical studies was shown [90, 91, 112-116], G_{D2}-targeting TRUCKs with inducible IL-18 were generated [111]. With this protocol, $CD4^+$ and $CD8^+$ cells were enriched from a leukapheresis product, since blast counts as well as cells, such as monocytes, known to hinder CAR-T-cell expansion were shown to be decreased by this selection [91]. After a total expansion of 12 days, the final yield was 4.7×10^9 transduced white blood cells resulting from an expansion rate of 67.2-fold and a transduction efficiency of 74.9%, which, exemplary, would allow administration of a dose of 5.9×10^7 engineered cells/kg to a recipient weighing 80 kg. Since transferred CAR-T-cell doses in clinical studies range from 1×10^4 to 1×10^{10} cells/kg [117] and, most likely, dose adjustments will be required for the more potent TRUCKs, this yield would, even in adults, enable a multi-dose application. The transduction efficiency and final yield was markedly higher when compared to other studies using the CliniMACS Prodigy® [90, 91, 112-116]. Further, the TRUCK vector copy number (VCN), which is considered as relevant risk factor for vector-mediated oncogenesis in a EMA reflection paper (EMA/CAT/190186/2012), was assessed to be 2.6 copies/cell lying below limit of 5 copies/cell recommended by the US Food and Drug Administration (FDA) [118]. Further manufacturing runs using leukapheresis products obtained from cancer patients would reveal how dependent the acquired expansion and transduction parameters are on the starting material, although another study showed that manufacturing from healthy and lymphoma or melanoma patient cells yielded final products that were comparable in their composition, phenotype and functionality [90].

In this study [111], the manufactured IL-18 TRUCKs targeting G_{D2} exhibited a favorable phenotype of mainly stem-cell memory T cells (T_{SCM}) and T_{CM} indicating an early differentiation stage. Additional to the described superior functionality of T_{CM} (see chapter 4.1), T_{SCM} were shown to mediate a robust immune response in the long-term after adoptive transfer of even low cell numbers [119, 120]. This, analogously to the TÜ165 TRUCKs with inducible IL-12 [83], can be

attributed to the presence of a 4-1BB co-stimulatory domain in the CAR construct and the usage of IL-7 and IL-15 for the expansion of TRUCKs in this study (see Chapter 4.1).

The here manufactured IL-18 TRUCKs targeting G_{D2} exhibited strong upregulation of CD25 and CD69, activation markers expressed on all $CD3^+$ T cells, CD137 on $CD8^+$ and CD154 on $CD4^+$ T cells upon specific recognition of G_{D2}-expressing target cells but not cells lacking G_{D2} indicating specificity and reactiveness of both T-cell groups [111]. Moreover, in the same specific manner, the release of several pro-inflammatory cytokines, such as IL-2, IFN-γ and TNF-α, as well as the cytotoxic mediators granzyme B and perforin was enhanced. Cytotoxicity of the modified cells was highly efficient and specific as shown by a reduced amount of G_{D2}^+ target cells upon co-cultivation with IL-18 TRUCKs targeting G_{D2} in several assays including flow cytometry, real-life impedance measurements and microscopy. Commercial CAR-T cells are cryopreserved after generation allowing for centralized manufacturing, storage of the manufactured cell product and flexibility in the timing of administration [121]. All parameters for T-cell functionality assessed in this study [111] were not impeded by cryopreservation indicating feasibility of this approach for potential clinical use in future. A retrospective study evaluating the effect of cryopreservation in six clinical trials confirmed that, although they exhibited an increased expression of markers for apoptosis and dysfunction, CAR-T cells that were frozen and thawed showed the same *in vivo* persistence and responsiveness as compared to fresh cells [121]. The clinical-scale manufactured IL-18 TRUCKs targeting G_{D2} moreover released IL-18 in a strictly target-dependent manner leading to the recruitment of a monocyte and NK cell line *in vitro*, which confirms the results from the laboratory-scale study [106] and, in addition, suggests that different endogenous immune cells could be recruited and affected in the tumor microenvironment of solid tumors.

In direct comparison, automatically-manufactured TRUCKs exhibited equal or even improved functionality as compared to TRUCKs generated in the laboratory scale showing the utilized manufacturing protocol on the CliniMACS Prodigy® yields a highly functional T cell product that reproduces functionality conducted in intensive pre-clinical studies. Others also observed comparable parameters between clinical- and laboratory-scale-manufactured CAR-T cells when utilizing the CliniMACS Prodigy®, although the target-induced release of the cytokines IFN-γ, TNF-α and IL-2 was slightly reduced for automatically compared to manually generated CAR-T cells targeting CD19 [89]. This evokes optimism that also the transfer of other CAR-T-cell or TRUCK products from research to clinically sufficient scales is easy to implement while maintaining functionality parameters assessed in intensive pre-clinical testing.

By using G_{D2}-targeting TRUCKs with inducible IL-18 as a proof-of-concept in this study [111], the feasibility to manufacture TRUCKs under GMP-compliant conditions was shown, suggesting validation of this process including a stringent quality control process might be the last remaining step to enter clinical testing. Since the utilized manufacturing protocol was designed and validated for the generation of several CAR-T cells [90, 91, 112-116] and, here, the possibility to smoothly transfer the gained experience to the persuasive manufacturing of next generation CAR-T-cell products was demonstrated, the ways may be paved for future clinical trials with various TRUCKs and related products with additional cell sources and targets.

5 Conclusion and Outlook

Although an adoptive transfer of CD19-specific CAR-T cells mediated anti-tumor efficacy in patients with different hematological diseases [19-22], the choice of further targets for CAR-T cell tumor therapies mediating differentiation between malignant and healthy cells remains challenging [44]. As approach for the treatment of PTLD, a CAR with specificity of the TCR-like mAb TÜ165 targeting an EBNA-3C-derived peptide of the EBV latency type III gene expression was developed in this study [83]. Thereby, targeting of an intracellular antigen was enabled, despite a CAR's inherent restriction to only recognize surface molecules [44].

Current CAR-T-cell approaches for the treatment of solid tumors mostly fail to achieve maintained responses, since these tumors are able to form an immunosuppressive TME restricting CAR-T-cell function [32, 33]. TRUCKs were developed to re-modulate the TME [36, 37], whereby TRUCKs with inducible IL-12 were especially shown to diminish suppressor cells [37] and recruit other immune cells enabling to eradicate solid tumors with heterogeneous antigen expression [36] and TRUCKs with inducible IL-18 expression converted T cells into potent, pro-inflammatory and enduring effector cells [42]. Combination of the TÜ165 CAR with an inducible IL-12 expression for the generation of TRUCKs directed the modified cells against an intracellular target with high coverage and specificity for EBV-infected B cells, whereby a low avidity of the TÜ165 CAR enabled to maintain target specificity and the inducible cytokine mediated an effective and highly improved functionality. The target-induced IL-12 moreover lead to the recruitment and activation of further immune cells *in vitro*, including NK cells that were shown to be involved in the control of EBV-infected cells [98-100], but not functional in context of PTLD [101]. Thereby, TÜ165 TRUCKs with inducible IL-12 may serve as an innovative therapeutic approach, enabling the specific elimination of malignant cells, while healthy immune cells are spared and recruited.

