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Bifunctional PD-1 x αCD3 x αCD33 fusion protein reverses adaptive immune escape in Acute Myeloid Leukemia

Running head: Bifunctional PD-1 fusion protein for AML treatment

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Key points

- Characterization of an αPD-L1 x αCD3 x αCD33 antibody construct with bifunctional activity against AML cells
- Strong cytotoxicity against primary AML cells *in vitro* and high selectivity in a xenograft mouse model

Abstract

The CD33-targeting bispecific T-cell engager (BiTE®) AMG 330 proved to be highly efficient in mediating cytolysis of acute myeloid leukemia (AML) cells *in vitro* and in mouse models. Yet, T-cell activation is correlated with upregulation of PD-L1 and other inhibitory checkpoints on AML cells that confer adaptive immune resistance. PD-1 and PD-L1 blocking agents may counteract T-cell dysfunction, however, at the expense of broadly distributed immune-related adverse events (irAEs). We developed a bifunctional checkpoint inhibitory T-cell-engaging (CiTE) antibody that combines T-cell redirection to CD33 on AML cells with locally restricted immune checkpoint blockade. This is accomplished by fusing the extracellular domain of PD-1 (PD-1_{ex}), which naturally holds a low affinity to PD-L1, to an αCD3.αCD33 BiTE®-like scaffold. By a synergistic effect of checkpoint blockade and avidity-dependent binding, the PD-1_{ex} attachment increases T-cell activation (3.3-fold elevation of IFN-γ) and leads to efficient and highly selective cytotoxicity against $CD33^+$PD-$L1^+$ cell lines (EC_{50} = 2.3 pM to 26.9 pM) as well as patient-derived AML cells (n=8). In a murine xenograft model, the CiTE induces complete AML eradication without initial signs of irAEs as measured by body weight loss. We conclude that our molecule preferentially targets AML cells, whereas high-affinity blockers, such as clinically approved anti-cancer agents, also address PD-$L1^+$ non-AML cells. By combining the high efficacy of T-cell engagers with immune checkpoint blockade in a single molecule, we expect to minimize irAEs associated with the systemic application of immune checkpoint inhibitors and suggest high therapeutic potential, particularly for patients with relapsed/ refractory AML.

Introduction

The treatment of acute myeloid leukemia (AML) remains challenging in 2018. Only half of the patients are eligible for curative intensive induction chemotherapy, and the majority will relapse due to the persistence of chemoresistant leukemic stem cells (LSCs). Allogeneic hematopoietic stem cell transplantation (HSCT) as post-remission therapy is able to lower this risk, yet it is correlated with a significant incidence of transplant-related morbidity and mortality.[1-4] Particularly patients with relapsed or refractory (r/r) disease as well as patients that are medically not fit for intensive treatment regiments urgently require new therapeutic approaches.

In acute lymphoblastic leukemia (ALL), several targeted immunotherapies already reached clinical implementation as standard treatment. With the approval of the bispecific T-cell engager (BiTE®) blinatumomab in 2014, the utilization of T cells as immune effectors also entered clinical mainstream.[5] This bispecific molecule addresses CD19 on B cells and thus redirects antigen-experienced T cells to leukemic cells.[6,7] In AML, the myeloid lineage antigen CD33 has been the focus of immunotherapeutic strategies for decades. Targeting CD33 by the antibody-drug conjugate gemtuzumab ozogamicin (GO, Mylotarg®) has proven to be safe and led to re-approval for the treatment of adults at primary diagnosis as well as adults and children with r/r disease.[8] Also the preclinical evaluation of the BiTE® antibody AMG 330 indicated efficient cytotoxic lysis of primary AML patient samples in allogeneic and autologous settings and entered clinical trials in August 2015 (NCT02520427).[9-12]

However, T-cell-recruiting immunotherapy is accompanied by the induction of adaptive immune escape mechanisms such as programmed cell death-ligand 1 (PD-L1) upregulation in response to proinflammatory cytokines.[13,14] Recent studies were able to directly correlate PD-L1 expression on cell lines with a decrease in AMG 330-mediated cytotoxicity and demonstrated that this effect could be abrogated by PD-L1 blockade.[15] We could underpin these findings *ex vivo* on primary AML patient cells, where the efficacy of AMG 330 was enhanced by complementation with PD-1/PD-L1 blocking monoclonal antibodies (mABs).[13]

PD-1 and PD-L1 inhibitors are approved for the treatment of solid cancers and clinical trials are currently exploring these agents in hematologic malignancies.[16-18] So far, monotherapy has shown limited clinical benefit and current strategies explore combinatorial approaches with hypomethylating agents. First data of a clinical phase IB/II study with αPD-1 mAB nivolumab in combination with azacytidine (AZA) in patients with r/r AML demonstrated encouraging median overall survival rates of 5.7 months (NCT02397720).[19]

Yet, the clinical application of PD-1 and PD-L1 blocking mABs is hampered by the frequent occurrence of immune-related adverse events (irAEs). These include skin disorders, colitis, hepatitis, endocrinopathies, pneumonitis and myocarditis and range from weak to severe or

fatal toxicity.[20-25] Medical intervention can require treatment interruption or discontinuation and immune suppression with corticosteroids.[26]

To combine the benefits of bispecific T-cell-engaging molecules with PD-1/PD-L1 checkpoint blockade and prevent on-target off-leukemia events, we have developed a novel immunotherapeutic format. Bifunctional checkpoint inhibitory T-cell-engaging (CiTE) antibodies consist of a high-affinity αCD33 single-chain variable fragment (scFv) fused to an αCD3ε scFv in one polypeptide chain. Additionally, we attached the extracellular domain of PD-1 (PD-1_{ex}), which intrinsically holds a low affinity to PD-L1. We hypothesized that the PD-1_{ex} domain is not sufficient to directly target PD-L1-expressing cells and does not block PD-1/PD-L1 interactions unspecifically. Instead, we aimed that PD-L1 blockade is thus dependent on αCD33 scFv-mediated targeting, which would consequently restrict checkpoint blockade to the surface of leukemic cells. A single-chain triplebody (sctb)[27], in which the PD-1_{ex} module is replaced by an αPD-L1 scFv, served as high-affinity control.

Our data reveal that the CiTE antibody binds to AML and T cells, increases T cell effector functions compared to a BiTE®-like molecule and induces efficient cancer cell eradication. Notably, *in vitro* the CiTE demonstrates a high selectivity for $CD33^+PD\text{-}L1^+$ cells while $PD\text{-}L1^+$ cells are not affected. This is further supported in a murine model system, where no indication for the development of irAEs due to on-target off-leukemia binding of the cross-reactive PD-1_{ex} could be detected. Contrarily, the sctb also leads to the depletion of $PD\text{-}L1^+$ cells *in vitro* as well as body weight loss and leukemia-unrelated PD-1 upregulation *in vivo*. Therefore, we consider the new CiTE format as promising therapeutic approach to treat patients with AML with high efficacy and minimize the risk to induce irAEs that are associated with systemic immune checkpoint blockade.

