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# *Augmenting bispecific T-cell engager (BiTE) therapy for the treatment of Acute Myeloid Leukemia*

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# List of abbreviations

| | |
|---|---|
| 2-HG | R-2-hydroxyglutarate |
| ALL | Acute lymphoblastic leukemia |
| AML | Acute myeloid leukemia |
| BCL-2 | B-cell lymphoma 2 |
| BiTE | Bispecific T-cell engager |
| BM | Bone marrow |
| BMMC | Bone marrow mononuclear cells |
| CiTE | Checkpoint inhibitory T cell-engaging |
| CLEC12A | C-type lectin domain family 12 member A |
| CML | Chronic myeloid leukemia |
| CR | Complete remission |
| CRh | Complete remission with partial hematologic recovery |
| CRi | Complete remission with incomplete hematologic recovery |
| CRS | Cytokine release syndrome |
| DC | Dendritic cell |
| DLI | Donor lymphocyte infusion |
| DNA | Deoxyribonucleic acid |
| FAB | French American British |
| FDA | Food and drug administration |
| ELN | European LeukemiaNet |
| EMA | European Medicines Agency |
| Fc | Fragment crystallizable |
| FLT3 | Fsm-like tyrosine kinase 3 |
| FLT3L | FLT3 ligand |
| Gilt | Gilteritinib |
| Glas | Glasdegib |
| GO | Gemtuzumab ozogamicin |
| GvHD | Graft-versus host disease |
| GvL | Graft-versus leukemia |
| HD | Healthy donor |
| Hh | Hedgehog |

**List of abbreviations**

| | |
|---|---|
| HSC | Hematopoietic stem cell |
| HSCT | Hematopoietic stem cell transplantation |
| IDH | Isocitrate dehydrogenase |
| IFNγ | Interferon gamma |
| irAE | Immune related adverse events |
| ITD | Internal tandem duplication |
| IV | Intravenous |
| kDa | kilodalton |
| LSC | Leukemic stem cell |
| Mido | Midostaurin |
| MRD | Minimal residual disease |
| NK cell | Natural killer cell |
| NOD/SCID | Non-obese diabetic/severe combined immuno- deficiency |
| PB | Peripheral blood |
| PD | Pharmacodynamic |
| PK | Pharmacokinetic |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed cell death protein 1 ligand 1 |
| RNA | Ribonucleic acid |
| r/r | Relapsed/refractory |
| scFv | Single chain fragment variable |
| sctb | Single-chain triplebody |
| TDCC | T-cell-dependent cellular cytotoxicity |
| allo-SCT | Allogeneic stem cell transplantation |
| TandAb | Bispecific tandem diabodies |
| TKD | Tyrosine kinase domain |
| Ven | Venetoclax |

# List of publications

This thesis includes two publications that have been published in peer-reviewed journals:

**Bettina Brauchle**\*, Rebecca L. Goldstein\*, Christine M. Karbowski, Anja Henn, Chi-Ming Li, Veit Bücklein, Christina Krupka, Michael C. Boyle, Priya Koppikar, Sascha Haubner, Joachim Wahl, Christoph Dahlhoff, Tobias Raum, Matthew J. Rardin, Christine Sastri, Dan Rock, Michael von Bergwelt-Baildon, Brendon Frank, Klaus H. Metzeler, Ryan Case, Matthias Friedrich, Mercedesz Balazs, Karsten Spiekermann, Angela Coxon, Marion Subklewe, Tara Arvedson:

**„Characterization of a Novel FLT3 BiTE Molecule for the Treatment of Acute Myeloid Leukemia"**

\*contributed equally

Monika Herrmann, Christina Krupka, Katrin Deiser, **Bettina Brauchle**, Anetta Marcinek, Ana Ogrinc Wagner, Felicitas Rataj, Ralph Mocikat, Klaus H. Metzeler, Karsten Spiekermann, Sebastian Kobold, Nadja C. Fenn, Karl-Peter Hopfner, Marion Subklewe:

