GMP-Compliant Manufacturing of TRUCKs: CAR T Cells targeting GD2 and Releasing Inducible IL-18

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Chimeric antigen receptor (CAR)-engineered T cells can be highly effective in the treatment of hematological malignancies, but mostly fail in the treatment of solid tumors. Thus, approaches using 4th advanced CAR T cells secreting immunomodulatory cytokines upon CAR signaling, known as TRUCKs (“T cells redirected for universal cytokine-mediated killing”), are currently under investigation. Based on our previous development and validation of automated and closed processing for GMP-compliant manufacturing of CAR T cells, we here present the proof of feasibility for translation of this method to TRUCKs. We generated IL-18-secreting TRUCKs targeting the tumor antigen GD2 using the CilniMACS Prodigy® system using a recently described “all-in-one” lentiviral vector combining constitutive anti-GD2 CAR expression and inducible IL-18. Starting with 0.84 x 10^8 and 0.91 x 10^8 T cells after enrichment of CD4+ and CD8+ we reached 68.3-fold and 71.4-fold T cell expansion rates, respectively, in two independent runs. Transduction efficiencies of 77.7% and 55.1% was obtained, and yields of 4.5 x 10^9 and 3.6 x 10^9 engineered T cells from the two donors, respectively, within 12 days. Preclinical characterization demonstrated antigen-specific GD2-CAR mediated activation after co-cultivation with GD2-expressing target cells. The functional capacities of the clinical-scale manufactured TRUCKs were similar to TRUCKs generated in laboratory-scale and were not impeded by cryopreservation. IL-18 TRUCKs were activated in an antigen-specific manner by co-cultivation with GD2-expressing target cells indicated by an increased expression of activation markers (e.g. CD25, CD69) on both CD4+ and CD8+ T cells and an enhanced release of pro-inflammatory cytokines and cytolytic mediators (e.g. IL-2,
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, anti-tumor activity, and in vivo persistence, advanced generations of CARs have been developed (1). A promising strategy to target solid tumors with their phenotypic heterogeneity has led to the fourth generation of CARs also known as TRUCKs or armed 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 cytokines, such as IL (interleukin)-12 and IL-18, but also enzymes and costimulatory ligands augmenting T cell activation. Innate immune cells are attracted and activated by IL-12 or IL-18 (4) to eliminate antigen-low expressing or antigen-negative cancer cells within the tumor (2).

CAR T cells engineered with inducible IL-18 release improve T cell effector functions towards superior activity against pancreatic and lung tumors in mice that were refractory to CAR T cells without cytokines (5, 6). 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 (7). GD2 is highly and consistently expressed in childhood cancer neuroblastoma and can be found on the cell surface of other solid cancer entities including breast cancer (8), osteosarcoma (9), melanoma (10), glioblastoma (11), small cell lung cancer (12), retinoblastoma (13), soft tissue sarcoma (14) and Ewing sarcoma (15, 16).

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 moderate or transient anti-tumor responses but failed to produce sustained remissions, emphasizing the need to modulate the T cell response (17–19). 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 (20). The CliniMACS Prodigy® (Miltenyi Biotec B.V. & Co. KG) allows ex vivo magnetic bead-based cell separation followed by activation, transduction, expansion, final formulation and sampling of T cells in one device leading to robust, reproducible and automated, supervised cost-effective manufacturing processes [Process of CAR T cell Therapy in Europe EHA Guidance Document, 2019 (21)]. 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, as shown by us and others (22–25) is here extended to the manufacturing of IL-18 TRUCKs targeting GD2 as an example. Preclinical characterization showed equivalent quality and function of the final clinical-scale products compared to manually produced IL-18 TRUCKs in laboratory-scale.

MATERIALS AND METHODS

Human Sample Materials

For the manufacturing of TRUCKs in a clinical-scale process (n=2) the CliniMACS Prodigy® (Miltenyi Biotec, Bergisch Gladbach, Germany) and for the manufacturing of laboratory-scale TRUCKs (n=3) lymphapheresis products from two healthy donors (D1 and D2) were obtained from the Institute for Transfusion Medicine of Hannover Medical School (MHH) after donors’ written informed consent. According to standard donation requirements, the donors had no signs of acute infection and no previous history of blood transfusion.