Methods

Expression and purification

The extracellular domain (amino acid 33-149) of human PD-1 (PD-1_{ex}) was amplified from human muscle cDNA (*PDCD1* gene). The αPD-L1 scFv was published before (YW243.55.S70, atezolizumab-derived) with variable light (V_L) and variable heavy (V_H) chains connected by a $(G_4S_4)_4$ linker.[28] The OKT3-based αCD3 scFv and hP67.6-derived αCD33 scFv were obtained from published sequences.[29,30] Coding sequences for CiTE, sctb and controls were cloned into the expression vector pSecTag2/HygroC (Thermo Fisher Scientific, Waltham, MA, USA) containing a His_6-tag. As control, PD-1_{ex} was fused to a C-terminal human IgG1-Fc. These molecules and the specificity control[27] were expressed in FreeStyle™ 293-F or Expi293F™ cells (Thermo Fisher Scientific). The αPD-L1 scFv was cloned into the pAK400[31] vector and expressed in *E.coli* BL21 (DE) cells (NEB, Ipswich, MA, USA). Proteins were purified by nickel affinity and size exclusion chromatography (SEC) using Superdex 200 increase 10/300 or Superdex 75 10/300 columns (GE Healthcare, Little Chalfont, UK) in 20 mM histidine, 300 mM NaCl (pH 6.5). Proteins were analyzed by SDS-PAGE and analytical SEC (Superdex 200 increase 5/150, GE Healthcare). For mouse studies, proteins were prepared in 1x DPBS (Thermo Fisher Scientific) and endotoxin levels were confirmed to be below 5 EU/kg per day.[32] Stability was measured by fluorescence-based thermal shift (ThermoFluor) assay using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).[33]

Cell lines

All cell lines were cultivated at standard conditions. Flp-In™ T-REx™ 293 cells (Thermo Fisher Scientific) were modified for expression of human PD-L1 and CD33 (HEK:PD-L1 and HEK:CD33:PD-L1), which could be enhanced by tetracycline induction (HEK:PD-L1_ind.). MOLM-13, OCI-AML3, BA/F3 and Jurkat cells were purchased from the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ, Leibniz-Institut DSMZ, Braunschweig, Germany). Stable PD-L1 expressing cells were generated by retroviral transduction with cDNA of human PD-L1 (MOLM-13:PD-L1 and OCI-AML3:PD-L1) or murine PD-L1 (Panc02-OVA:mPD-L1), BA/F3:CD33:PD-L1 cells by further transduction with cDNA of human CD33.[34]

Patient and healthy donor material

After written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilians-Universität (Munich, Germany), peripheral blood (PB) or bone marrow (BM) samples were collected from healthy donors

(HDs) and AML patients. At initial diagnosis or relapse, samples were analyzed at the Laboratory for Leukemia Diagnostics of the Klinikum der Universität München as described previously.[35-37] Patient characteristics are summarized in Supplementary Table S2.

Flow cytometry

Flow cytometry measurements were performed on a Guava easyCyte 6HT instrument (Merck Millipore, Burlington, MA, USA) and analyzed using GuavaSoft version 3.1.1 (Merck Millipore). or on a LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data were evaluated using FlowJo version 9.6 (Tree Star Inc., Ashland, OR, USA). Commercial antibodies are listed in Supplementary Methods. Surface antigen density of cell lines was evaluated with QIFIKIT (Agilent Dako, Santa Clara, CA, USA). Apparent dissociation constants (K_D) were determined by calibrated flow cytometry as described.[38] 3.0–3.4 µm Rainbow Calibration particles (BioLegend, San Diego, CA, USA) served as calibration control. Data points were normalized to the maximum MFI and fitted to a one-site specific binding model.

T-cell activation and cytotoxicity assays

HD T cells were incubated with target cell lines at an E:T ratio of 2:1, 1:3 or without targets in the presence of CiTE, sctb and control molecules. Assays were performed in RPMI1640 + GlutaMAX supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (100 U/mL) (Thermo Fisher Scientific). BA/F3 medium included 10% WEHI-3B supernatant and 2.5 µg/ml αCD28 mAB (BD Pharmingen). Bead-immobilized αCD3/αCD28 antibodies (Thermo Fisher Scientific) served as positive control. After 96 h, T-cell activation was assessed by flow cytometry quantifying the $CD2^+CD69^+$, $CD2^+CD25^+$ or $CD2^+PD\text{-}1^+$ population. For cytotoxicity readout, MOLM-13- and BA/F3-derived cells were directly applied, OCI-AML3:PD-L1 and OCI-AML3 were labeled with PKH67 (Sigma-Aldrich, St. Louis, MO, USA). After 72 h, total target cell numbers were assessed by flow cytometry as live $CD2^-CD33^+$ or $CD2^-PKH67^+$ population, respectively, and normalized to negative control. Data was transformed with a 4-parameter non-linear fit model. IFN-γ and Granzyme B release were determined after 72 h by Cytometric Bead Array (Human IFN-γ/Granzyme B Flex Set, BD Biosciences).

HEK:PD-L1 and HEK:CD33:PD-L1 cells were labeled with 15 µM Calcein AM (Thermo Fisher Scientific). Pre-activated T cells derived from an 18 days *ex vivo* PBMC expansion were incubated with a 1:1 mixture of unlabeled HEK:PD-L1 and labeled HEK:CD33:PD-L1 cells and vice versa at a total E:T ratio of 2:1 and increasing concentrations of molecules.[39] 2.5% Triton X-100 served as maximum lysis. Fluorescence intensity was measured using an

Infinite® M100 plate reader (TECAN, Männedorf, Switzerland) and specific lysis was calculated and analyzed with a 4-parameter non-linear fit model.

Specific lysis [%] = (fluorescence$_{(sample)}$ – fluorescence$_{(spontaneous\ lysis)}$)/(fluorescence$_{(maximum\ lysis)}$ – fluorescence$_{(background)}$) x 100

Ex vivo redirected lysis assay of co-cultured AML patient cells

Redirected lysis assays of AML patient samples were performed in α-MEM (Thermo Fisher Scientific) supplemented with 12.5% FCS, 12.5% horse serum, 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA, USA) and a distinct cytokine cocktail on irradiated MS-5 cells as described.[10,13,40-42] HD T cells and AML cells were incubated at an E:T ratio of 1:4 and addition of 10 nM of molecules or 10 µg/ml αPD-L1 mAB (eBioscience Thermo Fisher Scientific). Cell populations were assessed by flow cytometry. Cytotoxicity and T cell proliferation were evaluated as described.[10,13]

Murine AML xenograft studies

Female 170-265 days old non-obese diabetic SCID gamma (NSG) mice were housed under pathogen-free conditions at the research animal facility of the Helmholtz Zentrum München, Germany. Animal experiments were approved by regional regulating authorities (Regierung von Oberbayern) and conducted as described in a published protocol.[43] At day 0, $2x10^4$ MOLM-13:PD-L1 cells were injected intravenously (i.v.). At day 3, 10^7 *in vitro* pre-activated T cells were transferred intraperitoneally (i.p.) and mice were randomized into 5 groups: 3 treatment groups containing 6 mice each, a specificity control group of 4 mice and a 1x DPBS control group of 5 mice. At day 4, 1.7 pmol of molecules /g body weight or 1x DPBS were daily i.v. injected until day 12. At day 13, mice were sacrificed, spleens were removed and BM was obtained from femora of hind legs.