**„Bifunctional PD-1 × αCD3 × αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia."**

# 1 Introductory summary

## 1.1 Acute Myeloid Leukemia (AML)

Acute Myeloid Leukemia (AML) is a malignancy of the hematological compartment characterized by uncontrolled proliferation and impaired differentiation of myeloid progenitor cells.[1,2] In AML, malignant cells make up at least 20 % of total bone marrow nuclear cells and can be the predominant cell population.[3] The expansion of AML cells leads to the suppression of healthy hematopoietic cells. Subsequent lack of healthy immunocompetent cells is the origin of significant immunodeficiency in these patients. Hence, AML patients often suffer from potentially fatal infections.[4,5] AML is the most common form of acute leukemia in adults, with a prevalence of 4.3 newly diagnosed cases/100,000 people per year.[6] The prevalence is increasing with age leading to a median age of 68 years at diagnosis.[6] AML can be classified by several systems. The historic French American British (FAB)-classification is based on morphological and cytochemical characteristics of the malignant cells. The recently updated World Health Organization classification incorporates morphologic, cytogenetic and genetic properties.[3,7,8] The European LeukemiaNet (ELN) risk stratification system provides prognostic information for patients undergoing chemotherapy or hematopoietic stem cell transplantation and subdivides the patients in three risk groups predicting relapse free and overall survival. [9,10]

With the analysis of genetics, negative prognostic markers have been identified and can now serve as targets for novel therapeutic approaches.

Standard of care therapy remained unchanged for 40 years until 2017. It comprised of intensive induction chemotherapy followed by post-remission therapy, at least for patients deemed fit for such intensive treatment options.[11–13] Induction chemotherapy, often called the "7+3" regimen, consists of 3 days of an anthracycline and 7 days of cytarabine and leads to complete remission (CR) in 40-65 % of patients $\geq$ 60 years old and in 60-80 % of younger adults.[3,13,14] In the last four years several targeted therapies have been approved, leading to better standard of care therapy options at least for subgroups of AML patients. These options will be explained in detail in chapter 1.3.

Nevertheless, the risk of relapse is high due to chemo-refractory leukemic cells. The goal of post-remission therapy is to eliminate these cells.[15] After induction therapy, patients with a favorable genetic risk profile (according to ELN risk stratification) receive additional cycles of chemotherapy called consolidation therapy. Patients with a non-favorable genetic risk profile are assessed for eligibility of allogeneic stem cell transplantation (allo-SCT).[5,16]

## 1.2  Stem cell transplantation

Allo-SCT is the most effective anti-leukemic strategy in treatment of AML and the only curative treatment option in relapsed/refractory (r/r) AML patients.[15,17] As conditioning therapy, patients receive high doses of chemotherapy with or without total body irradiation.[18,19] This treatment also aims to reduce the leukemic burden and stops the recipients own hematopoiesis to allow engraftment of the donor hematopoietic cells. The transplanted hematopoietic stem and progenitor cells (HSPCs) reconstitute normal hematopoiesis.[15] The most important mechanism of the curative effect of allo-SCT is the graft-versus-leukemia (GvL) effect mediated by the donors allo-reactive T cells.[16,20–22] These T cells can also attack normal recipient tissues, resulting in the so-called graft-versus host disease (GvHD). The GvL effect has to be balanced against the GvHD risk.[23,24] Strategies to reduce GvHD are T-cell depletion of the transplant as well as treating the patient with immunosuppressive drugs after transplantation.[23,25,26] Unfortunately, by this, also the GvL response can be dampened, leading to treatment failures and increased relapse rates.[19,27]

Two options to reduce relapse rates are the infusion of donor T cells after the patient recovered from conditioning toxicity or the administration of targeted therapies.[27–29] Both concepts will be explained in more detail in the next chapters.

## 1.3 Targeted therapies in AML

After almost no changes in standard of care therapy for AML patients for 40 years, the treatment options started to change in 2017. Since then, several targeted drugs for the treatment of de-novo and r/r AML have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Drugs, indications, clinical effects, and date of approval can be found in Table 1.