IL-18 TRUCK Construct and Production of Lentiviral Supernatants

The generation of the lentiviral IL-18 TRUCK SIN vector was described previously (26). In brief, the lentiviral 3rd generation
particles were generated as described previously (26, 28). Lentiviral vector particles were generated and isolated from 293T cells used for calcium phosphate transfection in the presence of 25 μM chloroquine. For transfection, the following plasmids were used: lentiviral vector plasmid (10 μg), pcDNA3.HIV-1.GP.4×CTE (lentiviral gag/pol) (12 μg) (29), pRSV-Rev (5 μg; kindly provided by T. Hope, Northwestern University Chicago, IL), and VSVg-encoding pMD.G (1.5 μg) (30). For better standardization, pcDNA3.HIV-1.GP.4×CTE, pRSV-Rev and pMD.G were produced and purified by PlasmidFactory (Bielefeld, Germany). After 36 h and 48 h of transfection, supernatants were harvested and concentrated via ultracentrifugation at 4°C and 13,238×g or 82,740×g for 16 h or 2 h, respectively. The particles were resuspended in TexMACSTM GMP medium. Lentiviral supernatant was titrated in HT1080 fibroblasts via spinoculation-mediated transduction, i.e. 1 × 10^5 cells were seeded, the supernatant containing viral particles and 4 μg/ml protamine sulfate (Sigma-Aldrich, St. Louis, USA) were added and cells were centrifuged (1 h, 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 (26, 31).

**GMP-Compliant Manufacturing of IL-18 TRUCKs Targeting GD2 With CliniMACS Prodigy® (Clinical-Scale Process)**

GMP-compliant manufacturing of IL-18 TRUCKs was performed using the CliniMACS Prodigy® platform, which allows for automated cell processing in a closed system controlled by operating software version V.1.3 and process software for T cell transduction (TCT) version V.2.0 (released). For overview of clinical-scale process see Figure 1. Within the scope of the automatically running process, the input of different variable process parameters like time points of transduction, media exchange, culture wash, harvesting and volume of media exchange is possible. Buffer, media, starting cell material and vector were connected directly to TS520 via sterile tubing welder device (TSCDII Terumo BCT).

The applied materials were either medicinal products with a marketing approval (HSA, PEI.H.03272.01.1), GMP-grade reagents and tubing sets from Miltenyi Biotec (designed following the recommendations of USP <1043> on ancillary materials and/or compliant with the requirements laid down in the Ph. Eur. Chapter 5.2.12, where applicable), or approved medical devices (DMSO, Composol, SSP+, transfer bags, connections, syringes). The pool-Human Serum P-HS/Tü was purchased from the Centre for Clinical Transfusion Medicine Tübingen/ZKT and certified as suitable for manufacturing of pharmaceutical products. The single non-regulated reagent was the vector, designed and produced at the Institute of Experimental Hematology, Hannover Medical School, Division of Hematology/Oncology. Detailed information regarding the materials was recorded, including the supplier, lot number, and expiration date. 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 × 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 TexMACSTM 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 72 h (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 D1 and 10 D2 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 – 24 h after day 5 of culture. Hereby the concentration of AB serum in the culture was reduced continuously using supplemented TexMACSTM 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 (D1) or SSP+ (D2) (Maco Pharma International GmbH, Langen, Germany) with 3.33% HSA for later cryopreservation. During cultivation, the temperature and atmosphere was maintained at 37°C with 5% CO2. 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 pH during cell cultivation pH-indicator strips MColorpHast™ pH 6.5 - 10.0 (Merck Millipore, Darmstadt, Germany) were used.

**Flow Cytometric Characterization of IL-18 TRUCKs Manufactured in Clinical-Scale Process**

Flow cytometric analysis was performed by using anti-human monoclonal antibodies. Cellular composition antibody table is
shown in Supplementary Table 1. Transduction efficiency was determined by GD2-CAR detection with the antibody Ganglidiomab, which was conjugated with Phycoerythrin (PE) by Miltenyi Biotec, herein after referred to as Ganglidiomab-PE. The antigen-specific single-chain variable fragment (scFv) of the GD2-CAR is derived from the anti-GD2 antibody 14G2a (32, 33).