To approach the problem of lacking persistence of G_{D2} CAR-T cells and subsequent tumor reappearance in clinical studies with neuroblastoma patients [80], G_{D2}-specific TRUCKs with inducible cytokine expression were generated here [106]. Usage of modular "all-in-one" lentiviral vectors combining G_{D2}-specific CAR with inducible IL-12 or IL-18 expression to simplify manufacturing allowed for the generation TRUCKs with maintained target specificity and even improved effector functions compared to G_{D2}-specific CAR-T cells.

For all generated CAR-T cells and TRUCKs, further *in vivo* testing in clinically relevant, immune-competent mice models is required to confirm the observed target specificity and functionality. Although an adoptive transfer of EBNA-3C-peptide-specific T cells should be safe due to the restricted target expression on EBV-infected cells, especially for G_{D2}-specific TRUCKs, the occurrence of possible "on-target/off-tumor" effects should be one focus of the study, since G_{D2} is an antigen with high expression on several tumors [73], but in low densities also present on healthy cells. Yet, clinical studies with G_{D2}-specific CAR-T cells did not reveal such toxicities [80]. Another important focus is to study the effect of the target-induced cytokine. One aspect would be the evaluation of systemic toxicity due to baseline cytokine expression without CAR activation, which is of major interest for IL-12, since another study reported toxicity in mice models [97]. On the other hand, re-modulation of the TME as well as recruiting and activation of other immune cells should be studied, since IL-12, which was e.g. released by TÜ165 TRUCKs, was shown to induce cytokines mediating resistance to immunosuppressive factors often released by EBV^+ tumors [95]. The recruitment of immune cells would be also interesting to evaluate for G_{D2}-specific TRUCKs, as G_{D2} is heterogeneously expressed on some tumors (e.g. Ewing sarcoma) [73] and attracted and activated immune cells were shown to enable elimination of target-negative tumor variants [36]. Lastly, G_{D2}-specific TRUCKs with inducible IL-12 or IL-18 could be compared in an appropriate *in vivo* model, whereby especially long-term functionality and persistence of the modified cells should be assessed, as these were shown to be particularly improved for TRUCKs with inducible IL-18 [42].

Finally, GMP-compliant manufacturing of functional TRUCKs with a great proportion of transduced cells and high yields in an automated and closed system was found to be feasible in this study [111]. By using IL-18 TRUCKs targeting G_{D2} as an example, a smooth transfer to clinical-scale manufacturing was shown to maintain functionality gained in pre-clinical testing, which evokes optimism for the research and development of more TRUCK-related cell products. Thus, the next step is the validation of the applied manufacturing process with implemented quality control for microbiological, endotoxin and mycoplasma testing, which is one of the last remaining points required to enter early clinical studies. With this, TRUCK technology is brought one step closer to clinical application.

6 References

[1] Bonilla, F.A. and Oettgen, H.C., *Adaptive immunity.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.

[2] Klein, J. and Sato, A., *The HLA system. First of two parts.* N Engl J Med, 2000. **343**(10): p. 702-9.

[3] Stroncek, D.F., Reddy, O., Highfill, S., and Panch, S.R., *Advances in T-cell Immunotherapies.* Hematol Oncol Clin North Am, 2019. **33**(5): p. 825-837.

[4] Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A.V., Helman, L.J., Mackall, C.L., Kammula, U.S., Hughes, M.S., Restifo, N.P., Raffeld, M., Lee, C.C., Levy, C.L., Li, Y.F., El-Gamil, M., Schwarz, S.L., Laurencot, C., and Rosenberg, S.A., *Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1.* J Clin Oncol, 2011. **29**(7): p. 917-24.

[5] Rapoport, A.P., Stadtmauer, E.A., Binder-Scholl, G.K., Goloubeva, O., Vogl, D.T., Lacey, S.F., Badros, A.Z., Garfall, A., Weiss, B., Finklestein, J., Kulikovskaya, I., Sinha, S.K., Kronsberg, S., Gupta, M., Bond, S., Melchiori, L., Brewer, J.E., Bennett, A.D., Gerry, A.B., Pumphrey, N.J., Williams, D., Tayton-Martin, H.K., Ribeiro, L., Holdich, T., Yanovich, S., Hardy, N., Yared, J., Kerr, N., Philip, S., Westphal, S., Siegel, D.L., Levine, B.L., Jakobsen, B.K., Kalos, M., and June, C.H., *NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma.* Nat Med, 2015. **21**(8): p. 914-921.

[6] Robbins, P.F., Kassim, S.H., Tran, T.L., Crystal, J.S., Morgan, R.A., Feldman, S.A., Yang, J.C., Dudley, M.E., Wunderlich, J.R., Sherry, R.M., Kammula, U.S., Hughes, M.S., Restifo, N.P., Raffeld, M., Lee, C.C., Li, Y.F., El-Gamil, M., and Rosenberg, S.A., *A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response.* Clin Cancer Res, 2015. **21**(5): p. 1019-27.

[7] Ping, Y., Liu, C., and Zhang, Y., *T-cell receptor-engineered T cells for cancer treatment: current status and future directions.* Protein Cell, 2018. **9**(3): p. 254-266.

[8] Heemskerk, M.H., Hagedoorn, R.S., van der Hoorn, M.A., van der Veken, L.T., Hoogeboom, M., Kester, M.G., Willemze, R., and Falkenburg, J.H., *Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex.* Blood, 2007. **109**(1): p. 235-43.

[9] Bendle, G.M., Linnemann, C., Hooijkaas, A.I., Bies, L., de Witte, M.A., Jorritsma, A., Kaiser, A.D., Pouw, N., Debets, R., Kieback, E., Uckert, W., Song, J.Y., Haanen, J.B., and Schumacher, T.N., *Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy.* Nat Med, 2010. **16**(5): p. 565-70, 1p following 570.

[10] van Loenen, M.M., de Boer, R., Amir, A.L., Hagedoorn, R.S., Volbeda, G.L., Willemze, R., van Rood, J.J., Falkenburg, J.H., and Heemskerk, M.H., *Mixed T cell receptor dimers harbor potentially harmful neoreactivity.* Proc Natl Acad Sci U S A, 2010. **107**(24): p. 10972-7.

[11] Zhang, Y. and Li, Y., *T cell receptor-engineered T cells for leukemia immunotherapy.* Cancer Cell Int, 2019. **19**: p. 2.

[12] Sadelain, M., Brentjens, R., and Riviere, I., *The basic principles of chimeric antigen receptor design.* Cancer Discov, 2013. **3**(4): p. 388-98.

[13] Gross, G., Waks, T., and Eshhar, Z., *Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 10024-8.

[14] Eshhar, Z., Waks, T., Gross, G., and Schindler, D.G., *Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors.* Proc Natl Acad Sci U S A, 1993. **90**(2): p. 720-4.

[15] Clambey, E.T., Davenport, B., Kappler, J.W., Marrack, P., and Homann, D., *Molecules in medicine mini review: the alphabeta T cell receptor.* J Mol Med (Berl), 2014. **92**(7): p. 735-41.