Fsm like tyrosine kinase 3 (FLT3), a transmembrane ligand-activated tyrosine kinase is frequently mutated in AML. FLT3 mutations can be found in approximately 30 % of AML patients and occur as either internal tandem duplication (ITD, ~25 %) or as point mutations in the tyrosine kinase domain (TKD, 7-10 %).[30–34] FLT3-ITD is a common driver mutation associated with poor prognosis and both mutations constitutively activate FLT3 kinase leading to proliferation and survival of AML cells.[33–36] By binding of FLT3 inhibitors like midostaurin or gilteritinib to the kinase, the receptor is dephosphorylated and thereby the oncogenic signalling is disrupted.[35]

Further mutations that typically occur in AML patients are gain of function mutations in the isocitrate dehydrogenase (IDH) genes. Mutations in the two isoforms IDH 1 and IDH 2 occur in 20-30 % of AML patients and result in accumulation of the oncometabolite R-2-hydroxyglutarate (2-HG) and thereby inhibition of Deoxyribonucleic acid methylation and histone modification.[37–40] This contributes to AML pathogenesis and results in blocked hematopoietic cell differentiation.[37] R-2-hydroxyglutarate levels in patients can be used to assess the effectiveness of IDH-targeted therapies.[41,42]

Venetoclax, a selective B-cell lymphoma 2 (BCL-2) inhibitor in combination with chemotherapy is the new standard for elderly or unfit patients with newly diagnosed AML.[43] BCL-2 which is known to be upregulated in AML is an antiapoptotic protein that regulates outer membrane permeabilization and thereby intrinsic mitochondrial apoptosis.[44] Binding of venetoclax to the BCL-2 protein leads to the release of proapoptotic factors from the BCL-2 protein and restores the mitochondrial apoptotic pathway.[40]

The hedgehog (Hh) signalling pathway plays an important role in embryogenesis and fetal development. Abnormal signalling leads to proliferation of leukemic stem cells (LSCs). Glasdegib interacts with a transmembrane protein that regulates Hh signalling and inhibits the Hh pathway.

Gemtuzumab ozogamicin (GO) is an antibody drug conjugate consisting of a monoclonal antibody directed against CD33, a commonly expressed AML target, and a cytotoxic derivative of calicheamicin.[45,46]

## Targeted therapies in AML

Table 1: Targeted therapies approved for the treatment of AML

| Drug | | Indication (AML only) | Clinical effects | Approval FDA | Approval EMA | Ref |
|---|---|---|---|---|---|---|
| Midostaurin (Mido) | FLT3 inhibitor | • FLT3 mutation<br>• de novo | • Median OS: 74.7 mo (Mido) vs 25.6 mo (Pbo) | 04/17 | 09/17 | [47] |
| Gileritinib (Gilt) | | • FLT3 mutation<br>• r/r | • CR rate: 21.1 % (Gilt) vs 10.5 % (Cont)<br>• OS: 9.3 mo (Gilt) vs 5.6 mo (Cont) | 07/18 | 11/19 | [48] |
| Enasidenib | IDH2 inhibitor | • IDH2 mutation<br>• r/r | • CR rate: 19.6 %<br>• Overall survival: 8.8 mo<br>• ORR: 38.8 % | 08/17 | - | [49] |
| Ivosidenib | IDH1 inhibitor | • IDH1 mutation<br>• r/r | • ORR: 39.1 %<br>• CR/CRh rate: 30.2 % | 07/18 | | [38] |
| | | • IDH1 mutation<br>• de novo | • ORR: 54.5 %<br>• CR/CRh rate: 42.4 % | 05/19 | - | [39] |
| Venetoclax (Ven) | BCL-2 inhibitor | • ≥75 years old or ineligible for intensive chemotherapy<br>• de novo | • Median OS: 14.7 mo (Aza+Ven) vs 9.6 mo (Aza+Pbo)<br>• Composite response rate: 66.4 % (Aza+Ven) vs 28.3 % (Aza+Pbo)<br>• Median duration of Composite CR: 17.5 mo (Aza+Ven) vs 13.3 mo (Aza+Pbo) | 11/18 | - | [44] |
| Glasdegib (Glas) | Hedgehog inhibitor | • ≥75 years old or ineligible for intensive chemotherapy<br>• de novo | • CR rate: 17.0 (LDAC+Glas) vs 2.3 (LDAC)<br>• Median OS: 8.8 mo (LDAC+Glas) vs 4.9 mo (LDAC) | 11/18 | 06/20 | [50] |
| Gemtuzumab ozogamicin (GO) | CD33 antibody drug conjugate | • CD33$^+$<br>• De novo<br>• in the US also r/r | • ORR: 27.5 mo (D+A+GO) vs 21.8 mo (D+A)<br>• CR rate: 70.4 (D+A+GO) vs 69.9 (D+A)<br>• EFS: 17.3 mo (D+A+GO) vs 9,5 mo (D+A) | 09/17 | 04/18 | [46] |