Ganglidiomab is a monoclonal anti-idiotype antibody to 14G2a and therefore allows for a direct detection of the GD2-CAR on cells. Subsequently antibody staining (10 min at RT) cells were incubated with freshly prepared red blood cell lysis solution (Miltenyi Biotec). T cell phenotype antibody panel is shown in Supplementary Table 1. After antibody staining (10 min at RT in PBS supplemented with 4% FCS) cells were washed and resuspended with PBS supplemented with 4% FCS (both Merck, Darmstadt, Germany). Prior to flow cytometric analysis 7-AAD and Flow-Count Fluospheres (both Beckman Coulter) were added to the samples for dead cell discrimination and single platform cell quantification, respectively. Flow cytometric analysis was performed with the Navios flow cytometer (Navios 3L 10C, Software 1.3, Beckman Coulter). For gating strategies see Supplement Figures S6, 7.

**Cell Lines and Cell Culture**

The cell lines 293T, HT1080, HT1080-GD2 and SH-SY5Y were cultivated as recently described (26). NK-92 cells (human natural killer lymphoma #ACC 488; DSMZ, Braunschweig, Germany) were cultivated in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, 2 mM L-glutamine (c.c.pro, Oberdorla, Germany) and 400 IU/mL human IL-2 (Proleukin S, Novartis Pharma GmbH, Nürnberg, Germany). THP-1 (human acute monocytic leukemia, #ACC 16; DSMZ, Braunschweig, Germany) cells were cultivated in RPMI 1640 medium with 2 mM L-glutamine, 2-mercaptoethanol to a final concentration of 0.05 mM, 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS), and 50 IU/mL penicillin and 50 μg/ml streptomycin. All cells were tested for mycoplasma.
contamination on a regular basis using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland) according to the manufacturer’s protocol.

**Laboratory Manufacturing of IL-18 TRUCKs Targeting GD₂ (Laboratory-Scale Process)**

CD4⁺ and CD8⁺ T cells isolated by the CliniMACS Prodigy® (see clinical-scale manufacturing) were transduced and expanded as previously described (34). Briefly, they were activated with anti-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1:1 in TexMACS™ (Miltenyi Biotec) with 3% human serum (c.c.pro, Oberdorla, Germany) supplemented with 12.5 ng/ml IL-7 and IL-15 (PeproTech, Rocky Hill, NJ, USA). On the following day, T cells were either left untransduced or transduced with lentiviral particles by spinoculation using an MOI of 7 and addition of 5 μg/ml Polybrene Infection/Transfection Reagent (Merck Millipore, Burlington, MA, USA). The anti-CD3/CD28 beads were removed on the following day and cells were further cultivated in TexMACS™ medium supplemented with 3% human serum, 12.5 ng/ml IL-7 and IL-15 and splitting 1:2 every 2 - 3 days for a total expansion time of 12 days.

**Cryopreservation and Thawing of IL-18 TRUCKs Targeting GD₂**

Untransduced and transduced T cells were cryopreserved in Composol® FS Fresenius Kabi, Bad Homburg, Germany/2.86% (w/v) HSA (Biotest, Dreieich, Germany) (D1) and SSP+ (Maco Pharma International GmbH, Langen, Germany) supplemented with 3.33% (w/v) HSA (D2), respectively after manufacturing by adjusting the cell counts and addition of DMSO (CryoSure-DMSO, USP grade; WAK Chemie, Steinbach, Germany) to a final concentration of 10% (v/v). After cryopreservation in a < -80°C freezer overnight, the cells were stored in the vapor phase above liquid nitrogen at < -140°C. For T cell phenotype analysis cells were thawed in RPMI 1640 medium with 20% (v/v) FCS (both Merck, Darmstadt, Germany) and rested for 1 h in RPMI 1640 with 10% FCS (37°C, 5% (v/v) CO₂) before flow cytometric analysis. For functional analysis of the cryopreserved cells, they were thawed and seeded in TexMACS™ medium in a cell density of 2.5 x 10⁶ cells/ml and rested overnight.

**Co-Culture of Laboratory- and Clinical-Scale IL-18 TRUCKs With Target Cells**

Directly after expansion or after cryopreservation and thawing (cryo), functionality of the laboratory- and clinical-scale IL-18 TRUCKs in comparison to untransduced T cells was assessed by co-culturing them with target cells. For flow cytometry and soluble mediator measurements, 5 x 10⁴ target cells were seeded in 800 μl of their respective culture medium, which was removed after 4 - 24 h followed by 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 10⁸ 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 Gangliodiomab-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:

\[
\text{CD3⁻ cell killing} = 100 \% - \frac{\text{CD3⁻ cell frequency (co-culture)}}{\text{CD3⁻ cell frequency (respective co-culture with untransduced T cells)}}
\]

**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). Background impedance of all control wells served as maximum controls. Absorbance 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 10⁵ cells (SH-SY5Y) or 2 x 10⁴ cells (HT1080, HT1080-GD₂) in 200 μl 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 – 25 h. 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 chemo-attractive 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, migration assays with THP-1 or NK-92 cells were performed using a Boyden chamber (NeuroProbe, Gaithersburg, MD, USA) as recently described (26). The cell number was calculated using an Olympus IX71 microscope and imageJ 1.53k software. To normalize results from different plates, cell numbers of migrated cells towards untransduced T cells as background migration were subtracted from all values for each plate.

