CD33-directed immunotherapy with third-generation chimeric antigen receptor T cells and gemtuzumab ozogamicin in intact and CD33-edited acute myeloid leukemia and hematopoietic stem and progenitor cells

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Abstract
Immunotherapies, such as chimeric antigen receptor (CAR) modified T cells and antibody-drug conjugates (ADCs), have revolutionized the treatment of cancer, especially of lymphoid malignancies. The application of targeted immunotherapy to patients with acute myeloid leukemia (AML) has been limited in particular by the lack of a tumor-specific target antigen. Gemtuzumab ozogamicin (GO), an ADC targeting CD33, is the only approved immunotherapeutic agent in AML. In our study, we introduce a CD33-directed third-generation CAR T-cell product (3G.CAR33-T) for the treatment of patients with AML. 3G.CAR33-T cells could be expanded up to the end-of-culture, that is, 17 days after transduction, and displayed significant cytokine secretion and robust cytotoxic activity when incubated with CD33-positive cells including cell lines, drug-resistant cells, primary blasts as well as normal hematopoietic stem and progenitor cells.

Abbreviations: 2G.CAR33-T cell, second-generation CD33-directed CAR T cell; 3G.CAR33-T cell, third-generation CD33-directed CAR T cell; 3G.CAR19-T cell, third-generation CD19-directed CAR T cell; 51Cr, chromium-51; ADC, antibody-drug conjugate; AML, acute myeloid leukemia; AraC, cytarabine; ATC, activated T cell; CAR, chimeric antigen receptor; CAR T cells, chimeric antigen receptor T cells; CD, cluster of differentiation; CML, chronic myelogenous leukemia; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats with associated Caspase 9; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FC, flow cytometry; FDA, U.S. Food and Drug Administration; FLT-3, fms-like tyrosine kinase 3; FLT3-L, fms-like tyrosine kinase 3 ligand; G-CSF, granulocyte colony stimulating factor; GO, gemtuzumab ozogamicin; gRNA, guide RNA; HD, healthy donor; HSPCs, hematopoietic stem and progenitor cell; IC50, half-maximal inhibitory concentration; IFN, interferon; Ig, immunoglobulin; IgG2, C2-set Ig-like; IgGv, V-set Ig-like; IIT, Investigator initiated trial; IL, interleukin; IMDM, Iscove's Modified Dulbecco's Medium; MFI, mean fluorescent intensity; OS, overall survival; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20; PCR, polymerase chain reaction; RNP, ribonucleoprotein; RT, room temperature; SCF, stem cell factor; scFv, single chain variable fragment; Siglec, sialic acid-binding Ig-like lectin; SR1, stemregenin 1; TCR, T-cell receptor; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor; TPO, thrombopoietin.

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When compared to second-generation CAR33-T cells, 3G.CAR33-T cells exhibited higher viability, increased proliferation and stronger cytotoxicity. Also, GO exerted strong antileukemia activity against CD33-positive AML cells. Upon genomic deletion of CD33 in HSPCs, 3G.CAR33-T cells and GO preferentially killed wildtype leukemia cells, while sparing CD33-deficient HSPCs. Our data provide evidence for the applicability of CD33-targeted immunotherapies in AML and its potential implementation in CD33 genome-edited stem cell transplantation approaches.

**KEYWORDS**
acute myeloid leukemia, CD33, CD33-editing, chimeric antigen receptor T cells, CRISPR/Cas9, gemtuzumab ozogamicin, hematopoietic stem and progenitor cells, third generation CAR T cells

**What’s new?**
In the development of immunotherapy for acute myeloid leukemia (AML), a target of interest is CD33, which is expressed on blast cells in more than 90 percent of AML patients. CD33 is also expressed on healthy myeloid and progenitor cells, however, raising the risk for off-target effects with CD33 therapies. Here, the authors introduce a CD33-directed third-generation chimeric antigen receptor (CAR) T-cell product (3G.CAR33-T). 3G.CAR33-T cells were effective against CD33-positive cells, including AML blasts, and successfully overcame AML drug resistance. Genomic deletion of CD33 in hematopoietic stem and progenitor cells resulted in preferential killing of leukemia cells by 3G.CAR33-T cells.

**1 | INTRODUCTION**

Acute myeloid leukemia (AML) is a malignant proliferation of abnormal myeloid cells in the bone marrow, which interferes with normal hematopoiesis. Although treatment has improved the prognosis of AML patients, the 5-year overall survival (OS) among patients younger than 60 years is approximately 35% to 40%. In elderly patients, the 5-year OS rate further decreases to approximately 5% to 15% with median survival of only 5 to 10 months, particularly due to frailty and comorbidities that limit the applicability of intensive chemotherapeutic regimens. In addition, therapy with chemotherapeutic drugs can result in drug resistance further limiting available treatment options. Thus, novel therapies for AML patients are needed.

In fact, with recently approved small molecule inhibitors, for example, the fms-like tyrosine kinase 3 (FLT-3) inhibitor midostaurin and gilteritinib, new treatment approaches are available for AML patients. Nonetheless, in AML patients lacking these specific target mutations treatment options remain limited. Besides the development of small molecule inhibitors, immunotherapy has emerged as a promising therapeutic approach for hematologic malignancies, in particular with the development and improvement of chimeric antigen receptor (CAR) modified T cells and antibody-drug conjugates (ADCs).

CAR T cells combine the antigen-specific properties of a monoclonal antibody with the activating and effector function of a T cell. First-generation CARs contain the tyrosine-based ζ-signal-transducing subunit from the T-cell receptor (TCR)/CD3 complex as intracellular domain, whereas second-generation CARs carry costimulatory domains, for example, CD28, 4-1BB (CD137) or OX40 (CD134) adjacent to the ζ domain. Costimulation has been shown to enhance CAR T-cell activity and signaling and mediate higher rates of clinical responses. Third-generation CARs including CD28 and 4-1BB as costimulatory molecules have demonstrated superior proliferation, survival and antitumor activity as compared to second-generation CARs comprising either CD28 or 4-1BB.