Plotting and statistical analysis

Statistical evaluation was performed using GraphPad Prism version 6.07 (GraphPad Software Inc., San Diego, CA, USA) applying unpaired Student's *t*-test with Welch correction for cell line-based assays with the same T-cell donors, Wilcoxon test for different HDs and patient samples, and Mann-Whitney U test for mouse xenograft experiments. If $P < .05$, results were considered statistically significant.

Results

Generation and characterization of the CiTE antibody

To combine specific T-cell redirection to AML cells with a target cell-restricted PD-1/PD-L1 blockade, we generated a CiTE antibody by fusing the endogenous extracellular domain of human PD-1 (PD-1_{ex}) to an αCD3.αCD33 BiTE®-like molecule. The CiTE was compared to a sctb[27], in which PD-1_{ex} was replaced by a high-affinity αPD-L1 scFv. The BiTE®-like molecule αCD3.αCD33, PD-1_{ex}.αCD3 and αPD-L1.αCD3, as well as a non-targeting molecule served as controls (Figure 1A, Supplementary Figure S1A). Purified proteins were analyzed by SDS-PAGE and analytical SEC (Supplementary Figure S1B,C) and protein stability was assessed by fluorescence-based thermal shift assay (Supplementary Figure S1D).

The binding properties and apparent dissociation constants of CiTE and sctb to antigen-presenting cells were analyzed by flow cytometry (Supplementary Figure S2). When investigating CiTE and sctb as whole molecules, both bound similarly to $CD33^+PD\text{-}L1^+$ AML cell lines and HD T cells (Figure 1B). Since the unique feature of the CiTE format is the weak PD-1_{ex} affinity to PD-L1, we evaluated the binding abilities of PD-1_{ex} and the αPD-L1 scFv independently. To this end, MOLM-13 and tetracycline-inducible HEK293 cells both stably expressing PD-L1 (MOLM-13:PD-L1 and HEK:PD-L1) were quantified for their PD-L1 surface antigen density (Supplementary Table S1). As expected, our results showed weak physiological binding of PD-1_{ex} (described in low µM range)[44,45] and comparably strong binding of the αPD-L1 scFv (Figure 1C).

Consequently, CiTE-mediated checkpoint inhibition on AML cells depends on avidity contribution of the CD33 targeting module. We performed a blocking assay with a labeled αPD-L1 mAB that interferes with the binding of the checkpoint modules. Despite the weak interaction of PD-1_{ex} in comparison to the αPD-L1 scFv, the CiTE was able to block subsequent binding of the αPD-L1 mAB on $CD33^+PD\text{-}L1^+$ AML cells (Figure 1D). However, it was not as efficient as the sctb and the high-affinity αPD-L1.αCD3 control, which were able to completely occupy accessible PD-L1 surface molecules. In line with the binding studies, the low-affinity PD-1_{ex}.αCD3 control was displaced by the αPD-L1 mAB. Thus, PD-1_{ex} only interacts with its ligand on AML cells when it is covalently linked to a high-affinity leukemia-targeting arm.

CiTE-mediated activation of resting T cells

In vitro, BiTE®-mediated T-cell activation strictly depends on the crosslink to target cells.[46] To assess T-cell activation caused by sole CD3 engagement, we incubated HD T cells with CiTE and sctb in the absence or presence of MOLM-13:PD-L1 cells (Figure 2A). As expected, none of the molecules induced expression of CD25 and CD69 without target cells,

whereas T cells significantly upregulated both markers in the presence of MOLM-13:PD-L1 cells. As a further hallmark of T-cell activation, we quantified the IFN-γ and Granzyme B release (Figure 2B-D). On $CD33^+PD\text{-}L1^+$ cells both CiTE and sctb led to a significant increase in IFN-γ and Granzyme B levels compared to the BiTE®-like molecule. We also observed an upregulation of PD-1 upon T-cell activation (Supplementary Figure S3).

To effectively counteract adaptive immune resistance caused by PD-1/PD-L1 signaling, current clinical trials investigate combination therapies of targeting agents with checkpoint inhibitors.[47,48] Thus, INF-γ levels were measured upon T-cell activation by CiTE, BiTE®-like molecule or combinations of BiTE®-like and checkpoint inhibitors. Strikingly, the CiTE induced similar cytokine levels compared to high-affinity blocking agents plus BiTE®-like molecule, whereas the equimolar addition of $PD\text{-}1_{ex}$-Fc (low-affinity blocking module) triggered a weaker IFN-γ release (Figure 2C). We conclude that the fusion of $PD\text{-}1_{ex}$ to a BiTE®-like scaffold leads to similar T-cell activation as combination approaches, but with the advantage of local restriction to $CD33^+$ cells. We hypothesize that this effect is due to a synergy of avidity-dependent binding and PD-1/PD-L1 checkpoint blockade.

CiTE-mediated cytotoxicity is limited to $CD33^+$ cells

With the CiTE format, we provide a molecule that targets $CD33^+$ leukemic cells with high affinity and locally blocks PD-L1 due to the low affinity of $PD\text{-}1_{ex}$. Furthermore, we expect the CiTE to address $CD33^+PD\text{-}L1^+$ cells more efficiently than $CD33^+PD\text{-}L1^-$ cells due to avidity-dependent binding of the αCD33 scFv and $PD\text{-}1_{ex}$. To test this hypothesis, the molecules were incubated with non-stimulated HD T cells and MOLM-13 or MOLM-13:PD-L1 cells, expressing high levels of CD33 (Figure 3, Supplementary Figure S5, Supplementary Table S1). Both CiTE and sctb induced specific lysis of both cell lines. Yet, PD-L1 expression on AML cells increased the efficacy of target cell depletion (Figure 3A,C). Also T-cell proliferation was triggered more strongly on $CD33^+PD\text{-}L1^+$ target cells (Supplementary Figure S4). Interestingly, both molecules revealed similar EC_{50} values despite their different affinities for PD-L1. Consistent with the previous characterization, the low-affinity $PD\text{-}1_{ex}$.αCD3 control had a low impact on cytotoxicity, whereas the high-affinity αPD-L1.αCD3 control led to target cell lysis when PD-L1 was expressed. Since CD33 levels on AML cells exhibit a high inter- and intra-patient heterogeneity[10], the results were confirmed with OCI-AML3 and OCI-AML3:PD-L1 cells, which express low CD33 levels (Figure 3B,D, Supplementary Table S1). The advantage of the bifunctional CiTE and sctb in comparison to the standard BiTE®-like molecule was further investigated by T-cell induced cytotoxicity assays using MOLM13:PD-L1 or BA/F3:CD33:PD-L1 target cells at an E:T ratio of 1:3 (Figure 3E,F). In contrast to the BiTE®-like molecule, both CiTE and sctb significantly enhanced target cell lysis. Collectively, CiTE- and sctb-mediated cytolysis is strongest against $CD33^+PD\text{-}L1^+$ double-positive cells,