Isolation of Genomic DNA and Determination of the VCN by qPCR
Genomic DNA was isolated from 1 x 10⁶ transduced or untransduced cells (-20°C frozen cell pellets) with the QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The determination of the VCN by qPCR was performed as recently described (35).

Statistics
Statistical analysis was performed with GraphPad Prism V.9.1.2 using the Kruskal-Wallis and uncorrected Dunn’s test. Only mean ranks of preselected data sets were compared: large-scale TRUCKs cultured alone or co-cultured with different target cells, large-scale TRUCKs co-cultured with different target cells immediately after generation (indicated by black asterisks) or cryopreservation (indicated by grey asterisks), large-scale TRUCKs co-cultured with different target cells in comparison to the same co-cultures with all laboratory-scale manufactured cells. Significant differences are shown (*p ≤ 0.05, **p ≤ 0.01).

RESULTS
GMP-Compliant Manufacturing Process of IL-18 TRUCKs Targeting GD₂ Using Automated Cell Processing in a Closed System
To assess feasibility, we performed two complete GMP-compliant processes (D1 and D2 with two individual donor lymphocyte products) to manufacture IL-18 TRUCKs targeting GD₂ using an automated TCT protocol for CliniMACS Prodigy® as shown in Figure 1.

Recovery and Purity of CD4⁺ and CD8⁺ Cells Enriched in an Automated Process
Starting with the lymphapheresis, 1.14 x 10⁸ D1 and 1.17 x 10⁹ D2, respectively, CD4⁺ and CD8⁺ cells (20.6% D1 and 46.1% D2 of unstimulated short time lymphapheresis) were applied for enrichment. An amount of 0.67 x 10⁹ D1 and 0.88 x 10⁹ D2 CD4⁺ and CD8⁺ cells were obtained representing a recovery of 58.8% D1 and 75.2% D2. Thereby, the isolation of CD4⁺ cells with a recovery of 63.9% D1 and 84.0% D2 was more effective than enrichment of CD8⁺ cells with 46.8% D1 and 61.7% D2 recovery, respectively (Figure 2A). The achieved T cell purities and cell compositions are shown in Figure 2B. Due to the CD8 based isolation procedure, NK as well as NKT cells were not completely depleted releasing 7.6% D1 and 4.4% D2 contaminating NKT cells in the final cell product (Figure 2B). The CD4/CD8 ratio pre-enrichment was 2.1 D1 and 1.6 D2 and post-enrichment 2.9 D1 and 2.2 D2 reflecting a superior recovery of CD4⁺ cells (Figure 2D).

Expanded IL-18 TRUCKs Targeting GD₂ Display a High Purity and Favorable Phenotype
In accordance with the TCT protocol, the cell expansion started with 0.84 x 10⁸ D1 and 0.9 x 10⁹ D2 viable T cells (corresponding 1.18 x 10⁹ D1 and 1.0 x 10⁹ D2 viable WBC) after enrichment of CD4⁺ and CD8⁺ cells. The expansion rate during the 12-day manufacturing process was 68.3-fold D1 and 71.4-fold D2 for T cells. Viability of the cells was >96% during the whole process until final harvest (Figure 2C). Cell concentration increased in a constant culture volume from 3.8 x 10⁶/ml D1 on day 5 and 5.5 x 10⁶/ml D2 to 31.2 x 10⁶/ml D1 and 34.2 x 10⁶/ml D2 in the final products (Figure S1A). Monitoring of cell culture condition revealed a pH of 7.0 - 7.1 and a glucose level above the critical level of 100 mg/dl. (Figure S1B). T cell purity and cell composition of the final products is shown in Figure 2B. During expansion, the CD4/CD8 ratio increased in the first run from 2.9 to 3.3 D1 but decreased slightly in the second run from 2.2 to 2.0 D2 (Figure 2D). 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 at process day 0 and in the final product at process day 12 as shown in Figure 2F.