[16] Chmielewski, M. and Abken, H., *TRUCKs: the fourth generation of CARs.* Expert Opin Biol Ther, 2015. **15**(8): p. 1145-54.

[17] Kagoya, Y., Tanaka, S., Guo, T., Anczurowski, M., Wang, C.H., Saso, K., Butler, M.O., Minden, M.D., and Hirano, N., *A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects.* Nat Med, 2018. **24**(3): p. 352-359.

[18] Tokarew, N., Ogonek, J., Endres, S., von Bergwelt-Baildon, M., and Kobold, S., *Teaching an old dog new tricks: next-generation CAR T cells.* Br J Cancer, 2019. **120**(1): p. 26-37.

[19] Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K.L., Martin, P.L., Nemecek, E.R., Yanik, G.A., Peters, C., Baruchel, A., Boissel, N., Mechinaud, F., Balduzzi, A., Krueger, J., June, C.H., Levine, B.L., Wood, P., Taran, T., Leung, M., Mueller, K.T., Zhang, Y., Sen, K., Lebwohl, D., Pulsipher, M.A., and Grupp, S.A., *Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia.* N Engl J Med, 2018. **378**(5): p. 439-448.

[20] Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., Timmerman, J.M., Stiff, P.J., Friedberg, J.W., Flinn, I.W., Goy, A., Hill, B.T., Smith, M.R., Deol, A., Farooq, U., McSweeney, P., Munoz, J., Avivi, I., Castro, J.E., Westin, J.R., Chavez, J.C., Ghobadi, A., Komanduri, K.V., Levy, R., Jacobsen, E.D., Witzig, T.E., Reagan, P., Bot, A., Rossi, J., Navale, L., Jiang, Y., Aycock, J., Elias, M., Chang, D., Wiezorek, J., and Go, W.Y., *Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma.* N Engl J Med, 2017. **377**(26): p. 2531-2544.

[21] Schuster, S.J., Bishop, M.R., Tam, C.S., Waller, E.K., Borchmann, P., McGuirk, J.P., Jäger, U., Jaglowski, S., Andreadis, C., Westin, J.R., Fleury, I., Bachanova, V., Foley, S.R., Ho, P.J., Mielke, S., Magenau, J.M., Holte, H., Pantano, S., Pacaud, L.B., Awasthi, R., Chu, J., Anak, Ö., Salles, G., and Maziarz, R.T., *Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma.* N Engl J Med, 2019. **380**(1): p. 45-56.

[22] Wang, M., Munoz, J., Goy, A., Locke, F.L., Jacobson, C.A., Hill, B.T., Timmerman, J.M., Holmes, H., Jaglowski, S., Flinn, I.W., McSweeney, P.A., Miklos, D.B., Pagel, J.M., Kersten, M.J., Milpied, N., Fung, H., Topp, M.S., Houot, R., Beitinjaneh, A., Peng, W., Zheng, L., Rossi, J.M., Jain, R.K., Rao, A.V., and Reagan, P.M., *KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma.* N Engl J Med, 2020. **382**(14): p. 1331-1342.

[23] European Medicines Agency. *Kymriah*. Accessed March 22, 2021, https://www.ema.europa.eu/en/medicines/human/EPAR/kymriah.

[24] European Medicines Agency. *Yescarta*. Accessed March 22, 2021, https://www.ema.europa.eu/en/medicines/human/EPAR/yescarta#authorisation-details-section.

[25] European Medicines Agency. *Tecartus*. Accessed March 22, 2021, https://www.ema.europa.eu/en/medicines/human/EPAR/tecartus.

[26] Wang, X. and Rivière, I., *Clinical manufacturing of CAR T cells: foundation of a promising therapy.* Mol Ther Oncolytics, 2016. **3**: p. 16015.

[27] Tyagarajan, S., Spencer, T., and Smith, J., *Optimizing CAR-T Cell Manufacturing Processes during Pivotal Clinical Trials.* Mol Ther Methods Clin Dev, 2020. **16**: p. 136-144.

[28] Berger, C., Jensen, M.C., Lansdorp, P.M., Gough, M., Elliott, C., and Riddell, S.R., *Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates.* J Clin Invest, 2008. **118**(1): p. 294-305.

[29] Hinrichs, C.S., Borman, Z.A., Gattinoni, L., Yu, Z., Burns, W.R., Huang, J., Klebanoff, C.A., Johnson, L.A., Kerkar, S.P., Yang, S., Muranski, P., Palmer, D.C., Scott, C.D., Morgan, R.A., Robbins, P.F., Rosenberg, S.A., and Restifo, N.P., *Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy.* Blood, 2011. **117**(3): p. 808-14.

[30] Gattinoni, L., Lugli, E., Ji, Y., Pos, Z., Paulos, C.M., Quigley, M.F., Almeida, J.R., Gostick, E., Yu, Z., Carpenito, C., Wang, E., Douek, D.C., Price, D.A., June, C.H., Marincola, F.M., Roederer, M., and Restifo, N.P., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.

[31] Levine, B.L., *Performance-enhancing drugs: design and production of redirected chimeric antigen receptor (CAR) T cells.* Cancer Gene Ther, 2015. **22**(2): p. 79-84.

[32] Chmielewski, M., Hombach, A.A., and Abken, H., *Of CARs and TRUCKs: chimeric antigen receptor (CAR) T cells engineered with an inducible cytokine to modulate the tumor stroma.* Immunol Rev, 2014. **257**(1): p. 83-90.

[33] Hanahan, D. and Weinberg, R.A., *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

[34] Dunn, G.P., Old, L.J., and Schreiber, R.D., *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.

[35] Pagès, F., Galon, J., Dieu-Nosjean, M.C., Tartour, E., Sautès-Fridman, C., and Fridman, W.H., *Immune infiltration in human tumors: a prognostic factor that should not be ignored.* Oncogene, 2010. **29**(8): p. 1093-102.

[36] Chmielewski, M., Kopecky, C., Hombach, A.A., and Abken, H., *IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression.* Cancer Res, 2011. **71**(17): p. 5697-706.

[37] Chinnasamy, D., Yu, Z., Kerkar, S.P., Zhang, L., Morgan, R.A., Restifo, N.P., and Rosenberg, S.A., *Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice.* Clin Cancer Res, 2012. **18**(6): p. 1672-83.

[38] Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nat Rev Immunol, 2003. **3**(2): p. 133-46.

[39] Car, B.D., Eng, V.M., Lipman, J.M., and Anderson, T.D., *The toxicology of interleukin-12: a review.* Toxicol Pathol, 1999. **27**(1): p. 58-63.

[40] Nguyen, K.G., Vrabel, M.R., Mantooth, S.M., Hopkins, J.J., Wagner, E.S., Gabaldon, T.A., and Zaharoff, D.A., *Localized Interleukin-12 for Cancer Immunotherapy.* Front Immunol, 2020. **11**: p. 575597.