Gemtuzumab ozogamicin (GO, Mylotarg), the only ADC approved for the treatment of AML, consists of a humanized immunoglobulin (Ig)G4 antibody directed against CD33 that is conjugated to the cytotoxic drug calicheamicin. After binding of the antibody to CD33 and subsequent internalization, calicheamicin mediates cytotoxicity to CD33-positive myeloid cells primarily through induction of DNA damage and subsequent apoptosis. GO was initially approved in 2000 for the treatment of relapsed AML patients older than 60 years of age, but was withdrawn from the market in 2010 due to a lack of benefit. Subsequent trials, however, supported safety and efficacy of GO as first-line, single agent for the treatment of older adults with AML unsuitable for intensive chemotherapy, as well as for newly diagnosed AML patients in combination with induction therapy. Based on these trials, GO was approved in September 2017 for the treatment of adults with CD33-positive newly diagnosed AML and adults and children aged 2 years or older with relapsed or refractory CD33-positive AML. In June 2020, the FDA extended the approval of GO to newly diagnosed pediatric patients ≥1 month of age. Further studies applying optimized treatment strategies including fractionated GO doses confirmed a lower risk of relapse and prolonged survival for AML patients treated with GO. CD33 (Siglec-3), the target molecule of GO, is a member of the sialic acid-binding Ig-like lectin (Siglec) family. It is expressed as a
transmembrane glycoprotein on healthy and malignant myeloid cells.\textsuperscript{31,32} In AML, which accounts for 70% of acute leukemias in adults,\textsuperscript{33} CD33 is expressed on blasts of >90% patients and importantly also on leukemic stem cells.\textsuperscript{34-36} Accordingly, CD33 is a promising target for immunotherapy of AML, particularly with antibody-based treatment\textsuperscript{37-39} and CAR T cells.\textsuperscript{30,41} Full-length CD33 consists of two extracellular domains, the V-set Ig-like (IgV) and C2-set Ig-like (IgC2), a transmembrane, and an intracellular domain. In addition to the full-length isoform, CD33 is expressed as a shorter IgV-lacking isoform (CD33-D2) generated by alternative splicing in some patients.\textsuperscript{42,43} CD33 is not only expressed on leukemic cells but also on virtually all healthy myeloid and progenitor cells.\textsuperscript{44,45} The on-target off-leukemia toxicity is a major side effect observed in the clinical practice and in clinical trials investigating CD33-targeting therapies. Thus, efforts have focused on the optimization of CD33-targeting therapies. Recent studies demonstrated that CD33-deficient hematopoietic stem and progenitor cells (HSPCs) maintain their full function in terms of engraftment, differentiation ability to all lineages, morphology and response to proinflammatory stimuli while conferring resistance to CD33-directed treatment.\textsuperscript{44,45}

In our study, we evaluated anti-CD33 immunotherapies in AML, using both CAR T cells and the recently reapproved ADC GO. We generated a novel CD33-specific third-generation CAR (3G.CAR33) and performed in-depth analysis of 3G.CAR33 expressing T cells (3G.CAR33-T) regarding their functionality towards both, AML cell lines and primary patient-derived AML cells. We demonstrate superior efficacy of our 3G.CAR33-T cells compared to second-generation CD33-directed CAR T cells (2G.CAR33-T). We further provide evidence that CD33-deletion in healthy and malignant hematopoietic cells confers resistance to CD33-targeted immunotherapies, 3G.CAR33-T cells and GO. Consistent with previous work, we confirm CD33-deletion in primary HSPCs to be an efficient and feasible approach to reduce off-tumor targeting of normal myeloid progenitor cells, while preserving on-tumor efficacy.

2 | MATERIAL AND METHODS

2.1 | Cells

Leukemia cell lines MV4-11 (RRID:CVCL_0064), HL60 (RRID:CVCL_0002), Kasumi-1 (RRID:CVCL_0589), as well as HEK293 T cells (RRID:CVCL_0003) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Drug resistant MV4-11 cells (MV4-11\textsuperscript{R}) were generated by continuous exposure of parental MV4-11 to increasing concentrations of PKC412 (Midostaurin/N-benzoylstaurosporin, LC Laboratories Woburn, Massachusetts) for 3 months. Drug resistant HL60 cells (HL60\textsuperscript{R}) were generated by continuous exposure of parental HL60 to increasing concentrations of venetoclax and azacitidine (Selleckchem, Houston, Texas) twice weekly for 3 months. All human cell lines have been authenticated using STR or SNP profiling within the last 3 years.

For CAR T-cell manufacturing, peripheral blood (PB) mononuclear cells (PBMCs) of three healthy donors (HDs) were collected at the Heidelberg University Hospital, Heidelberg, Germany. Primary AML blasts were isolated from patient PB (n = 9) by Ficoll density gradient centrifugation. CD34-positive primary HSPCs (n = 4) from HDs were selected from cord blood using a commercial isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany).