Transduction of T Cells With the “All-In-One” Lentiviral Vector Is Highly Efficient
The recently described “all-in-one” lentiviral vector (26) encoding constitutive GD₂ CAR and inducible IL-18 expression was used for cell transduction on day 1 of the manufacturing process with a multiplicity of infection (MOI) of 29 for D1 and 10 for D2 derived T cells. The percentage of transduced CD3⁺ cells in the final product, was 74.9% D1 and 52.2% D2, while CD4⁺ cells exhibited a higher transduction efficiency (80.6% D1 and 65.8% D2) compared to CD8⁺ cells (57.2% D1 and 26.5% D2) (Figure 2E). The VCN determined by qPCR of genomic DNA in the final products were 2.6 D1 and 2.4 D2 copies/cell, respectively.

Preclinical In Vitro Characterization Using Clinical- and Laboratory-Scale Manufactured TRUCKs
IL-18 TRUCKs targeting GD₂ manufactured using the CliniMACS Prodigy® (referred to as clinical-scale TRUCKs)
were compared to the respective TRUCKs generated under laboratory conditions (referred to as laboratory-scale TRUCKs). These cells were manufactured manually as previously described (34) using the same isolated CD4+ and CD8+ T cell starting population, lentiviral vector, expansion media, cytokines, and experimental timing. Untransduced T cells as well as GD2 TRUCKs with inducible EGFP expression (referred to as EGFP-TRUCKs) were generated in laboratory scale and served as controls. From a starting fraction of 0.6 x 10^6 cells, laboratory-scale TRUCKs expanded to a cell number of 48 x 10^6 cells, which was slightly lower compared to untransduced T cells and EGFP-TRUCKs with final cell numbers of 53 x 10^6 and 54 x 10^6 cells, respectively (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 2.2 for clinical- and 2.1 for laboratory-scale TRUCKs, whereas it was
CD25 and CD69 expression on CD3+ T cells (45% and 24%; CD28 stimulation and IL-7 and IL-15 supplementation, revealed TRUCKs, assessed after expansion of T cells with anti-CD3/

Increase of Activation Marker Expression

Respond to GD+

of Cytokines Upon Target Recognition

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 69 - 88% of HT1080-GD2 and 65 - 86% of SH-SY5Y cells at different E:T ratios, whereas the frequency of HT1080 in co-cultures with the clinical-scale TRUCKs was on the level of untransduced T cells or laboratory-scale manufactured cells, likely representing expected allo-reactivity (Figure 6A). Compared to TRUCKs generated in the laboratory-scale, clinical-scale-manufactured TRUCKs exhibited a similar cytotoxic ability to eliminate GD2 target cells, which was moreover not impeded by cryopreservation. The release of lactate dehydrogenase (LDH) into the supernatant as parameter for cytosis confirmed the result; cytotoxicity was enhanced in co-cultures of both IL-18 TRUCKs with HT1080-GD2 cells (18-23% and 0-17% for large- and laboratory-scale TRUCKs, respectively) compared to the respective co-cultures with unmodified HT1080 cells, in which LDH was not released above background of cells cultured alone (Figure S5A). Target cell death by LDH release in co-cultures of clinical-scale TRUCKs with SH-SY5Y was also enhanced (6-16%) and similar to the cytotoxicity by laboratory-scale TRUCKs (2-8%). LDH measurements in the co-cultures with cryopreserved T cells revealed a similar cytotoxic capability towards GD2 target cells. Real-time measurement of target cell viability confirmed these results. 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 with laboratory- or clinical-scale TRUCKs for 60 h in different ratios (Figures 6B, C). 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 6D). 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 tended to be even larger and already appeared at low E:T ratios in co-cultures with SH-SY5Y cells (Figure S5B).
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 freshly-generated or cryopreserved clinical-scale TRUCKs was induced in a target-specific manner up to 41 pg/ml upon HT1080-GD2 or 18 pg/ml upon SH-SY5Y encounter (Figure 7A). 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 TRUCKs with inducible EGFP expression (EGFP-TRUCK) as control. The manufactured cells were either characterized directly after the generation process (d12) or after cryopreservation and thawing (cryo). (A, B) Frequency of CD4+ and CD8+ T cells in the final cell product. (C, D) Percentage of CAR+ cells of 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 Gangliosidoma antibody after expansion. (E–H) Expression of the activation markers CD25 on CD3+, CD69 on CD3+, CD137 on CD8+ and CD154 on CD4+ T cells. (A, B, D–H) Data are shown as mean ± SD. Statistical differences of large-scale TRUCKs directly after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn’s test, significant differences are shown (*p ≤ 0.05).
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 QlinMACS Prodigy (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) 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). (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. (B–E) A dashed line indicates background levels of the respective expression by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. Data is shown as mean ± SD. Statistical differences of clinical-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn’s test, significant differences are shown (*p \leq 0.05, **p \leq 0.01).
FIGURE 5 | 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 TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) 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 in an effector-to-target (E:T) ratio of 8:1 or cultivation of T cells only. The concentration of released cytokines (A) IL-2, (B) IL-4, (C) IL-10, (D) granzyme B, (E) IFN-γ, (F) perforin, and (G) TNF-α in the cell culture supernatants after 48 h was assessed by LEGENDPlex™. A dashed line indicates background levels of the respective cytokine release by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. (A–G) Data are shown as mean ± SD. Statistical differences of clinical-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to all laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn’s test, significant differences are shown (*p ≤ 0.05, **p ≤ 0.01).
untransduced T cells, EGFP-TRUCKs or upon co-culture with GD2 target cells, likely as a result of induced IL-18 cytokine secretion (Figures 7C, 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 IL-18 biological activity.