independent of the absolute affinity of the checkpoint blocking module, and the fused PD-L1 blocking module increases lysis of double-positive cells.

We next evaluated whether the CiTE molecule is able to induce elimination of $CD33^+PD\text{-}L1^+$ cells selectively in the presence of $PD\text{-}L1^+$ cells. To this end, preferential lysis was analyzed in a mixed target cell population (Figure 4, Supplementary Figure S6). While the CiTE triggered preferential lysis $CD33^+PD\text{-}L1^+$ cells, molecules with high affinity to PD-L1 revealed dose-dependent elimination of both $CD33^+PD\text{-}L1^+$ and $PD\text{-}L1^+$ cell lines. This indicates that the low-affinity $PD\text{-}1_{ex}$ module is not sufficient to redirect T cells to $PD\text{-}L1^+$ non-AML cells, which might provide an important safety feature for the CiTE platform.

CiTE and sctb increase specific cytotoxicity against patient-derived AML cells and enhance T-cell proliferation

In ALL, relapse after blinatumomab treatment was suggested to originate from PD-L1 expressing leukemic cells, which are resistant to T cell-mediated cytotoxicity.[14] A similar mechanism was identified in AML, where AMG 330-induced T-cell activation was accompanied by PD-L1 upregulation on patient-derived AML cells as well as PD-1 expression on T cells *ex vivo*.[13] Also CiTE-mediated T-cell activation led to the upregulation of PD-L1 on primary AML patient cells (Supplementary Figure S7A). In 7 out of 8 patients, the CiTE was able to induce equal or enhanced redirected lysis of target cells compared to the BiTE®-like molecule (62 ± 9% compared to 55 ± 6%), and the sctb triggered similar or higher lysis in all patients (76 ± 6%) (Figure 5A,B, Supplementary Figure S7B). An increase in T-cell proliferation was induced by the CiTE and sctb in contrast to the BiTE®-like molecule through prolongation of co-culture time to 6-7 days (Figure 5D). Furthermore, elevated T-cell activity was demonstrated by virtue of PD-1 expression as well as IFN-γ release (Figure 5C, Supplementary Figure S7C). Interestingly, addition of a PD-L1 blocking mAB to the BiTE®-like molecule had a lower impact on cytotoxicity and T-cell proliferation than the sctb. Thus, we hypothesize that CiTE and sctb are able to efficiently counteract PD-L1-mediated resistance mechanisms and to induce specific lysis of AML cells by a synergy of avidity-dependent binding and checkpoint blockade.

CiTE induces leukemia eradication *in vivo* without on-target off-leukemia events

As T cell-based immunotherapies such as BiTE®s, chimeric antigen receptor (CAR) T cells and HSCT rely on T-cell activation, the induced proinflammatory response will consistently evoke PD-L1 upregulation on AML cells.[13,49-52] In order to mimic this physiological situation *in vivo*, we engrafted MOLM-13:PD-L1 cells into NSG mice followed by transfer of *in vitro* activated human HD T cells (Figure 6A). Evaluation of the residual $hCD45^+CD33^+$ AML population in the BM after 9 days of treatment revealed complete eradication of leukemic

cells in all three treatment groups (Figure 6B). In contrast, the control cohort showed 1 - 3% AML cells in the BM, which resembles MRD criteria of < 5% myeloblasts in humans (Supplementary Figure S8A).[53]

Besides efficient eradication of AML cells, the main purpose of the CiTE antibody is to avoid irAEs that originate from systemic binding to PD-L1$^+$ tissue. To investigate potential targeting of non-AML cells, we took advantage of the cross-reactivity of both PD-1$_{ex}$ and PD-L1 checkpoint blocking modules to murine PD-L1, which bound murine PD-L1 with comparable affinities than human PD-L1 (Supplementary Figure S8B). Mice treated with the high-affinity sctb lost body weight compared to the other treatment groups (Figure 6D, Supplementary Figure S8C). PD-1 was significantly upregulated on CD4$^+$ and CD4$^-$ T cells in the BM (Figure 6C) and a similar T-cell phenotype was noted when splenic T cells were analyzed (data not shown). We hypothesize that this observation is due to sctb-mediated T-cell redirection to PD-L1$^+$ murine cells and represents on-target off-leukemia events. Most importantly, no such effects were observed for the CiTE antibody. These findings demonstrate that the CiTE efficiently induces specific AML eradication *in vivo* without affecting the body weight as indication for systemic PD-L1 targeting. Thus, we consider the new CiTE format as favorable post-remission approach in AML, which is particularly suited to counteract PD-L1-mediated adaptive immune resistance.

Discussion

The BiTE® technology is a successful immunotherapeutic approach in ALL, and with AMG 330 a first T-cell engager recently entered the clinics for AML treatment. However, it has been shown that BiTE®-mediated T-cell activation and the associated release of proinflammatory cytokines trigger the upregulation of the inhibitory ligand PD-L1 on AML and ALL cells.[13,14] As reflected in *ex vivo* experiments using human patient samples, the combination of AMG 330 and PD-1/PD-L1 inhibitors might abrogate this axis and restore T-cell activity.[13] Yet, PD-1 and PD-L1 blocking mABs that have hitherto been approved by regulatory authorities are limited by their risk to induce irAEs. Although adverse events are often successfully managed, they can develop into a severe state and require therapy interruption or discontinuation. Thus, new approaches are urgently needed.