A: cytarabine; D: daunorubicin; Cont: control; CR: complete remission; CRh: complete remission with partial hematologic recovery; CRi: complete remission with incomplete hematologic recovery; EFS: Event free survival; EMA: European Medicines Agency, FDA: Food and Drug Administration; LDAC: low dose cytarabine, mo: months; ORR: overall response rate; OS: overall survival; Pbo: placebo; Ref: reference

## 1.4    Donor lymphocyte infusion

Another way to increase or prolong the GvL effect is the aforementioned donor lymphocyte infusion (DLI). In 1990 Kolb et al were the first to present DLI as a treatment for relapsed chronic myeloid leukemia (CML) after allo-SCT.[51] All three patients achieved (partly long-lasting) remission.[51] With these results, DLI represented the first highly effective cellular immunotherapy.
In AML, however, DLI as individual therapy has only limited benefit. This is most likely based on the lower impact of GvHD-independent GvL in AML as compared to CML.[52]

Although the overall response rate is 34 % of patients with relapsed AML after allo-SCT, 2-year overall survival is less than 20 %.[53] Due to the fast regrowth of leukemic cells at relapse, a DLI must on the one hand be delivered early but on the other hand increases treatment related morbidity and mortality.[27] DLI cannot only be used as a therapeutic strategy, also prophylactic or preemptive studies have been performed.[29,54]

One of the biggest downsides of DLI therapy is the high incidence of GvHD (up to 50 % of patients develop or show relapse of a pre-existing GvHD).[55,56] Several ways to overcome this are currently being tested in preclinical or clinical studies. Options include the transplantation of natural killer (NK) cells instead of T cells or the depletion of CD8$^+$ T cells from the T cell product.[57–59]
Other studies try to genetically manipulate the T cells to be able to turn off T-cell proliferation in case of severe GvHD or to direct them against tumor antigens.[60–63]
There are also efforts to separate host-reactive from leukemia-reactive T cells to be able to solely transplant antileukemic T cells.[64–66]

Another method utilizing the anti-leukemic potential of T cells currently being investigated in clinical studies is the use of T-cell recruiting antibodies to direct the patients' own T cells or donor T cells against the leukemic cells. These cannot only be administered in the context of DLI but also as an independent therapy.