**DISCUSSION**

In general, CAR and TRUCK vector design is a critical aspect for the generation of engineered T cells. Especially, the costimulatory domains within the CAR might be of high interest to improve CAR T-cell activity and long-lasting persistence with reduced T cell exhaustion, also with respect to the targeted tumor antigen
TRUCKs additionally combine the redirected CAR T cell attack with the on-site release of a biologically active protein while avoiding its systemic toxicity, thereby holding promise to modulate the environment of the targeted solid tumor. The cytokine of choice within the TRUCK concept should be chosen with respect to the desired immune response within the tumor microenvironment (TME). The IL-18 cytokine creates a proinflammatory environment, recruits bystander effector cells to the tumor site and enhances cytolytic activity (4–6). Therefore, IL-18 was an attractive cytokine for the development of the “all-in-one” lentiviral vector combining constitutive anti-GD2 CAR expression and inducible IL-18 (26) as well as for the automated and closed processing for GMP-compliant manufacturing process for CAR T cells (24, 25).

TRUCKs combine the redirected CAR T cell attack with the on-site release of a biologically active protein while avoiding its systemic toxicity, thereby holding promise to modulate the environment of the targeted solid tumor. Further development of the strategy is based on design and broad in vitro characterization of the “all-in-one” lentiviral vector combining constitutive anti-GD2 CAR expression and inducible IL-18 (26) as well as on an automated and closed processing for GMP-compliant manufacturing process for CAR T cells (24, 25). We present the proof of feasibility for translation of the 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. The fully integrated modular system allows for 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+ and CD8+ T cell subset followed by activation, transduction and expansion of effector cells. CD4+ and CD8+ enrichment is regarded as a safety procedure to decrease blast counts in the culture (38). CD4+ and CD8+ enrichment also decreases contaminating cells such as monocytes, which inhibit CAR T cell expansion (39). The primary objective was the feasibility of cell production for 3 dose levels (e.g. 5 x 10^5, 1 x10^6, and 3 x 10^6 anti-GD2 IL-18 TRUCKs/kg). For enrichment of CD4+ and CD8+ cells the TCT process is limited to a maximum of 3 x 10^6 target cells. This means for our two processes that only a part (20.6% D1 and 46.1% D2) of the lymphapheresis from a healthy donor was used for enrichment. Likewise, only part of enriched cells (12.5% D1 and 10.0% D2) could be applied for activation and expansion. Due to the limited culture volume and growth area, it is recommended to start with 1 x 10^5 cells. Any remainder may be frozen as backup for the patient.