The presented CiTE format is able to combine T-cell redirection with a restricted PD-1/PD-L1 blockade to the surface of AML cells and thereby to sustain immune tolerance against healthy tissue. This is achieved by fusing the extracellular domain of human PD-1, which naturally holds a low affinity to PD-L1, to a BiTE®-like scaffold. $PD\text{-}1_{ex}$ is not sufficient to bind PD-L1 alone but only linked to a high-affinity leukemia-targeting module. As a consequence, the CiTE exclusively induces lysis of $CD33^{+}PD\text{-}L1^{+}$ cells *in vitro*, whereas $PD\text{-}L1^{+}$ non-AML cells are not affected. *In vivo* the CiTE did not lead to on-target off-leukemia events indicated by body weight loss and leukemia-unrelated T-cell activation. Thus, the bifunctional format displays a promising therapeutic strategy to lower irAEs compared to high-affinity PD-1 and PD-L1 blocking agents.

Since CD3-addressing approaches by T-cell-engaging molecules are effective at very low protein concentrations (picomolar or even sub-picomolar),[9,54,55] an obvious question is whether fusing checkpoint ligands to CD3-binding modules in a single molecule would be sufficient to block PD-1/PD-L1 interactions. However, TCR and PD-1 are suggested to be closely associated in the immunological synapse.[56] Consequently, a locally restricted full or even partial inhibition of PD-1/PD-L1 interactions at the TCR could lead to a more efficient T-cell activation even at low antibody concentrations.

Stimulation of CD3ε on T cells with monoclonal antibodies was shown to induce T-cell activation.[46,57] Accordingly, patients treated with muronomab (Orthoclone OKT3®) frequently experience cytokine release syndrome (CRS).[58,59] In contrast, monovalent CD3 stimulation by the CiTE does not *per se* trigger upregulation of T-cell activation markers such as CD69 or CD25 *in vitro*. In concordance to preclinical studies of BiTE® antibodies[46], T cells are exclusively activated by crosslinking to leukemic cells that express the targeted CD33. Nevertheless, blinatumomab does induce CRS in some patients.[60] Intensive investigations in animal models are therefore indispensable.

Similar to BiTE® molecules[9,54,55], the CiTE is able to induce redirected lysis of cancer cells at very low concentrations with EC_{50} values in the low picomolar range. Due to avidity-dependent binding, the targeting efficacy of $CD33^+$ AML cells that express PD-L1 is increased. This might provide the possibility to preferentially address double-positive cells, which is especially important since CD33 is also expressed on $CD34^+CD38^-$ hematopoietic stem cells and healthy myeloid cells, and the general depletion of $CD33^+$ cells by CD33 monotargeting agents such as GO consequentially results in neutropenia.[10,61-66]

As the upregulation of immune checkpoints in response to T-cell activation is a general mechanism of adaptive immune resistance, combination therapies of targeting agents, chemotherapies or kinase inhibitors with blocking mABs are currently under intensive investigation.[16,47,48,67] We were able to demonstrate that the CiTE molecule, despite the low-affinity $PD\text{-}1_{ex}$ domain, induces similar IFN-γ levels in comparison to the combination of the BiTE®-like molecule and PD-1 or PD-L1 inhibitors. However, most importantly and in contrast to high-affinity PD-L1 binders applied in combination therapies, the CiTE preferentially and highly selectively eliminated $CD33^+PD\text{-}L1^+$ double-positive target cells. This is expected to translate into a decreased incidence of irAE as was observed in our xenograft mouse model.

Collectively, we showed that the CiTE antibody reveals a high potency to activate resting T cells and to induce efficient cytotoxicity against AML cells. It features a high specificity for $CD33^+PD\text{-}L1^+$ target cells *in vitro* and does not show adverse events *in vivo*. Due to its beneficial performance compared to the BiTE® format, we consider the CiTE as a promising candidate to reverse immune resistance in AML. Future studies will have to examine efficacy and tolerance in more advanced *in vivo* models before applying the CiTE format into a clinical setting.

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Authorship Contributions

M.H., N.C.F., M.S. and K.P.H. designed the experiments and interpreted the data, M.H. generated and characterized the molecules and performed cell line-based assays. F.R. and S.K. contributed to cell line generation. C.K. and B.B. performed the evaluation on AML patient samples and analyzed the results, A.O. and A.M. contributed to the cell line-based assays and patient sample evaluation. K.D. performed the *in vivo* studies and evaluated the data. K.H.M., K.S. and M.S. contributed to patient characterization including cytogenetic and molecular data. R.M. wrote animal test proposal and supervised animal experiments. K.P.H. and M.S. supervised the project. M.H. and N.C.F. wrote the manuscript with input from all authors.

Conflict of interest

The authors declare no conflicts of interest.

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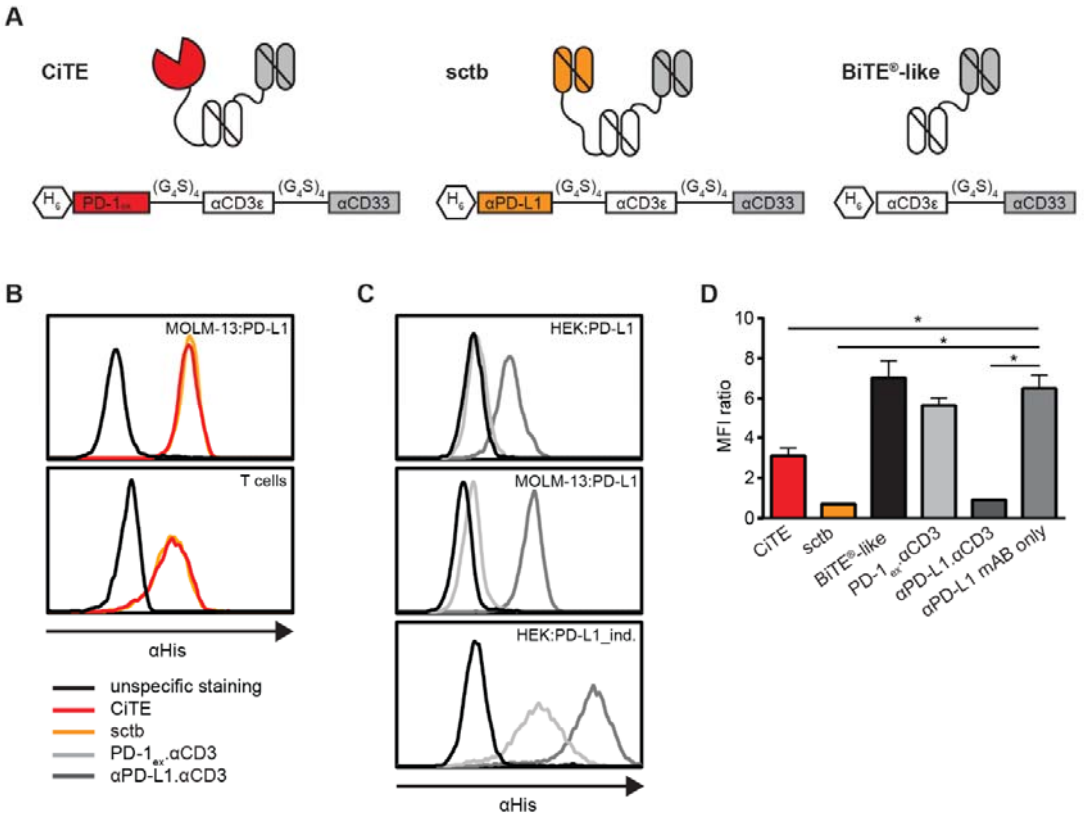