MV4-11, MV4-11\textsuperscript{R}, HL60, HL60\textsuperscript{R} and Daudi cells were cultured in RPMI-1640 (Life Technologies, Darmstadt, Germany) supplied with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany). Kasumi-1 cells were cultured in RPMI-1640 supplemented with 20% FBS. The 293T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific) containing 10% FBS. CD34-positive HSPCs were cultured in IMDM containing 20% serum substitute B/T 9500 (Stem Cell Technologies, Vancouver, Canada), 50 ng/mL recombinant human thrombopoietin (TPO; Miltenyi Biotec), 100 ng/mL stem cell factor (SCF; Miltenyi Biotec), 100 ng/mL–FLT3-ligand (FLT3-L, Miltenyi Biotec), 100 μM beta-mercaptopoethanol (Thermo Fisher Scientific), 50 μg/mL gentamicin (Carl Roth, Karlsruhe, Germany), 1× penicillin/streptomycin (pen/strep; Biochrom) and 35 nM UM171 (Miltenyi Biotec). AML blasts were cultured in IMDM (Thermo Fisher Scientific) containing 15% BIT 9500, 20 ng/mL interleukin (IL)-3 (Miltenyi Biotec), 20 ng/mL granulocyte colony-stimulating factor (G-CSF) (Miltenyi Biotec), 50 ng/mL FLT3-L, 100 ng/mL SCF, 50 μg/mL gentamicin, 10 μg/mL ciprofloxacin (Carl Roth), 100 μM beta-Mercaptopoethanol, 500 nM stemregenin 1 (SR1, Stem Cell Technologies) and 500 nM U7379 (Miltenyi Biotec). CAR T cells were cultured in 45% RPMI-1640, 45% Click's Medium (EHAA) (Irvine Scientific, Santa Ana, California) supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), 10% FBS, 10 ng/mL IL7 (R&D Systems, Minneapolis, Minnesota) and 5 ng/mL IL15 (R&D Systems). All cells were cultured in a humid incubator with 5% CO\textsubscript{2} at 37°C. All experiments were performed with mycoplasma-free cells.

2.2 | Generation of 3G.CAR33 and 2G.CAR33s

The coding sequence of the CD33-specific single chain variable fragment (scFv) was amplified via polymerase chain reaction (PCR) based on the sequence of the anti-CD33 GO antibody clone p67.7. The purified PCR product was inserted into the retroviral vector RV-SFG. CD19.CD28.4-1BB.CD3. The coding sequence of the CD33-specific single chain variable fragment (scFv) was amplified via polymerase chain reaction (PCR) based on the sequence of the anti-CD33 GO antibody clone p67.7. The purified PCR product was inserted into the retroviral vector RV-SFG.
2.3 | Generation of CAR T cells

Details of transfection, retrovirus production and manufacturing of CD19-specific CAR T cells have been described previously and were used for generation of CD33-specific CAR T cells. In brief, 293T cells were cotransfected with RV-SFG.CD33.CD28.4-1BB.CD3ζ (3G.CAR33; 3.75 μg), RV-SFG.CD33.CD28.4-1BB.CD3ζ (2G.CD28.CAR33; 3.75 μg), RV-SFG.CD33.4-1BB.CD3ζ (2G.4-1BB.CAR33; 3.75 μg), RV-SFG.CD19.CD28.4-1BB.CD3ζ (3G.CAR19; 3.75 μg), packaging plasmid PegPam3 (3.75 μg) and the envelope plasmid RDF (2.5 μg) (RV-SFG.CD19.CD28.4-1BB.CD3ζ, PegPam3 and the envelope plasmid RDF) were kindly provided by Malcolm Brenner (Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas). The retroviral supernatant was collected after 48 and 72 hours. Cryopreserved PBMCs from HDs were thawed and activated using anti-CD3 and anti-CD28 coated 24-well plates (Corning, Wiesbaden, Germany). On day 3, activated T cells (ATCs) were transduced with 3G.CAR19, 2G.CD28.CAR33, 2G.4-1BB.CAR33 and 3G.CAR33 retroviral supernatant in 24-well plates (Corning) previously coated with retronectin (Takara Bio). The resulting CAR T cells, that is, 3G.CAR19-T, 2G.CD28.CAR33-T, 2G.4-1BB.CAR33-T and 3G.CAR33-T were evaluated by flow cytometry (see below).

2.4 | Flow cytometry analysis

CD33 expression in cells was measured by flow cytometry (FC; LSRII; BD Biosciences, Franklin Lakes, New Jersey) using an APC conjugated anti-CD33 antibody (clone 67.7; BD Biosciences). CAR T cells were distinguished from nontransduced T cells with antihuman goat F(ab) IgG (H + L) PE antibody (Dianova, Hamburg, Germany). The following antibodies were used for surface marker staining: anti-CD3 and anti-CD28 and anti-CD33 antibodies (BD Biosciences, Franklin Lakes, New Jersey). For all staining procedures, dead cells were excluded using the LIVE/DEAD fixable near-infrared (IR) dead cell stain kit (Thermo Fisher Scientific).

2.4.1 | Intracellular staining

For assessment of cytokine release, 3G.CAR33-T, 2G.CD28.CAR33-T, 2G.4-1BB.CAR33-T or 3G.CAR19-T cells were incubated with target cells in 96-well U-bottom microplates (Greiner Bio-One, Frickenhausen, Germany) in the presence of Brefeldin A (Biolegend, San Diego, California) for 6 hours. Subsequently, cell mixture was fixed and permeabilized using FoxP3 buffer set (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by intracellular staining with anti-interferon (IFN)-γ-Alexa Fluor 488 (Biolegend, San Diego, California) and antitumor necrosis factor (TNF)-α-BV421 (BD Biosciences). All samples were measured on LSRII (BD Biosciences) and data were analyzed using FlowJo software (Ashland, Oregon).