## 1.5 Bispecific T-cell engaging antibodies

Bispecific T-cell engaging antibodies (BiTEs) comprise 2 binding sites, one to bind a tumor antigen and the other to bind T cells.[67–69] There are several different formats of bispecific T-cell engaging antibodies, depicted in Figure 1.[70]
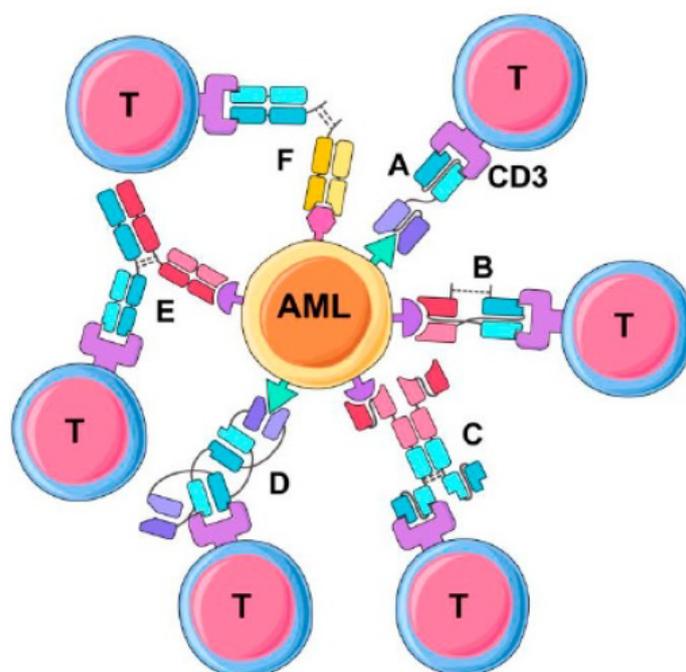


Figure 1: Different T-cell engaging; (A) Bispecific tandem fragment variable format (BiTE,), (B) Dual Affinity Re-targeting Antibody (C) Bispecific single-chain Fv immunofusion, (D) Bispecific tandem diabodies (TandAb); (E) Duobody; (F) Chemically conjugated Fab (fragment antigen binding).[70]

With the help of bispecific antibodies, T cells can be recruited to target antigen expressing cells irrespective of their TCR specificity.[71] In principle, by binding to the tumor antigen and the T cell simultaneously, a cytolytic synapse is formed, and the T cell secretes granzyme B, perforin and cytokines. This induces the lysis of the tumor cell, and the activated T cells start to proliferate.[72,73]

Proof of efficacy of BiTE antibodies (Figure1A) was shown in 2014. Blinatumomab, a bispecific tandem fragment variable format antibody (BiTE) which is engineered by combining the VL and VH domains of a monoclonal antibody into a single chain fragment variable (scFv) specific to CD3 linked to a second scFv specific to the target antigen CD19, was the first bispecific antibody construct to be approved by the FDA. It is indicated for the treatment of r/r B-cell acute lymphoblastic leukemia (ALL))[74,75]. Later, approval by the EMA (2015) for r/r disease as well as FDA and EMA (2018 and 2019 respective) approval for minimal residual disease (MRD) followed. In a phase II trial, CR was achieved in 44 % of r/r patients after two cycles of Blinatumomab (CR + CR with incomplete hematologic recovery (CRi) + CR with partial hematologic recovery (CRh)[76]. Even

better response rates were achieved in MRD patients (78 % achieved complete MRD response)[77].

The molecule was translated into the setting of AML by exchanging the target-antigen specific CD19 binding site with a CD33 binding site. AMG330, the CD33xCD3 bispecific antibody manufactured by AMGEN, is under clinical investigation since 2015 (NCT02520427), and the clinical trial currently is still in the phase of dose escalation. In the latest available reports on the ongoing trial, three of 42 evaluable patients achieved a CR, four patients achieved CRi and in one patient, a morphologic leukemia free state was the best response to therapy.[78–80]

Due to the small size of approximately 55 kilodalton (kDa), AMG330 has a short half-life of about 1 - 4 hours and is renally excreted[81–83]. By adding a fragment-crystallizable (fc) domain to the antibody construct the size is doubled, leading to a slower renal excretion of about 7 days[81]. The larger size might also allow for a once weekly intravenous (iv) infusion instead of a continuous iv infusion.[84]

The half-life extended CD33xCD3 bispecific antibody AMG673 is under clinical investigation since 2017 (NCT03224819) and preliminary results show 1/27 evaluable patients achieved a CRi and 6 showed a ≥50 % blast reduction compared to baseline. In total 44 % (12/27) show a decrease in bone marrow (BM) blasts.[85,86] Dose escalation is also ongoing for this molecule. So far, treatment with CD33 directed bispecific antibodies falls short of expectations. Therefore, there are several strategies to enhance the efficacy of bispecific antibodies. Other antibody formats as well as other target antigens are under evaluation.