[41] Poorebrahim, M., Melief, J., Pico de Coaña, Y., S, L.W., Cid-Arregui, A., and Kiessling, R., *Counteracting CAR T cell dysfunction.* Oncogene, 2021. **40**(2): p. 421-435.

[42] Chmielewski, M. and Abken, H., *CAR T Cells Releasing IL-18 Convert to T-Bet(high) FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors.* Cell Rep, 2017. **21**(11): p. 3205-3219.

[43] Hu, B., Ren, J., Luo, Y., Keith, B., Young, R.M., Scholler, J., Zhao, Y., and June, C.H., *Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18.* Cell Rep, 2017. **20**(13): p. 3025-3033.

[44] Wei, J., Han, X., Bo, J., and Han, W., *Target selection for CAR-T therapy.* J Hematol Oncol, 2019. **12**(1): p. 62.

[45] Petrara, M.R., Giunco, S., Serraino, D., Dolcetti, R., and De Rossi, A., *Post-transplant lymphoproliferative disorders: from epidemiology to pathogenesis-driven treatment.* Cancer Lett, 2015. **369**(1): p. 37-44.

[46] Martinez, O.M. and Krams, S.M., *The Immune Response to Epstein Barr Virus and Implications for Posttransplant Lymphoproliferative Disorder.* Transplantation, 2017. **101**(9): p. 2009-2016.

[47] Cockfield, S.M., *Identifying the patient at risk for post-transplant lymphoproliferative disorder.* Transpl Infect Dis, 2001. **3**(2): p. 70-8.

[48] Dierickx, D., Tousseyn, T., Sagaert, X., Fieuws, S., Wlodarska, I., Morscio, J., Brepoels, L., Kuypers, D., Vanhaecke, J., Nevens, F., Verleden, G., Van Damme-Lombaerts, R., Renard, M., Pirenne, J., De Wolf-Peeters, C., and Verhoef, G., *Single-center analysis of biopsy-confirmed posttransplant lymphoproliferative disorder: incidence, clinicopathological characteristics and prognostic factors.* Leuk Lymphoma, 2013. **54**(11): p. 2433-40.

[49] Trappe, R.U., Dierickx, D., Zimmermann, H., Morschhauser, F., Mollee, P., Zaucha, J.M., Dreyling, M.H., Duhrsen, U., Reinke, P., Verhoef, G., Subklewe, M., Huttmann, A., Tousseyn, T., Salles, G., Kliem, V., Hauser, I.A., Tarella, C., Van Den Neste, E., Gheysens, O., Anagnostopoulos, I., Leblond, V., Riess, H., and Choquet, S., *Response to Rituximab Induction Is a Predictive Marker in B-Cell Post-Transplant Lymphoproliferative Disorder and Allows Successful Stratification Into Rituximab or R-CHOP Consolidation in an International, Prospective, Multicenter Phase II Trial.* J Clin Oncol, 2017. **35**(5): p. 536-543.

[50] Shannon-Lowe, C. and Rickinson, A., *The Global Landscape of EBV-Associated Tumors.* Front Oncol, 2019. **9**: p. 713.

[51] Morscio, J., Dierickx, D., and Tousseyn, T., *Molecular pathogenesis of B-cell posttransplant lymphoproliferative disorder: what do we know so far?* Clin Dev Immunol, 2013. **2013**: p. 150835.

[52] Reshef, R., Vardhanabhuti, S., Luskin, M.R., Heitjan, D.F., Hadjiliadis, D., Goral, S., Krok, K.L., Goldberg, L.R., Porter, D.L., Stadtmauer, E.A., and Tsai, D.E., *Reduction of immunosuppression as initial therapy for posttransplantation lymphoproliferative disorder.* Am J Transplant, 2011. **11**(2): p. 336-47.

[53] Elstrom, R.L., Andreadis, C., Aqui, N.A., Ahya, V.N., Bloom, R.D., Brozena, S.C., Olthoff, K.M., Schuster, S.J., Nasta, S.D., Stadtmauer, E.A., and Tsai, D.E., *Treatment of PTLD with rituximab or chemotherapy.* Am J Transplant, 2006. **6**(3): p. 569-76.

[54] Bollard, C.M., Rooney, C.M., and Heslop, H.E., *T-cell therapy in the treatment of post-transplant lymphoproliferative disease.* Nat Rev Clin Oncol, 2012. **9**(9): p. 510-9.

[55] Schultze-Florey, R.E., Tischer, S., Kuhlmann, L., Hundsdoerfer, P., Koch, A., Anagnostopoulos, I., Ravens, S., Goudeva, L., Schultze-Florey, C., Koenecke, C., Blasczyk, R., Koehl, U., Heuft, H.G., Prinz, I., Eiz-Vesper, B., and Maecker-Kolhoff, B., *Dissecting Epstein-Barr Virus-Specific T-Cell Responses After Allogeneic EBV-Specific T-Cell Transfer for Central Nervous System Posttransplant Lymphoproliferative Disease.* Front Immunol, 2018. **9**: p. 1475.

[56] Tischer, S., Priesner, C., Heuft, H.G., Goudeva, L., Mende, W., Barthold, M., Kloess, S., Arseniev, L., Aleksandrova, K., Maecker-Kolhoff, B., Blasczyk, R., Koehl, U., and Eiz-Vesper, B., *Rapid generation of clinical-grade antiviral T cells: selection of suitable T-cell donors and GMP-compliant manufacturing of antiviral T cells.* J Transl Med, 2014. **12**: p. 336.

[57] Priesner, C., Esser, R., Tischer, S., Marburger, M., Aleksandrova, K., Maecker-Kolhoff, B., Heuft, H.G., Goudeva, L., Blasczyk, R., Arseniev, L., Kohl, U., Eiz-Vesper, B., and Kloss, S., *Comparative Analysis of Clinical-Scale IFN-gamma-Positive T-Cell Enrichment Using Partially and Fully Integrated Platforms.* Front Immunol, 2016. **7**: p. 393.

[58] Andersen, P.S., Stryhn, A., Hansen, B.E., Fugger, L., Engberg, J., and Buus, S., *A recombinant antibody with the antigen-specific, major histocompatibility complex-restricted specificity of T cells.* Proc Natl Acad Sci U S A, 1996. **93**(5): p. 1820-4.

[59] Froscher, B.G. and Klinman, N.R., *Immunization with SV40-transformed cells yields mainly MHC-restricted monoclonal antibodies.* J Exp Med, 1986. **164**(1): p. 196-210.

[60] Sastry, K.S., Too, C.T., Kaur, K., Gehring, A.J., Low, L., Javiad, A., Pollicino, T., Li, L., Kennedy, P.T., Lopatin, U., Macary, P.A., and Bertoletti, A., *Targeting hepatitis B virus-infected cells with a T-cell receptor-like antibody.* J Virol, 2011. **85**(5): p. 1935-42.

[61] Sergeeva, A., Alatrash, G., He, H., Ruisaard, K., Lu, S., Wygant, J., McIntyre, B.W., Ma, Q., Li, D., St John, L., Clise-Dwyer, K., and Molldrem, J.J., *An anti-PR1/HLA-A2 T-cell receptor-like antibody mediates complement-dependent cytotoxicity against acute myeloid leukemia progenitor cells.* Blood, 2011. **117**(16): p. 4262-72.