2.5 | Cytotoxicity assays

Chromium-51 (51Cr) release assays were performed as previously described. Coculturing experiments of 3G.CAR33-T, 2G.CD28.CAR33-T, 2G.4-1BB.CAR33-T or 3G.CAR19-T cells (effector cells) and target cells, that is, CD33-positive tumor cell lines (HL60, Kasumi-1, MV4-11, MV4-117) and primary cells (AML blasts and HSPCs), were performed in effector to target cell ratios of 30:1, 15:1, 7.5:1, 3.75:1 and 1:1. Daudi cells were used as CD33-negative controls. Target cells were labeled with 51Cr (Hartmann Analytic, Braunschweig, Germany) for 2 hours in a humidified incubator at 37°C and 5% CO2. Subsequently, the labeled cells were added to the CAR T cells in 96-well U-bottom microplates (Greiner Bio-One) and cocultured for 4 hours. Supernatant was collected and radioactivity was measured in a liquid scintillation counter Tri-Carb 2800TR (Perkin Elmer, Shelton, Connecticut) as described previously.

2.6 | CAR33-T cells and AML cell coculture assay

1.5 × 10⁶ 3G.CAR33-T, 2G.CD28.CAR33-T or 2G.4-1BB.CAR33-T cells were separately cocultured with 3 × 10⁴ HL60 cells in a 48-well flat-bottom microplate with 800 μL of medium, that is, 45% RPMI-1640, 45% Click’s medium (EHAA), 2 mM L-glutamine, 10% FBS, 10 ng/mL IL7 and 5 ng/mL IL15. On days 3, 5 and 7, 3 × 10⁴ HL60 cells were added for rechallenge.

2.7 | CRISPR/Cas9-mediated CD33 deletion

2.7.1 | Cloning

CRISPR/Cas9 was used to generate CD33 knockout cells. Guide ribonucleic acids (gRNAs) were designed using the online gRNA design tool Off-spotter. Six different gRNAs targeting exon 2 (V domain) or 3 (C2 domain) of CD33 were selected. All selected gRNAs showed the highest score of inverse likelihood of off-target binding. Two scramble gRNAs without targeting site within the genome were designed as controls. gDNAs (sequences provided in Table S2) were synthesized by Biolegio (Biolegio, Nijmegen, The Netherlands). Prior to step-wise annealing, sense and antisense-strand gDNAs were denatured at 95°C for 10 minutes. The pl-CRISPR.EFS. GFP (Addgene: 57818) vector was used for digestion using the restriction enzymes FastDigest Esp3I (Thermo Fisher Scientific) at 37°C for 15 minutes and gel-purified on a 1% agarose gel (Biozyme, St. Joseph, Missouri). Annealed double-stranded gDNA and the digested vector were ligated at a molar ratio of five to one using T4 DNA ligase (Thermo Fisher Scientific). One microliter of the ligation reaction was transformed into 50 μL of Stbl3 competent cells according to the manufacturer’s protocol. After expansion on agarose plates, clones were picked and correct clones confirmed by Sanger sequencing.
Plasmid pL-CRISPR.EFS.GFP-gDNA (10.4 µg) and helper plasmids pLP1 (7.2 µg), pLP2 (2.4 µg) and VSVG (4 µg) (Thermo Fisher Scientific) were transfected into 10 cm dishes of 293T cells using Turbofect reagent (Thermo Fisher Scientific). After 72 hours, the supernatant containing lenti-virions was filtered through a 0.45 µm low protein binding membrane (Merck Millipore, Darmstadt, Germany) for cell debris removal. Virus was concentrated by ultracentrifugation at 29,000 rpm at 4°C for 2 hours and then resuspended in phosphate-buffered saline (PBS) supplemented with 2% FBS (Biochrom). Aliquots were used immediately or stored at −80°C. Leukemia cell lines were transduced with lentivirus via spinfection in the presence of 8 µg/mL polybrene (Sigma-Aldrich, Taufkirchen, Germany). Positively transfected cells were selected 72 hours after transduction by fluorescence-activated cell sorting (FACS) and knockouts were validated 24 hours later.

2.7.3 | gRNA in vitro transcription and electroporation

DNA templates for gRNA in vitro transcription were generated by PCR with forward primers containing a T7 promoter sequence (TTAA TACGACTCTATAG), the specific 20-nt protospacer sequence and a sequence (GTGTAGAGCTAGAAATAGC) corresponding to the 5′ end of the gRNA scaffold and reverse primer (AGCACCGACTCGGT GCCACT). Plasmid pSpCas9(BB)-2A-GFP (PX458, Addgene: 48138) was used as PCR template. PCR products were purified with Zymoclean Gel DNA Recovery Kit (ZymoResearch, Irvine, California). In vitro transcription was performed with HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany) following the manufacturer instructions. Transcribed gRNAs were purified with RNA Clean & Concentrator-25 (Zymo Research, Freiburg, Germany). One microgram of purified gRNA was incubated with 1 µg of Cas9 protein (PNA bio, California) at room temperature (RT) for 15 minutes prior to electroporation into cells with Neon transfection system (Thermo Fisher Scientific). Electroporation parameters used were 1600 V, 10 pulse width and 3 pulses. Knockout efficiencies were analyzed 96 hours after electroporation by FC.

2.8 | Cell viability assay

2.5 × 10⁶ cells in 100 µL respective culture medium were seeded as triplicates in 96-well plates and treated with GO (Pfizer, New York, New York) at increasing concentrations of 0, 1, 5, 10, 25, 50, 100 and 250 ng/mL. After 72 hours of incubation, cells were stained with acridine orange and propidium iodide (AO/PI; BioCat, Heidelberg, Germany). Cell number and cell viability were counted using the Luna cell counter (logos Biosystems, Gyeonggi-do, South Korea).