Figure 1. Schematic drawing and binding characteristics of CiTE, sctb and control molecules. (A) Modular composition of CiTE, sctb and BiTE®-like molecule. (B) Binding analysis of CiTE and sctb to MOLM-13:PD-L1 cells and HD T cells at 15 ng/µl concentration by flow cytometry. (C) Binding analysis of PD-1_{ex} (in PD-1_{ex}.αCD3) and αPD-L1 scFv (in αPD-L1.αCD3) to HEK:PD-L1, MOLM-13:PD-L1 and HEK:PD-L1_ind. cells at 1.5 ng/µl concentration. The black line indicates unspecific staining by the secondary antibody. Histograms show one out of 3 experiments with similar results. (D) Binding of αPD-L1 mAB (clone MIH1) to MOLM-13:PD-L1 cells in the presence or absence of CiTE, sctb or controls at 150 nM concentration as measured by flow cytometry. Mean values show n=3 independent experiments with standard error of the mean (SEM) as error bars. For statistical analysis unpaired Student's *t*-test with Welch correction was applied. * $P < .05$, ** $P < .01$, *** $P < .001$.

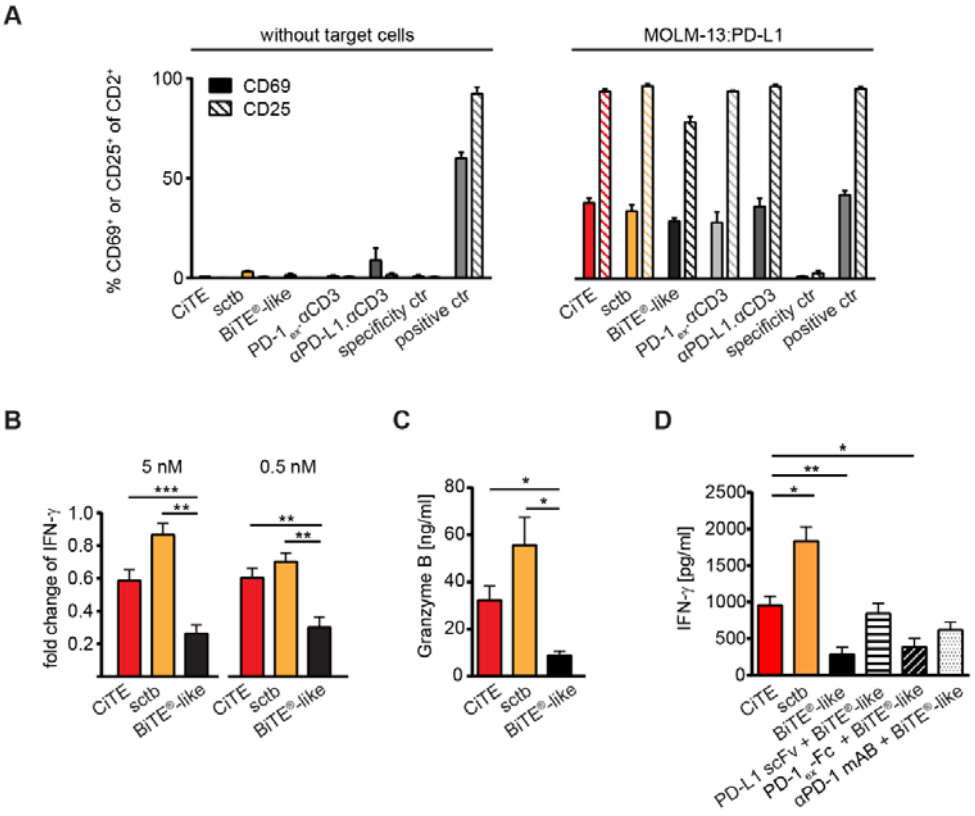

Figure 2. CiTE- mediated T-cell activation depends on crosslink to target cells and is enhanced compared to BiTE®-like molecule. (A) CiTE- and sctb-induced upregulation of CD69 and CD25 on HD T cells in comparison to controls as analyzed by flow cytometry in the absence or presence of MOLM-13:PD-L1 cells after 96 h and E:T ratio of 2:1. Bar charts show mean values of n=3 independent experiments at 5 nM concentration with SEM as error bars. (B) Fold change of IFN-γ release in the presence of MOLM-13:PD-L1 compared with MOLM-13 cells at 5 nM and 0.5 nM protein concentration. (C) Granzyme B release in the presence of MOLM-13:PD-L1 cells at 0.5 nM protein concentration. (D) IFN-γ release in the presence of MOLM-13:PD-L1 cells upon addition of CiTE, sctb or BiTE®-like molecule at 5 nM protein concentration and combination of BiTE®-like molecule with PD-1/PD-L1 blocking agents at equimolar concentration. Cytokine and Granzyme B release were measured after 72 h at an E:T ratio of 2:1 using non-stimulated HD T cells. Bar charts represent mean values of n=4-5 (B,D) or n=4-6 (C) independent experiments with SEM as error bars. For statistical analysis unpaired Student's *t*-test with Welch correction was applied. * $P < .05$, ** $P < .01$, *** $P < .001$.