[62] Dao, T., Yan, S., Veomett, N., Pankov, D., Zhou, L., Korontsvit, T., Scott, A., Whitten, J., Maslak, P., Casey, E., Tan, T., Liu, H., Zakhaleva, V., Curcio, M., Doubrovina, E., O'Reilly, R.J., Liu, C., and Scheinberg, D.A., *Targeting the intracellular WT1 oncogene product with a therapeutic human antibody.* Sci Transl Med, 2013. **5**(176): p. 176ra33.

[63] Zhao, Q., Ahmed, M., Tassev, D.V., Hasan, A., Kuo, T.Y., Guo, H.F., O'Reilly, R.J., and Cheung, N.K., *Affinity maturation of T-cell receptor-like antibodies for Wilms tumor 1 peptide greatly enhances therapeutic potential.* Leukemia, 2015. **29**(11): p. 2238-47.

[64] Sergeeva, A., He, H., Ruisaard, K., St John, L., Alatrash, G., Clise-Dwyer, K., Li, D., Patenia, R., Hong, R., Sukhumalchandra, P., You, M.J., Gagea, M., Ma, Q., and Molldrem, J.J., *Activity of 8F4, a T-cell receptor-like anti-PR1/HLA-A2 antibody, against primary human AML in vivo.* Leukemia, 2016. **30**(7): p. 1475-84.

[65] Lai, J., Tan, W.J., Too, C.T., Choo, J.A., Wong, L.H., Mustafa, F.B., Srinivasan, N., Lim, A.P., Zhong, Y., Gascoigne, N.R., Hanson, B.J., Chan, S.H., Chen, J., and MacAry, P.A., *Targeting Epstein-Barr virus-transformed B lymphoblastoid cells using antibodies with T-cell receptor-like specificities.* Blood, 2016. **128**(10): p. 1396-407.

[66] Ahmed, M., Lopez-Albaitero, A., Pankov, D., Santich, B.H., Liu, H., Yan, S., Xiang, J., Wang, P., Hasan, A.N., Selvakumar, A., O'Reilly, R.J., Liu, C., and Cheung, N.V., *TCR-mimic bispecific antibodies targeting LMP2A show potent activity against EBV malignancies.* JCI Insight, 2018. **3**(4).

[67] Mareeva, T., Lebedeva, T., Anikeeva, N., Manser, T., and Sykulev, Y., *Antibody specific for the peptide.major histocompatibility complex. Is it T cell receptor-like?* J Biol Chem, 2004. **279**(43): p. 44243-9.

[68] Zhang, G., Wang, L., Cui, H., Wang, X., Zhang, G., Ma, J., Han, H., He, W., Wang, W., Zhao, Y., Liu, C., Sun, M., and Gao, B., *Anti-melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor.* Sci Rep, 2014. **4**: p. 3571.

[69] Maus, M.V., Plotkin, J., Jakka, G., Stewart-Jones, G., Riviere, I., Merghoub, T., Wolchok, J., Renner, C., and Sadelain, M., *An MHC-restricted antibody-based chimeric antigen receptor requires TCR-like affinity to maintain antigen specificity.* Mol Ther Oncolytics, 2016. **3**: p. 1-9.

[70] Rafiq, S., Purdon, T.J., Daniyan, A.F., Koneru, M., Dao, T., Liu, C., Scheinberg, D.A., and Brentjens, R.J., *Optimized T-cell receptor-mimic chimeric antigen receptor T cells directed toward the intracellular Wilms Tumor 1 antigen.* Leukemia, 2017. **31**(8): p. 1788-1797.

[71] Uchanska-Ziegler, B., Nossner, E., Schenk, A., Ziegler, A., and Schendel, D.J., *Soluble T cell receptor-like properties of an HLA-B35-specific monoclonal antibody (TU165).* Eur J Immunol, 1993. **23**(3): p. 734-8.

[72] Menssen, R., Orth, P., Ziegler, A., and Saenger, W., *Decamer-like conformation of a nona-peptide bound to HLA-B*3501 due to non-standard positioning of the C terminus.* J Mol Biol, 1999. **285**(2): p. 645-53.

[73] Rossig, C., Kailayangiri, S., Jamitzky, S., and Altvater, B., *Carbohydrate Targets for CAR T Cells in Solid Childhood Cancers.* Front Oncol, 2018. **8**: p. 513.

[74] Todeschini, A.R., Dos Santos, J.N., Handa, K., and Hakomori, S.I., *Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway.* Proc Natl Acad Sci U S A, 2008. **105**(6): p. 1925-30.

[75] Furukawa, K., Hamamura, K., Ohkawa, Y., Ohmi, Y., and Furukawa, K., *Disialyl gangliosides enhance tumor phenotypes with differential modalities.* Glycoconj J, 2012. **29**(8-9): p. 579-84.

[76] Whittle, S.B., Smith, V., Doherty, E., Zhao, S., McCarty, S., and Zage, P.E., *Overview and recent advances in the treatment of neuroblastoma.* Expert Rev Anticancer Ther, 2017. **17**(4): p. 369-386.

[77] Dhillon, S., *Dinutuximab: first global approval.* Drugs, 2015. **75**(8): p. 923-7.

[78] Mujoo, K., Kipps, T.J., Yang, H.M., Cheresh, D.A., Wargalla, U., Sander, D.J., and Reisfeld, R.A., *Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal antiganglioside GD2 antibody 14.18.* Cancer Res, 1989. **49**(11): p. 2857-61.

[79] Rossig, C., Bollard, C.M., Nuchtern, J.G., Merchant, D.A., and Brenner, M.K., *Targeting of G(D2)-positive tumor cells by human T lymphocytes engineered to express chimeric T-cell receptor genes.* Int J Cancer, 2001. **94**(2): p. 228-36.

[80] Heczey, A., Louis, C.U., Savoldo, B., Dakhova, O., Durett, A., Grilley, B., Liu, H., Wu, M.F., Mei, Z., Gee, A., Mehta, B., Zhang, H., Mahmood, N., Tashiro, H., Heslop, H.E., Dotti, G., Rooney, C.M., and Brenner, M.K., *CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma.* Mol Ther, 2017. **25**(9): p. 2214-2224.

[81] Long, A.H., Highfill, S.L., Cui, Y., Smith, J.P., Walker, A.J., Ramakrishna, S., El-Etriby, R., Galli, S., Tsokos, M.G., Orentas, R.J., and Mackall, C.L., *Reduction of MDSCs with All-trans Retinoic Acid Improves CAR Therapy Efficacy for Sarcomas.* Cancer Immunol Res, 2016. **4**(10): p. 869-880.

[82] Kailayangiri, S., Altvater, B., Lesch, S., Balbach, S., Göttlich, C., Kühnemundt, J., Mikesch, J.H., Schelhaas, S., Jamitzky, S., Meltzer, J., Farwick, N., Greune, L., Fluegge, M., Kerl, K., Lode, H.N., Siebert, N., Müller, I., Walles, H., Hartmann, W., and Rossig, C., *EZH2 Inhibition in Ewing Sarcoma Upregulates G(D2) Expression for Targeting with Gene-Modified T Cells.* Mol Ther, 2019. **27**(5): p. 933-946.