2.9 | Western blot

Western blot was performed as previously described. In brief, 1 × 10⁶ cells were lysed in 200 µL radio-immunoprecipitation assay buffer (RIPA buffer; Thermo Fisher Scientific) with 1× complete protease inhibitor cocktail (Roche, Basel, Switzerland) at RT for 10 minutes followed by centrifugation at 12 000g at 4°C for 10 minutes. Protein-containing supernatant was collected and loaded on SDS-PAGE gels. After electrophoresis, separated proteins were immediately blotted onto nitrocellulose membranes. Prior to incubation with primary anti-CD33 antibody (clone 67.7; BD Biosciences) at RT for 2 hours, the membranes were blocked for 1 hour at RT or overnight at 4°C with 5% milk powder in phosphate-buffered saline with Tween 20 (PBST). Horseradish peroxidase-conjugated secondary antibodies (Agilent, Santa Clara, California) were used in a dilution of 1:500. Proteins were visualized in an Amersham Imager 600 (GE life sciences, Massachusetts).

2.10 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, La Jolla, California). P-values were calculated using the unpaired parametric t-test. IC₅₀s were calculated with GraphPad Prism 8.

3 | RESULTS

3.1 | Generation of 3G.CAR33-T cells

We generated a novel 3G.CAR33 construct that contains an N-terminal leader directing the hybrid protein to the cell surface. The extracellular antigen-specific scFv derived from the anti-CD33 clone p67.7 antibody is fused to the leader. The variable heavy chain (VH) and variable light chain (VL) of the scFv are connected by a (GGGSG)₃ linker. The hinge and transmembrane domain of the CAR33 are derived from human immunoglobulin G (IgG) 1. Intracellular, the CD28- and 4-1BB-costimulatory domains are fused to the CD3ζ domain, the signal-transducing subunit derived from the TCR/CD3 receptor complex (Figure 1A). This generated 3G.CAR33 construct was delivered into HEK293T cells to produce 3G.CAR33 retroviral particles for infection of activated T cells. Transduction was performed in triplicates. The median percentage of 3G.CAR33 expressing T cells on day 7 was 58.9% (range 50.9%-74.5%) and increased to 70.1% (range 60.6%-79.7%) and 74.1% (range 62.2%-86.6%) on day 10 and 14, respectively (Figure 1B,C). 3G.CAR33-T cells displayed a viability of 83.2% (range 75.1%-89.4%), which was comparable to nontransduced T cells with a median of 7.0% (Figure 1E).
3.2 | 3G.CAR33-T cells exhibit potent cytotoxic activity against leukemia cell lines, primary AML samples and healthy HSPCs

To investigate the functionality of 3G.CAR33-T cells, we first analyzed CD33 surface expression on leukemia cell lines and primary cells. CD33 was expressed at varying levels on the myelogeneous cell lines HL60, Kasumi-1, MV4-11, HL60R and MV4-11R (Figure 2A). MV4-11R is a generated cell line that is resistant to varying tyrosine kinase inhibitors (TKI) such as PKC412 (Midostaurine) and AC220 (Quizartinib), as well as standard chemotherapeutic agents like cytarabine (AraC) and daunorubicin, whereas HL60R is resistant to venetoclax and azacytidine (Figure S1).

Besides of expression on cell lines, CD33 was also detectable on most AML blast cells (n = 9, patients 1, 4, 9, 14, 29, 30, 2604, 6880 and 9187: CD33-positive AML blasts; patient 14: CD33-negative AML blasts) (Figure 2B; Table S1) as well as normal CD33-positive HSPCs (n = 4; HSPCs from donors 45, 57, 416 and 521) (Figures 2C and S2). The Burkitt lymphoma cell line Daudi was negative for CD33 expression and in the following used as negative control (Figure 2A).

Next, we assessed the cytolytic capacity of 3G.CAR33-T cells using a standardized chromium-51 (51Cr) release assay. 3G.CAR33-T cells mediated cytotoxicity against all myelogenous cell lines in a dose-dependent manner (Figure 2D). CAR-mediated lysis of MV4-11 and MV4-11R cells, as well as HL60 and HL60R was comparable suggesting that CD33-targeting therapy with 3G.CAR33-T cells can overcome AML drug resistance. In contrast, CD33-negative Daudi cells were not eliminated by 3G.CAR33-T cells, but were lysed by CD19-directed CAR T cells (Figures 2D and S3). These 3G.CAR19-T cells, however, were unable to kill HL60 cells (Figure S3), indicating that the CAR T cell cytotoxicity of the 3G.CAR33-T cell was antigen-dependent. Further, we confirmed cytolytic capacity of 3G.CAR33-T cells against primary AML blasts (n = 6) (Figure 2E) and primary HSPCs (n = 4) (Figure 2F), although lysis of HSPCs was less pronounced when compared to AML cell lines and primary CD33-positive AML blasts. Of note, the CD33-negative AML blasts derived from patient 14 (Figure 2B) were not eliminated by 3G.CAR33-T cells (Figure 2E), further highlighting the antigen-specificity and antigen-dependency of 3G.CAR33-T cells. In addition, stimulation of 3G.CAR33-T cells with CD33-positive AML cells resulted in significantly higher cytokine secretion when compared to stimulation with CD33-negative Daudi cells and primary AML cells from patient 14 (Figures 2G, H and S4). In line with the results from the short-term killing assay (Figure 2D-F), cytokine secretion was lower when 3G.CAR33-T cells were incubated with HSPCs (Figure 2I).
3.3 3G.CAR33-T cells exhibit increased proliferation and cytotoxic activity compared to 2G.CAR33-T cells