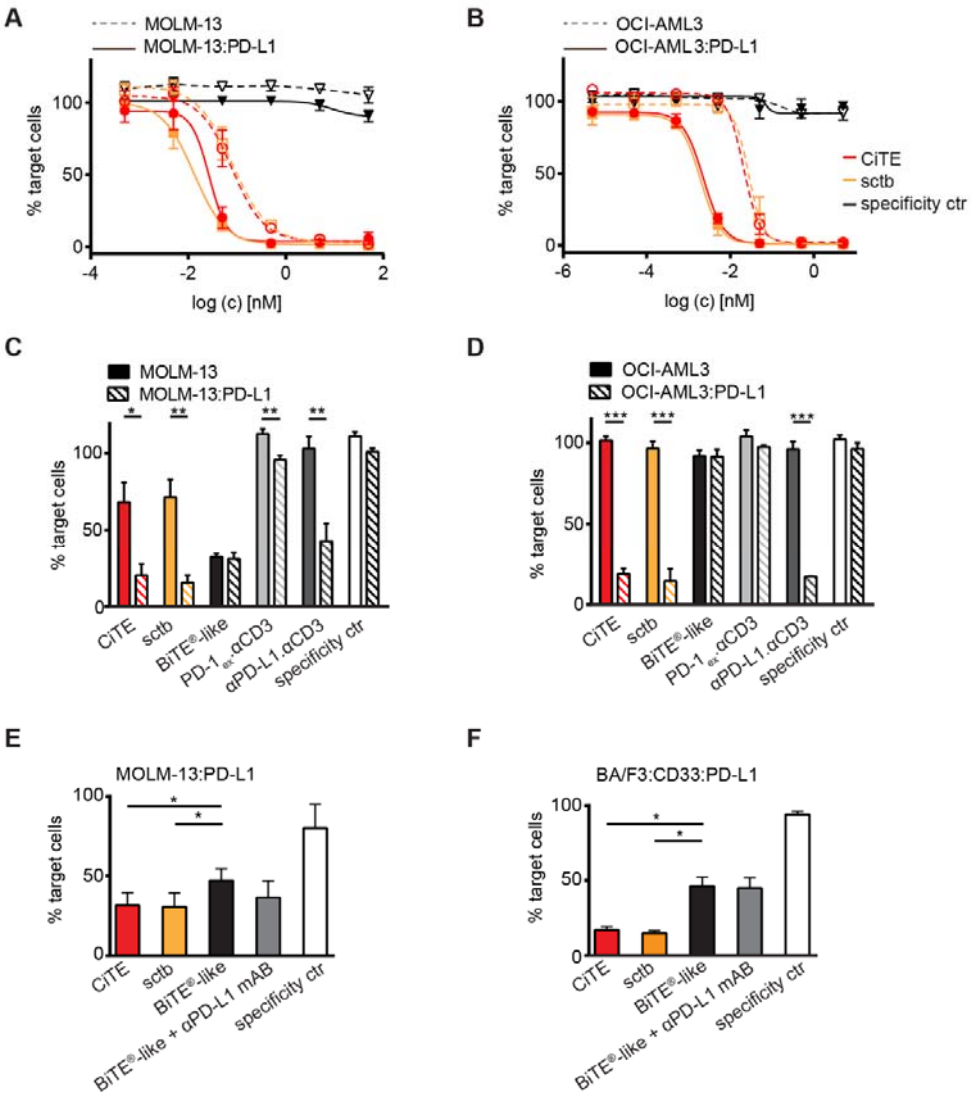

Figure 3. CiTE and sctb enhance lysis of CD33$^+$PD-L1$^+$ target cells. (A) Dose-dependent lysis of MOLM-13 vs. MOLM-13:PD-L1 cells or (B) OCI-AML3 vs. OCI-AML3:PD-L1 cells by non-stimulated HD T cells. Calculated EC_{50} values for CiTE and sctb on MOLM-13 cells were 79.1 pM and 81.6 pM, on MOLM-13:PD-L1 cells 26.9 pM and 13.2 pM, on OCI-AML3 cells 20.8 pM and 27.4 pM, and on OCI-AML3:PD-L1 cells 2.3 pM and 1.9 pM, respectively. (C) Lysis of MOLM-13 vs. MOLM-13:PD-L1 cells at 50 pM protein concentration, and (D) OCI-AML3 vs. OCI-AML3:PD-L1 cells at 5 pM concentration at an E:T ratio of 2:1 after 72 h. (E) Lysis of MOLM-13:PD-L1 cells and (F) BA/F3:CD33:PD-L1 cells at 0.5 nM concentration, E:T ratio of 1:3, and culture time of 72 h. Graphs display mean values of n=4-5 (A-D), n=7 (E) or n=6 (F) independent experiments. For statistical analysis unpaired Student's *t*-test with Welch correction (C,D) or Wilcoxon test (E,F) was applied. * $P < .05$, ** $P < .01$, *** $P < .001$.

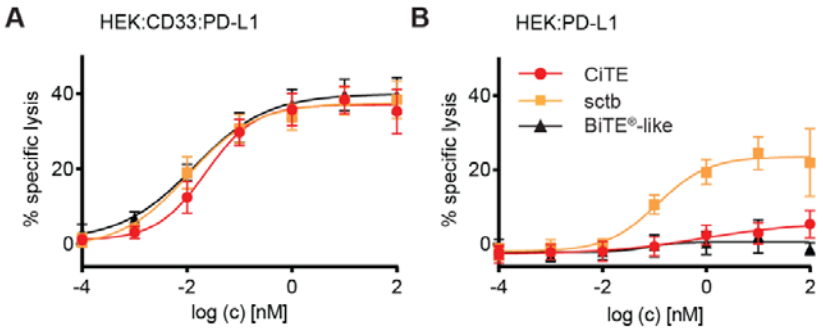

Figure 4. CiTE induces preferential lysis of CD33$^+$PD-L1$^+$ cells and has no activity against CD33$^-$PD-L1$^+$ cells. Dose-dependent preferential lysis of HEK:CD33:PD-L1 (A) over HEK:PD-L1 cells (B) induced by CiTE, sctb or BiTE®-like molecule and vice versa. Pre-activated HD T cells were incubated with target cells and CiTE, sctb or BiTE®-like molecule for 4 h at a total E:T ratio of 2:1. EC_{50} values for CiTE, sctb and BiTE®-like molecule were 22.8 pM, 10.4 pM and 13.5 pM for HEK:CD33:PD-L1 cells. For HEK:PD-L1 cells, the EC_{50} value for the sctb was 114.4 pM. Graphs represent mean values of n=4 independent experiments with SEM as error bars.