[83] Dragon, A.C., Zimmermann, K., Nerreter, T., Sandfort, D., Lahrberg, J., Klöß, S., Kloth, C., Mangare, C., Bonifacius, A., Tischer-Zimmermann, S., Blasczyk, R., Maecker-Kolhoff, B., Uchanska-Ziegler, B., Abken, H., Schambach, A., Hudecek, M., and Eiz-Vesper, B., *CAR-T cells and TRUCKs that recognize an EBNA-3C-derived epitope presented on HLA-B*35 control Epstein-Barr virus-associated lymphoproliferation.* J Immunother Cancer, 2020. **8**(2).

[84] Allele Frequency Net Database. *Allele Frequency Search: HLA-B*35*. Accessed March 02, 2021, http://allelefrequencies.net/hla6006a.asp?hla_locus_type=Classical&hla_locus=B&hla_allele1=B*35&hla_allele2=B*35&hla_selection=&hla_pop_selection=&hla_population=&h

la_country=&hla_dataset=&hla_region=&hla_ethnic=&hla_study=&hla_order=order_1&hla_sample_size_pattern=bigger_equal_than&hla_sample_size=1000&hla_sample_year_pattern=equal&hla_sample_year=&hla_level_pattern=equal&hla_level=&standard=a&hla_show=.

[85] DTU Bioinformatics Department of Bio and Health Informatics. *NetMHCpan 4.1*. Accessed July 07, 2020, http://www.cbs.dtu.dk/services/NetMHCpan/.

[86] Allele Frequency Net Database. *Allele Frequency search: HLA-A*02*. Accessed March 02, 2021, http://allelefrequencies.net/hla6006a.asp?hla_locus_type=Classical&hla_locus=A&hla_allele1=A*02&hla_allele2=A*02&hla_selection=&hla_pop_selection=&hla_population=&hla_country=&hla_dataset=&hla_region=&hla_ethnic=&hla_study=&hla_order=order_1&hla_sample_size_pattern=bigger_equal_than&hla_sample_size=100&hla_sample_year_pattern=equal&hla_sample_year=&hla_level_pattern=equal&hla_level=&standard=a&hla_show=.

[87] Klebanoff, C.A., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A.R., Finkelstein, S.E., Palmer, D.C., Antony, P.A., Hwang, S.T., Rosenberg, S.A., Waldmann, T.A., and Restifo, N.P., *Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9571-6.

[88] Kawalekar, O.U., O'Connor, R.S., Fraietta, J.A., Guo, L., McGettigan, S.E., Posey, A.D., Jr., Patel, P.R., Guedan, S., Scholler, J., Keith, B., Snyder, N.W., Blair, I.A., Milone, M.C., and June, C.H., *Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells.* Immunity, 2016. **44**(2): p. 380-90.

[89] Mock, U., Nickolay, L., Philip, B., Cheung, G.W., Zhan, H., Johnston, I.C.D., Kaiser, A.D., Peggs, K., Pule, M., Thrasher, A.J., and Qasim, W., *Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy.* Cytotherapy, 2016. **18**(8): p. 1002-1011.

[90] Lock, D., Mockel-Tenbrinck, N., Drechsel, K., Barth, C., Mauer, D., Schaser, T., Kolbe, C., Al Rawashdeh, W., Brauner, J., Hardt, O., Pflug, N., Holtick, U., Borchmann, P., Assenmacher, M., and Kaiser, A., *Automated Manufacturing of Potent CD20-Directed Chimeric Antigen Receptor T Cells for Clinical Use.* Hum Gene Ther, 2017. **28**(10): p. 914-925.

[91] Blaeschke, F., Stenger, D., Kaeuferle, T., Willier, S., Lotfi, R., Kaiser, A.D., Assenmacher, M., Döring, M., Feucht, J., and Feuchtinger, T., *Induction of a central memory and stem cell memory phenotype in functionally active CD4(+) and CD8(+) CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19(+) acute lymphoblastic leukemia.* Cancer Immunol Immunother, 2018. **67**(7): p. 1053-1066.

[92] Cieri, N., Camisa, B., Cocchiarella, F., Forcato, M., Oliveira, G., Provasi, E., Bondanza, A., Bordignon, C., Peccatori, J., Ciceri, F., Lupo-Stanghellini, M.T., Mavilio, F., Mondino, A., Bicciato, S., Recchia, A., and Bonini, C., *IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors.* Blood, 2013. **121**(4): p. 573-84.

[93] Oren, R., Hod-Marco, M., Haus-Cohen, M., Thomas, S., Blat, D., Duvshani, N., Denkberg, G., Elbaz, Y., Benchetrit, F., Eshhar, Z., Stauss, H., and Reiter, Y., *Functional comparison of engineered T cells carrying a native TCR versus TCR-like antibody-based chimeric antigen receptors indicates affinity/avidity thresholds.* J Immunol, 2014. **193**(11): p. 5733-43.

[94] Srivastava, S. and Riddell, S.R., *Engineering CAR-T cells: Design concepts.* Trends Immunol, 2015. **36**(8): p. 494-502.

[95] Wagner, H.J., Bollard, C.M., Vigouroux, S., Huls, M.H., Anderson, R., Prentice, H.G., Brenner, M.K., Heslop, H.E., and Rooney, C.M., *A strategy for treatment of Epstein-Barr virus-positive Hodgkin's disease by targeting interleukin 12 to the tumor environment using tumor antigen-specific T cells.* Cancer Gene Ther, 2004. **11**(2): p. 81-91.

[96] Zhang, L., Kerkar, S.P., Yu, Z., Zheng, Z., Yang, S., Restifo, N.P., Rosenberg, S.A., and Morgan, R.A., *Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment.* Mol Ther, 2011. **19**(4): p. 751-9.

[97] Kunert, A., Chmielewski, M., Wijers, R., Berrevoets, C., Abken, H., and Debets, R., *Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors.* Oncoimmunology, 2017. **7**(1): p. e1378842.

[98] Munz, C., *Epstein-Barr Virus-Specific Immune Control by Innate Lymphocytes.* Front Immunol, 2017. **8**: p. 1658.

[99] Lunemann, A., Vanoaica, L.D., Azzi, T., Nadal, D., and Munz, C., *A distinct subpopulation of human NK cells restricts B cell transformation by EBV.* J Immunol, 2013. **191**(10): p. 4989-95.

[100] Hatton, O., Strauss-Albee, D.M., Zhao, N.Q., Haggadone, M.D., Pelpola, J.S., Krams, S.M., Martinez, O.M., and Blish, C.A., *NKG2A-Expressing Natural Killer Cells Dominate the Response to Autologous Lymphoblastoid Cells Infected with Epstein-Barr Virus.* Front Immunol, 2016. **7**: p. 607.