To compare the third generation CAR33 to the second generation CAR33, we constructed two second generation CAR33, either with CD28 (2G.CD28.CAR33) or with 4-1BB (2G.4-1BB.CAR33) as costimulatory domain (Figure 3A). 2G.CD28.CAR33-T cells displayed similar viability to 3G.CAR33-T cells, but the viability of 2G.4-1BB.CAR33-T cells decreased dramatically from 88% on day 14 to 45% on day 16 and subsequently remained at a lower level (Figure 3B). Of the three constructs, 2G.4-1BB.CAR33-T cells demonstrated the lowest proliferation ability, from $7 \times 10^6$ on day 3 to $1.3 \times 10^6$ on day 14 and decreased to $3.6 \times 10^6$ on day 18 due to an increased mortality rate (Figure 3B,C). 2G.CD28.CAR33-T cells also showed a reduced expansion ($20.1 \times 10^6$ on day 18) when compared to 3G.CAR33-T cells ($36.6 \times 10^6$ on day 18) and...
nontransduced T cells \( \left( 35.8 \times 10^6 \text{ on day 18} \right) \) (Figure 3C). Overall, 3G.CAR33-T cells exhibited a proliferation capacity comparable to nontransduced T cells.

With regard to cytotoxic activity, an 1.4-fold increase of killing capacity was observed for 2G.4-1BB.CAR33-T cells, while a 3.2-fold and 3.1-fold increase was observed for 3G.CAR33-T cells and 2G.
CD28.CAR33-T cells, respectively, when compared to nontransduced T cells (Figure 3D). Interestingly, when stimulated with HL60 cells, CD4-positive 3G.CAR33-T cells released the highest cytokines levels. For CD8-positive CAR T cells, the highest cytokine secretion was observed for 2G.CD28.CAR33-T cells (Figure 3E). Consistent with the cytotoxic activity, 2G.4-1BB.CAR33-T cells displayed the lowest cytokine release levels (Figure 3E). Taken altogether, 3G.CAR33-T cells displayed optimized properties in terms of proliferation and short-term antileukemia activity when compared to second-generation constructs.

To assess the long-term killing capacity of different CAR T cells, we cocultured third-generation and second-generation CAR T cells separately with the same amount of HL60 cells for up to 10 days. CAR T cells were continuously rechallenged with HL60 cells (on day 3, 5 and 7) (Figures 3F, S5 and S6). For the 2G.4-1BB.CAR33-T/HL60 coculture, the ratio of HL60 cells continuously increased from 26.1% on day 0 to 82.3% on day 10, whereas the ratio of CD3-positive T cells decreased similar to nontransduced T cells, from 73.1% on day 0 to 15.0% on day 10. This indicates that HL60 cells could not be effectively killed by 2G.4-1BB.CAR33-T cells (Figures 3G,H and S6).

**FIGURE 4** Knockout of CD33 in AML cell lines and primary HSPCs via CRISPR/Cas9. (A) Schematic representation of the CD33 gene. Guide RNAs (gRNAs) V1-V3 and C1-C3 with their corresponding targeting sites within the respective IgV and IgC2 domains of CD33 are displayed. (B) Flow cytometry (FC) analysis of MV4-11 cells transduced with GFP-Cas9-gRNA lentivirus. Cells were analyzed 96 hours after transduction. Prior to FC, cells were stained with APC-antiCD33 antibody (clone 67.7) for 15 minutes. GFP positive cells were gated for CD33 expression. Two nontargeting scramble gRNAs were used as negative controls. (C) Western blot of transduced cells. The anti-CD33 antibody clone 67.7 was used. gRNA C1 targeting C2 domain of CD33 (comprised within all CD33 isoforms) was chosen for further experiments. (D) Sanger sequencing results of MV4-11 and HSPCs after CRISPR/Cas9 application. gRNA scramble 1 was used as positive control. Arrows highlight the mutation starting point. (E) FC analysis of primary CD34+ cells (n = 4) 96 hours after electroporation with Cas9/gRNA-CD33 ribonucleoprotein (RNP) complex. Prior to FC, cells were stained with APC-antiCD33 antibody (clone 67.7). CD33 KO efficiencies (%) are indicated. CD33− cells were sorted for further experiments [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 5 Treatment of CD33-positive and CD33-deleted cells with 3G.CAR33-T cells. (A) and (F): Flow cytometry (FC) analysis of CD33 expression on HL60, HL60R, Kasumi-1, MV4-11, MV4-11R cell lines (A) and HSPCs (F) with (lower 5 histograms, labeled “KO”) and without (histograms 2-6, labeled “WT”) CD33-deletion mediated by Cas9/gRNA CD33_C1 (CD33+/C0) and Cas9/scramble gRNA (CD33+), respectively. CD33+ cells were sorted prior to the performance of Cr51-release and cytokine secretion assays. A control without staining is depicted in gray. Data of WT cells are also shown in Figure 2A,C. (B-E,G-K) Cytotoxicity of 3G.CAR33-T cells incubated with CD33-positive (filled boxes and solid lines) and CD33-negative (nonfilled boxes and dashed lines) cell lines (B-E) and HSPCs (G-K) were measured by intracellular staining (B and G) and 51Cr release assay (C-E and H-K). Data of WT cells are also shown in Figure 2D,F,G,I. Experiments were performed in triplicates. Mean values were calculated for each group; error bars indicate SD. ns, not significant (P > .05); **P < .01; ***P < .001 (unpaired t-test). E, effector cells; T, target cells. [Color figure can be viewed at wileyonlinelibrary.com]
In contrast, 3G.CAR33-T and 2G.CD28.CAR33-T cells exhibited opposite changes with reduced HL60 cell population and increased CAR-T cell ratios (Figures 3G,H and S6) demonstrating that 3G.CAR33-T and 2G.CD28.CAR33-T cells efficiently killed HL60 cells. Of note, the ratios of 3G.CAR33-T and 2G.CD28.CAR33-T cells were strongly promoted by HL60 stimulation, from 26.7% to 88.7% and 27.5% to 86.6%, respectively, indicating rapid proliferation of 3G.CAR33-T and 2G.CD28.CAR33-T cells upon tumor cell stimulation (Figure 3I).