After CD4+ and CD8+ enrichment we found high T cell purities with low contaminating cell populations of CD8+ NK and NKT cells which are not removed during the CD4+ and CD8+ enrichment step also described by other groups (23, 38).
FIGURE 7 | 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; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab-scale untransduced; n=3) as well as GD2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3 [except for D, in which n=1]) 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 in the indicated effector-to-target (E:T) ratios or cultivation alone (T cells only). (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 48 h was placed in a Boyden chamber, covered with an 8 µm polycarbonate membrane and incubated for another 4 h. 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. To normalize results from different plates, cell numbers of migrated cells towards untransduced T cells only were subtracted from all values. (A, C, D) Data is shown as mean ± SD. A dashed line indicates background levels of the respective cytokine release by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. Statistical differences of large-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn’s test, significant differences are shown (*p ≤ 0.05).
IL-18 in an antigen-dependent manner and mediated a very low background secretion upon co-cultivation with target-negative cells or spontaneous release without target cells. The risk of toxicity for IL-18 TRUCKs is different to IL-12 TRUCKs that revealed severe toxicity of T cells engineered with inducible IL-12 in a melanoma mouse model due to off-target cytokine secretion (4). However, other studies with IL-12 TRUCKs reported a safe administration into mice and such toxicities were not observed for IL-18 TRUCKs (2, 3, 5, 51). GMP-compliant manufactured IL-18 TRUCKs targeting GD2 showed a high cytotoxic capability and were able to eliminate tumor cells while forming clusters around target cells. IL-18 TRUCKs show higher toxicities against high GD2-expressing target cells confirming the in vitro studies by Wiebel et al. (52).

To accurately quantify and confirm cytotoxicity towards target cells, we combined different methodologies including the label-free, real-time monitoring by impedance measurements, flow cytometric analysis allowing for concurrent phenotypic evaluation of TRUCKs, and detection of pro-inflammatory cytokines as indirect and cytotoxic mediators as direct parameter of cell lysis in the co-culture. Kiesgen (53) et al. comprehensively compare the power and limitation of different cytotoxicity assays and emphasize that especially impedance-based assays display a superior sensitivity and signal-to-background ratio over the “gold standard” 51chromium-release assay making it possible to evaluate even low E:T ratios as most appropriate to resemble physiological conditions. Such low E:T ratios would be interesting to evaluate in further experiments, since the manufactured TRUCKs exhibited rapid elimination of SH-SY5Y cells in the lowest E:T ratio of 1:1.

Moreover, in vitro tests using repeated antigen stimulation or inclusion of immunosuppressive factors present in the TME are increasingly being used and could give an insight about persistence and exhaustion level of the generated TRUCKs upon high antigen stress.

We show similar manufacturing of clinical-scale and laboratory-scaled IL-18 TRUCKs concerning transduction and amplification efficiency and cellular functionality. After cryopreservation of the T cell products, the specificity and cytotoxicity of TRUCKs was maintained. Attempts to treat solid tumors with redirected T cells have largely failed so far, with very few patients responding and with only transient and partial tumor regression (17, 18, 48, 54–57). The poor clinical outcome is thought to be due at least in part to an unfavorable environment in the tumor tissue that suppresses CAR T cell responses. TRUCK-secreted cytokine IL-18 led to increased recruitment of monocytes and NK cells in an in vitro cell migration assay. This may contribute to reprogramming the tumor stroma towards a more favorable environment for CAR T cell function, thereby enhancing their efficacy in the treatment of solid tumors. Furthermore, IL-18 was shown to polarize TRUCKs towards more potent pro-inflammatory effector cells that do not drive into functional exhaustion in the long term (5).

In conclusion, GMP-compliant manufacturing of IL-18 TRUCKs targeting GD2 using the automated closed CliniMACS Prodigy® system is feasible and enables the manufacturing of a sufficient number of cells for clinical application. The automatic mode of operation improves standardization and robustness of the manufacturing process. This benefits the manufacturing at different sites for an academia-initiated multicenter trial. The smooth adaption of the process established and validated for the manufacturing of CAR T cells to generate IL-18 TRUCKs encourages the translation of the procedure to other cells and targets.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

**ETHICS STATEMENT**

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

**AUTHOR CONTRIBUTIONS**

Conceptualization: RE, WG, BE-V, AD, UK, KZ, and ASc. Methodology: AD, WG, RE, MM, AM-E, CK, ASt, KZ, and ASc. Formal analysis: WG, AD, RE, AM-E, MM, CK, ASt, KZ, and KA. Resources: HL, NS, and CR. Writing original draft application: WG, AD, RE, and AM-E. Writing review and editing: all authors. Supervision: HA, LA, TM, RB, LG, ASc, BE-V, and UK. Funding acquisition: BE-V, HA, TM, RB, ASc, UK, and CR. All authors have read and agreed to the published version of the manuscript.

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