[101] Wiesmayr, S., Webber, S.A., Macedo, C., Popescu, I., Smith, L., Luce, J., and Metes, D., *Decreased NKp46 and NKG2D and elevated PD-1 are associated with altered NK-cell function in pediatric transplant patients with PTLD.* Eur J Immunol, 2012. **42**(2): p. 541-50.

[102] Yu, A.L., Gilman, A.L., Ozkaynak, M.F., London, W.B., Kreissman, S.G., Chen, H.X., Smith, M., Anderson, B., Villablanca, J.G., Matthay, K.K., Shimada, H., Grupp, S.A., Seeger, R., Reynolds, C.P., Buxton, A., Reisfeld, R.A., Gillies, S.D., Cohn, S.L., Maris, J.M., and Sondel, P.M., *Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma.* N Engl J Med, 2010. **363**(14): p. 1324-34.

[103] European Medicines Agency. *Qarziba*. Accessed February 16, 2021, https://www.ema.europa.eu/en/medicines/human/EPAR/qarziba.

[104] Cheung, N.K., Kushner, B.H., Cheung, I.Y., Kramer, K., Canete, A., Gerald, W., Bonilla, M.A., Finn, R., Yeh, S.J., and Larson, S.M., *Anti-G(D2) antibody treatment of minimal residual stage 4 neuroblastoma diagnosed at more than 1 year of age.* J Clin Oncol, 1998. **16**(9): p. 3053-60.

[105] Cheung, N.K., Cheung, I.Y., Kushner, B.H., Ostrovnaya, I., Chamberlain, E., Kramer, K., and Modak, S., *Murine anti-GD2 monoclonal antibody 3F8 combined with granulocyte-macrophage colony-stimulating factor and 13-cis-retinoic acid in high-risk patients with stage 4 neuroblastoma in first remission.* J Clin Oncol, 2012. **30**(26): p. 3264-70.

[106] Zimmermann, K., Kuehle, J., Dragon, A.C., Galla, M., Kloth, C., Rudek, L.S., Sandalcioglu, I.E., Neyazi, B., Moritz, T., Meyer, J., Rossig, C., Altvater, B., Eiz-Vesper, B., Morgan, M.A., Abken, H., and Schambach, A., *Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-GD2 CAR Expression and Inducible Cytokines.* Cancers, 2020. **12**(2): p. 375.

[107] Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Bartholomae, C.C., Ranzani, M., Benedicenti, F., Sergi, L.S., Ambrosi, A., Ponzoni, M., Doglioni, C., Di Serio, C., von Kalle, C., and Naldini, L., *The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy.* J Clin Invest, 2009. **119**(4): p. 964-75.

[108] McGarrity, G.J., Hoyah, G., Winemiller, A., Andre, K., Stein, D., Blick, G., Greenberg, R.N., Kinder, C., Zolopa, A., Binder-Scholl, G., Tebas, P., June, C.H., Humeau, L.M., and Rebello, T., *Patient monitoring and follow-up in lentiviral clinical trials.* J Gene Med, 2013. **15**(2): p. 78-82.

[109] Avanzi, M.P., Yeku, O., Li, X., Wijewarnasuriya, D.P., van Leeuwen, D.G., Cheung, K., Park, H., Purdon, T.J., Daniyan, A.F., Spitzer, M.H., and Brentjens, R.J., *Engineered Tumor-Targeted T Cells Mediate Enhanced Anti-Tumor Efficacy Both Directly and through Activation of the Endogenous Immune System.* Cell Rep, 2018. **23**(7): p. 2130-2141.

[110] Levine, B.L., Miskin, J., Wonnacott, K., and Keir, C., *Global Manufacturing of CAR T Cell Therapy.* Mol Ther Methods Clin Dev, 2017. **4**: p. 92-101.

[111] Glienke, W., Dragon, A.C., Zimmermann, K., Martyniszyn-Eiben, A., Mertens, M., Abken, H., Rossig, C., Altvater, B., Aleksandrova, K., Arseniev, L., Kloth, C., Moritz, T., Lode, H.N., Siebert, N., Blasczyk, R., Goudeva, L., Schambach, A., Köhl, U., Eiz-Vesper, B., and Esser, R., *GMP-compliant manufacturing of TRUCKS: CAR T cells targeting GD2 and releasing inducible IL-18*. 2021: Manuscript in revision.

[112] Aleksandrova, K., Leise, J., Priesner, C., Melk, A., Kubaink, F., Abken, H., Hombach, A., Aktas, M., Essl, M., Bürger, I., Kaiser, A., Rauser, G., Jurk, M., Goudeva, L., Glienke, W., Arseniev, L., Esser, R., and Köhl, U., *Functionality and Cell Senescence of CD4/ CD8-Selected CD20 CAR T Cells Manufactured Using the Automated CliniMACS Prodigy® Platform.* Transfus Med Hemother, 2019. **46**(1): p. 47-54.

[113] Zhu, F., Shah, N., Xu, H., Schneider, D., Orentas, R., Dropulic, B., Hari, P., and Keever-Taylor, C.A., *Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS Prodigy device at an academic medical center.* Cytotherapy, 2018. **20**(3): p. 394-406.

[114] Zhang, W., Jordan, K.R., Schulte, B., and Purev, E., *Characterization of clinical grade CD19 chimeric antigen receptor T cells produced using automated CliniMACS Prodigy system.* Drug Des Devel Ther, 2018. **12**: p. 3343-3356.

[115] Castella, M., Boronat, A., Martín-Ibáñez, R., Rodríguez, V., Suñé, G., Caballero, M., Marzal, B., Pérez-Amill, L., Martín-Antonio, B., Castaño, J., Bueno, C., Balagué, O., González-Navarro, E.A., Serra-Pages, C., Engel, P., Vilella, R., Benitez-Ribas, D., Ortiz-Maldonado, V., Cid, J., Tabera, J., Canals, J.M., Lozano, M., Baumann, T., Vilarrodona, A., Trias, E., Campo, E., Menendez, P., Urbano-Ispizua, Á., Yagüe, J., Pérez-Galán, P., Rives, S., Delgado, J., and Juan, M., *Development of a Novel Anti-CD19 Chimeric Antigen Receptor: A Paradigm for an Affordable CAR T Cell Production at Academic Institutions.* Mol Ther Methods Clin Dev, 2019. **12**: p. 134-144.

[116] Castella, M., Caballero-Baños, M., Ortiz-Maldonado, V., González-Navarro, E.A., Suñé, G., Antoñana-Vidósola, A., Boronat, A., Marzal, B., Millán, L., Martín-Antonio, B., Cid, J., Lozano, M., García, E., Tabera, J., Trias, E., Perpiña, U., Canals, J.M., Baumann, T., Benítez-Ribas, D., Campo, E., Yagüe, J., Urbano-Ispizua, Á., Rives, S., Delgado, J., and Juan, M., *Point-Of-Care CAR T-Cell Production (ARI-0001) Using a Closed Semi-automatic Bioreactor: Experience From an Academic Phase I Clinical Trial.* Front Immunol, 2020. **11**: p. 482.

[117] Pettitt, D., Arshad, Z., Smith, J., Stanic, T., Holländer, G., and Brindley, D., *CAR-T Cells: A Systematic Review and Mixed Methods Analysis of the Clinical Trial Landscape.* Mol Ther, 2018. **26**(2): p. 342-353.