3.4 Editing of CD33 using CRISPR/Cas9 in myeloid cell lines and HSPCs

To prevent on-target off-leukemia toxicity of CD33-targeted therapies, we disrupted the CD33 gene in human myeloid cell lines as well as in HSPCs using CRISPR/Cas9 mediated gene editing. Three gRNAs targeting the extracellular IgV domain of CD33 (gRNA V1-V3) and three gRNAs targeting the IgC2 domain (gRNA C1-C3) were designed (Figure 4A; Table S2). The human CD33-positive myeloid cell line MV4-11 was used to test the knockout (KO) efficiency of the six gRNAs resulting in functional loss of CD33. Lentiviruses carrying Cas9, gRNA and GFP genes were used to transduce target cells. The transduction efficiencies as determined by the percentage of GFP-positive cells ranged between 10% and 27% (Figure S7). As measured by flow cytometry, CD33 KO was achieved in 22.9% to 73.7% of transduced cells (Figure 4B). We confirmed the KO of CD33 by western blot (Figure 4C) and Sanger sequencing (Figure 4D). All transductions were performed independently in triplicates, generating consistent data and thus highlighting reproducibility of the knockout. The gRNAs C1, C2 and V2 showed the highest KO efficiency. As all
CD33 isoforms contain the C2 domain, we chose gRNA C1 to delete CD33 in subsequent experiments and verified its efficient KO capacity in further cell lines, that is, Kasumi-1, HL60, HL60R, MV4-11 and MV4-11R (Figure 5A).

Next, we addressed CD33 KO in CD34-positive HSPCs (n = 4). As random integration of transgenes via lentiviral transduction is of concern in primary cells, we used a nonviral genome editing approach. We electroporated in vitro assembled Cas9/gRNA ribonucleoproteins (RNPs) into HSPCs. Ninety-six hours later, CD33 KO efficiencies of 79.8%, 93.9%, 66.6% and 82.7% (Figure 4E) and cell viability of 88% to 97% (data not shown) were achieved.

3.5 | Cytotoxicity of 3G.CAR33-T cells toward CD33-edited cells

We confirmed CD33 KO (Figure 5A,F), before we assessed the effect of 3G.CAR33-T cells on CD33-edited cells. In fact, compared to their respective wildtype counterparts, we observed a lower cytokine secretion of 3G.CAR33-T cells after incubation with CD33-edited cells (Figure 5B,G). In addition, CD33-negative cells were protected from cytotoxicity of 3G.CAR33-T cells (Figure 5C,E-H-K). CD33-edited HL60, HL60R, followed by MV4-11R and Kasumi-1 cells were least affected by 3G.CAR33-T cells, whereas the difference of cytotoxicity of 3G.CAR33-T cells was less pronounced for CD33-edited MV4-11 and primary HSPCs (Figure 5C,E-H-K).

3.6 | CD33 knock-out cells are tolerant to GO

To verify GO cytotoxicity, we incubated CD33-positive and CD33-deficient cells (HL60, HL60R, Kasumi-1, MV4-11, MV4-11R, primary CD34-positive HSPCs [n = 3] and primary AML blasts [n = 6]) separately with various concentration of GO for 3 days. All CD33-positive cells, including wildtype cell lines, drug-resistant cells, primary HSPCs and primary AML blasts were sensitive to GO (Figure 6). Among the tested cell lines, wildtype HL60 exhibited the highest sensitivity to GO (half-maximal inhibitory concentration [IC50] of 1.37 ng/mL). At 5 ng/mL GO concentration, almost all HL60 cells died (Figure 6A). HL60R, Kasumi-1, MV4-11 and MV4-11R showed similar sensitivity to GO, with IC50 of 2.28, 3.19, 3.63 and 5.36 ng/mL, respectively (Figure 6B-E). The IC50 of GO in primary AML blasts varied, from 1.51 [AML blasts from patient 29] to 119.9 ng/mL (AML blasts from patient 14). This observation was consistent with the level of CD33 expression (Figure 2B) as higher CD33 expression resulted in higher sensitivity to GO and vice versa (Figure 6F). In general, tested primary HSPCs displayed higher IC50 (17.99, 13.07 and 7.77 ng/mL) than cell lines (Figure 6G-I). In contrast, CD33-deficient cells (KO) displayed a significant survival benefit to treatment with GO (Figure 6A-I). The IC50 in CD33-deleted HL60 increased dramatically up to 450 ng/mL (Figure 6A). IC50 in CD33-negative HL60R, Kasumi-1, MV4-11 and MV4-11R cells also increased significantly to 95.53, 50.78, 72.74 and 66.8 ng/mL, respectively (Figure 6B-E). Of note, CD33-deleted HSPCs displayed tolerance toward GO treatment with increased IC50 of 87.82, 167.2 and 100.9 ng/mL (Figure 6G-I).

4 | DISCUSSION

At the Heidelberg University Hospital patients with CD19-positive lymphoid diseases are treated with third-generation CD19-directed CAR T cells within the framework of the investigator-initiated (IIT) Heidelberg CAR T cell trial 1 (HD-CAR-1; Eudra-CT No. 2016-0048; NCT03676504). HD-CAR-1 CAR T cells have shown clinical efficacy while providing an extraordinarily favorable toxicity profile with regards to cytokine release syndrome (CRS) and neurotoxicity. Extrapolation of these results to other hematologic disease entities is of significant interest. Prior studies evaluating CAR T cells directed against CD33 in the context of AML have primarily used second-generation CAR33-T cells. In our study, we generated a third-generation CD33-directed anti-AML CAR construct using the RV-SFG. CD28-1BBzeta backbone of the HD-CAR-1 trial, hence introducing a new third-generation CAR33-T construct with a backbone that is already successfully applied in the clinic.