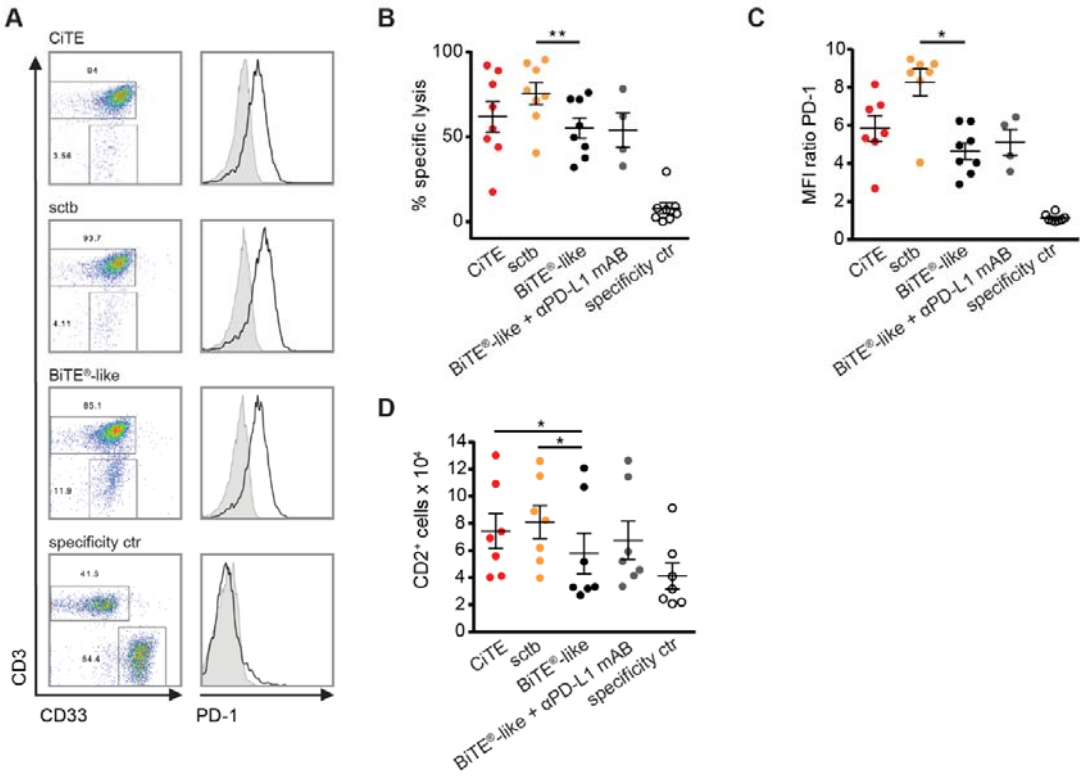

Figure 5. CiTE and sctb enhance lysis of patient-derived primary AML cells and increase T-cell proliferation. (A) Cytotoxic lysis of primary AML cells from an exemplary patient and PD-1 expression on T cells after 3-4 days of co-cultivation. (B) Mean cytotoxic lysis of primary AML cells induced by respective molecules after 3-4 days. (C) PD-1 expression on T cells after 3-4 days. (D) T-cell proliferation after 6-7 days measured by flow cytometry. CiTE, sctb, BiTE®-like molecule and specificity control were applied at 10 nM, the αPD-L1 mAB at 10 µg/ml concentration. Assays were performed at an initial E:T ratio of 1:4. Error bars display SEM. For statistical analysis Wilcoxon test was applied. * $P < .05$, ** $P < .01$, *** $P < .001$.

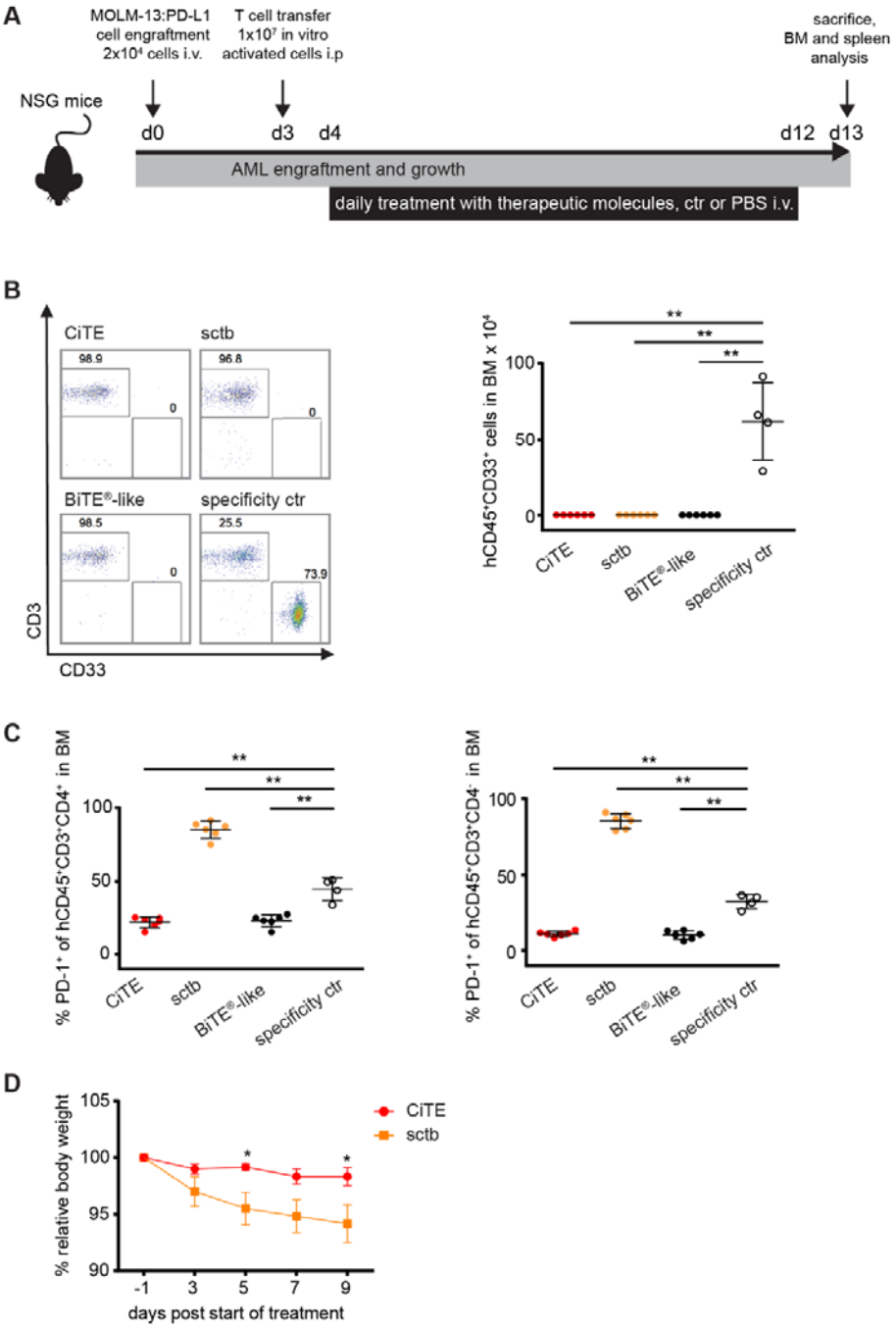

Figure 6. CiTE leads to eradication of AML cells in a murine NSG xenograft model without indication of enhanced PD-1 upregulation or body weight loss. (A) Experimental design of mouse studies. (B) Remaining engrafted MOLM-13:PD-L1 (live hCD45$^+$CD33$^+$) cells in BM of exemplary mice per cohort (left) and as mean (right) after 13 days. (C) PD-1 upregulation on human CD45$^+$CD3$^+$CD4$^+$ and CD45$^+$CD3$^+$CD4$^-$ T cells. (D) Relative body weight of mice as scored during treatment with CiTE and sctb. Cohorts contained 4-6 mice. Error bars in (B)-(C) indicate SD, in (D) SEM. For statistical analysis Mann-Whitney U test was applied. * $P < .05$, ** $P < .01$, *** $P < .001$.

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Bifunctional PD-1 x αCD3 x αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia

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