[118] Zhao, Y., Stepto, H., and Schneider, C.K., *Development of the First World Health Organization Lentiviral Vector Standard: Toward the Production Control and Standardization of Lentivirus-Based Gene Therapy Products.* Hum Gene Ther Methods, 2017. **28**(4): p. 205-214.

[119] Graef, P., Buchholz, V.R., Stemberger, C., Flossdorf, M., Henkel, L., Schiemann, M., Drexler, I., Höfer, T., Riddell, S.R., and Busch, D.H., *Serial transfer of single-cell-derived immunocompetence reveals stemness of CD8(+) central memory T cells.* Immunity, 2014. **41**(1): p. 116-26.

[120] Stemberger, C., Graef, P., Odendahl, M., Albrecht, J., Dössinger, G., Anderl, F., Buchholz, V.R., Gasteiger, G., Schiemann, M., Grigoleit, G.U., Schuster, F.R., Borkhardt, A., Versluys, B., Tonn, T., Seifried, E., Einsele, H., Germeroth, L., Busch, D.H., and Neuenhahn, M., *Lowest numbers of primary CD8(+) T cells can reconstitute protective immunity upon adoptive immunotherapy.* Blood, 2014. **124**(4): p. 628-37.

[121] Panch, S.R., Srivastava, S.K., Elavia, N., McManus, A., Liu, S., Jin, P., Highfill, S.L., Li, X., Dagur, P., Kochenderfer, J.N., Fry, T.J., Mackall, C.L., Lee, D., Shah, N.N., and Stroncek, D.F., *Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics.* Mol Ther, 2019. **27**(7): p. 1275-1285.

7 Appendix

7.1 List of abbreviations

AP-1	activator protein 1
B-ALL	B cell precursor acute lymphoblastic leukemia
BART	BamH1 rightwards transcript
B-LCL	B-lymphoblastoid cell line
BRLF1	BamHI R fragment leftward open reading frame 1
BZLF1	BamHI Z fragment leftward open reading frame 1
CAR	chimeric antigen receptor
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLBCL	diffuse large B cell lymphoma
EBER	Epstein-Barr virus-encoded small RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescent protein
EMA	European Medicines Agency
FDA	Food and Drug Administration
FoxO1	Forkhead box protein O1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GOI	gene of interest
HLA	human leukocyte antigen
hPGK	human phosphoglycerate kinase
HSCT	hematopoietic stem cell transplantation
IFN-γ	interferon-γ
IgM	immunoglobulin M
IL	interleukin

IL-2Rβ	IL-2 receptor subunit β
JAK	Janus kinase
LMP	latent membrane protein
mAb	monoclonal antibody
MCL	mantle cell lymphoma
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κb
NK cell	natural killer cell
NKT cell	natural killer T cell
NY-ESO-1	New York esophageal squamous cell carcinoma 1
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death protein 1
PMBCL	primary mediastinal large B-cell lymphoma
PTLD	post-transplant lymphoproliferative disorder
scFv	single-chain fragment variable
SIN	self-inactivating
SOT	solid organ transplantation
STAT3/5	signal transducer and activator of transcription 3/5
TAM	tumor-associated macrophage
T-bet	T-box expressed in T cells
TCR	T cell receptor
TCT	T cell transduction
TGF-β	transforming growth factor β
TME	tumor microenvironment
TNF-α	tumor necrosis factor α
Treg	regulatory T cell
TRUCK	T cells redirected for universal cytokine-mediated killing
VCN	vector copy number
WT1	Wilms tumor suppressor gene 1

7.3 Danksagung

Zuerst möchte ich mich bei meiner Arbeitsgruppenleiterin und Erstgutachterin, Frau Prof. Dr. Britta Eiz-Vesper, bedanken, dir mir ermöglicht hat, an diesem tollen Projekt zu arbeiten. Vielen Dank für die Unterstützung, Motivation und Diskussionen über neue Ideen und Strategien, die mein Projekt sehr vorangebracht haben. Insbesondere bedanke ich mich für das entgegengebrachte Vertrauen, dass es mir ermöglicht hat, eine wissenschaftliche Eigenständigkeit zu entwickeln.

Weiterhin möchte ich mich bei Herrn Professor Rainer Blasczyk für die Möglichkeit bedanken, meine Doktorarbeit am Institut für Transfusionsmedizin und Transplantat Engineering anzufertigen und an verschiedenen interessanten Projekten mitzuarbeiten. Besonderen Dank dafür, dass ich die Möglichkeit hatte, meine Arbeit auf diversen Konferenzen vorzustellen und dabei über die aktuellen Forschungsentwicklungen informiert zu werden.

Prof. Dr. Thomas Moritz und Prof. Dr. Britta Maecker-Kolhoff danke ich für die wissenschaftliche Zweitbetreuung, die Unterstützung und die hilfreichen Fragen und Anregungen während der Supervisor-Meetings, die mir neue Sichtweisen auf mein Projekt ermöglichten.

Ich möchte mich auch bei all den Kooperationspartnern bedanken, die meine Projekte bereichert haben: Danke an Michael Hudecek und Thomas Nerreter aus Würzburg, Hinrich Abken aus Regensburg, Axel Schambach und dem ganzen Institut für Experimentelle Hämatologie, sowie Wolfgang Glienke, Ruth Esser und weiteren Mitarbeitern des Instituts für Zelltherapeutika der Medizinischen Hochschule Hannover.

Ein ganz besonderer Dank geht an alle Mitarbeiter des Instituts für Transfusionsmedizin und Transplantat Engineering und vor allem an meine Arbeitsgruppe für die gute Arbeitsatmosphäre und die zahlreichen hilfreichen (auch nicht-wissenschaftlichen) Gespräche. Besonders möchte ich mich bei Agnes Bonifacius, Sabine Tischer-Zimmermann, Caroline Mangare, Szilvia Bak, Sarina Lukis, Marina Krämer und Elvira Schulde für die Unterstützung auf verschiedenste Weise bedanken. Vielen Dank an alle Mitdoktoranden für die schöne gemeinsame Zeit.

Von ganzem Herzen möchte ich mich zuletzt bei meinen Eltern und Geschwistern, meinem Freund und all den Freunden bedanken, die mich während der Studien- und Promotionszeit liebevoll unterstützt und motiviert haben. Danke, dass ihr immer für mich da wart!

7.4 Erklärung

Hiermit erkläre ich, dass ich die Dissertation „**Genetic engineered T cells for adoptive T-cell therapy: From CARs to TRUCKs and behind**“ selbstständig verfasst habe. Bei der Anfertigung wurden keine Hilfen Dritter in Anspruch genommen.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Ich habe die Dissertation am Institut für Transfusionsmedizin und Transplantat Engineering an der Medizinischen Hochschule Hannover angefertigt.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hiermit erkläre ich außerdem, dass die promotionsrelevanten Originaldaten und -aufzeichnungen einschließlich der elektronischen Daten verfügbar sind.

Hannover, 31.07.2021 _____

(Anna Christina Dragon)