In the context of CD19-directed CAR T cell therapy, third-generation CAR T cells have shown significantly superior engraftment, a 23-fold higher expansion and prolonged in vivo persistence when compared to second-generation CAR constructs. Clinically, superior expansion and longer persistence of third-generation CAR T cells was observed when second-generation (CD28 costimulatory domain) and third-generation (CD28 and 4-1BB) CAR T cells were administered simultaneously to lymphoma patients. Here, we demonstrate that also third-generation CD33-directed CAR T cells are associated with enhanced cytotoxic efficacy and prolonged proliferation capacity when compared to second-generation CD33-directed CAR T cells. In addition, our third-generation CAR33-T cells were effective against CD33-positive cells including AML blasts and were even able to overcome AML drug resistance as demonstrated by efficacy against MV4-11R and HL60R cells, thus indicating probable efficacy also in AML patients with refractory disease.

The target antigen density on malignant cells has been related to CAR T cell cytotoxicity and several preclinical studies have indicated that an antigen density threshold may provide a strategy to overcome on-target/off-tumor effects. In our study, we used various cells including cell lines derived from AML and Burkitt lymphoma, as well as primary AML blasts and healthy HSPCs. 3G.CAR33-T cells displayed higher cytotoxicity and cytokine release towards highly CD33 antigen-expressing HL60, HL60R, Kasumi-1, MV4-11, MV4-11R cells and primary AML blasts from patient 29, whereas absence of CD33 expression on Daudi cells and primary AML blasts from patient 14 was associated with lower lytic CAR T cell activity and cytokine secretion. With regards to tested HSPCs, those cells displayed moderate CD33 expression and consequently cytotoxicity as well as cytokine release of CAR33-T cells were also decreased.
Knocking-out the target antigen in healthy HSPCs can render myeloprotection to healthy myeloid tissue from CD33-directed treatment. Here, we evaluated CD33-editing on HSPCs. Six different gRNAs for CD33-editing using CRISPR/Cas9 mediated significantly diverse CD33-KO results ranging from 23% to 73%, highlighting that a systematic screening of effective gRNAs was required. Following selection gRNA C1 displaying high KO efficiency and targeting also the C2 domain of CD33, we observed that CD33-KO cells were less sensitive to 3G.CAR33-T cells compared to their wildtype counterparts. In line with other reports on CD33-deletion,45,55,59 we conclude that CD33-KO in HSPCs might enable the use of CD33-directed therapy including CAR T cells in AML.

With the recent approval of GO for the treatment of AML, therapy improvements are important. In our study, we investigated the efficacy of GO in different cells, including AML cell lines, drug resistant cells and primary cells. CD33-positive cells were sensitive, whereas CD33-deleted counterparts were resistant to GO. Similar to what we observed with 3G.CAR33-T cells, sensitivity to GO correlated with CD33 expression. Nonetheless, in light of our data, measurement of CD33 density on patient cells and CD33 density-dependent dose optimization of anti-CD33 treatment with GO seems advisable.

Our study is limited by the fact that the effects of GO and CAR T cells were only assessed under artificial two-dimensional cell culture conditions. Further in vivo studies are required to precisely characterize the features and optimized conditions for GO and CAR T cell treatments in AML in general, as well as in the context of CD33 target-depletion in particular.

In summary, our study confirms the feasibility of targeted immunotherapy, GO and 3G.CAR33-T cells against AML and provides valuable new insight into CAR T therapy in AML. We provide evidence that third-generation CD33-directed CAR T cells are associated with improved clinical efficacy over second-generation CAR constructs. Based on our clinical data on third-generation CD19 CAR T cells we expect manageable toxicity in the clinical setting. CRISPR/Cas9-mediated CD33 KO in CD33-positive tumor cells as well as primary HSPCs was feasible and CD33-edited cells were resistant to GO and CAR33-directed T cells.

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CONFLICT OF INTEREST

Yi Liu: Pfizer (provision of GO). Maximilian Felix Blank: Molecular Health GmbH (consultant). Maria-Luisa Schubert: Kite/Gilead, Takeda (consultant). Michael Schmitt: Apogenix, Hexal and Novartis (research support). Hexal, Kite/Gilead (travel grants). Bluebird bio, Kite, Novartis (financial support for educational activities and conferences). MSD (advisory board member). MSD, GSK, Kite, BMS (co-PI of clinical trials). TolerogenixX Ltd. (co-founder and shareholder). Carsten Müller-Tidow: Bayer AG (research support). Pfizer, Janssen-Cilag GmbH (advisory board member). Pfizer, Daiichi Sankyo, BioLineRx (grants and/or provision of investigational medicinal products). Pfizer (clinical trial financial support). Christian Kleist: TolerogenixX Ltd. (co-founder and shareholder). Tim Sauer: Pfizer, Gilead, Amgen, Takeda, Astellas, BMS, AbbVie, Matterhorn Biosciences, Ridgellen Discovery. Annika Laut, Chunhong Cui, Christina Schmidt, Fengbiao Zhou, Hao Yao, Jens-Ulrich Rahfeld, Maike Janssen, Stefanie Göllner, Sanmei Wang: none.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Sample collection and analysis of primary cells were approved by the Ethics Committee of the University of Heidelberg (ethics approval S-169/2017 and S-254/2016) and all patients had signed a written consent prior to experiment initiation. All experiments were performed in accordance with the Declaration of Helsinki.

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SUPPORTING INFORMATION
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