## 1.6    Target antigens for the treatment of AML

The identification of the most suitable tumor-associated target antigen to be used with bispecific antibodies is the greatest challenge in establishing novel immunotherapeutic treatment options in AML. To minimize immune related adverse events (irAE), preventing on-target off-leukemia toxicity is of utmost importance. Therefore, the optimal target antigen should be expressed to a high degree on AML bulk cells and LSCs but not on healthy cells, particularly not on hematopoietic stem cells (HSCs).[87]

CD33 is the most prominent target antigen that has been evaluated as an AML target using several different antibody formats over the past decade. Currently, there are six different clinical trials for CD33 targeting bispecific antibodies ongoing (NCT03915379, NCT03224819, NCT03647800, NCT02520427, NCT03144245, NCT03516760).

The first bispecific antibody for the treatment of AML which reached clinical phase I was the already mentioned AMG330. AMG330 and the half-life extended AMG673 both target CD33, which is expressed on more than 90 % of AML bulk cells and LSCs[68,83,85]. However, CD33 is also highly expressed on healthy HSPCs, granulocytes, monocytes and resident macrophages in the liver, lung and kidney[88,89]. In a phase I clinical trial with AMG330 (NCT02520427), the most commonly observed treatment related adverse event was cytokine release syndrome (CRS) with a prevalence of 67 %.[79,90] CRS can be mitigated by a low lead-in dose followed by several dose steps and the administration of corticosteroids or an interleukin 6 receptor agonist.[90–92] As a potential correlation of myelotoxicity mediated by activity against healthy hematopoietic cells, 20 % (8/40) of patients treated with AMG330 experienced febrile neutropenia grade 3 or 4.[78] To overcome potential on-target off-leukemia effects, like myelotoxicity, other target antigen are currently under clinical investigation.

**CD123**

The Interleukin 3 receptor CD123 is expressed homogeneously at high levels in bulk AML cells and in 75 % of CD34$^+$/CD38$^-$ leukemic stem/progenitor cells, whilst healthy CD34$^+$/CD38$^-$ HSCs lack CD123 expression.[93–95] Compared to CD33, CD123 expression is higher at relapse.[88] Transplanting CD34$^+$/CD38$^-$/CD123$^+$ cells into immunodeficient mice induces outgrowth of AML which makes CD123 a marker for LSCs and a possible target to prevent relapse[96].

CD123 as a target for T-cell recruiting antibodies is currently being evaluated in several clinical trials either alone (NCT02152956, NCT02730312, NCT03594955 and NCT02715011) or in combination with cytarabine (NCT04158739) or anti Programmed cell death protein 1 (PD-1) checkpoint blockade.[97]

19 % (5/27) patients treated with 500 ng/kg/day of the dual-affinity re-targeting antibody Flotetuzumab (NCT02152956) achieved a CR/CRi. Four CR/CRi were among patients with primary chemotherapy refractory AML (n=13) while no relapsed patient achieved CR/CRi.[98,99] During therapy 49/66 (77 %) of treated patients showed CRS grade 3 and 4.[99] Another antibody construct

directed against CD123, XmAb14045 (NCT02730312) is under clinical evaluation since 2016 and preliminary results showed a CR/CRi rate of 14 % (7/51) treated at 0.75 µg/kg. 62 of all 106 treated patients (58 %) encountered CRS with 15 % grade 3 or 4.[100]

### CLEC12A/CLL-1

C-type lectin domain family 12 member A (CLEC12A) is expressed on almost all immune cells of the myeloid lineage (except erythrocytes) and their precursor/progenitor cells.[101] 61.8 % granulo-cyte and monocyte precursor cells as well as 41.6 % progenitors show high to medium CLEC12A expression, whereas only 2.5 % of healthy $CD34^+/CD38^-$ HSCs express CLEC12A.[102] CLEC12A is highly expressed on primary AML (77.5 – 92 %) and $CD34^+/CD38^-/CLEC12A^+$ AML cells have the potential to induce leukemia in non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice.[102–104]

CLEC12A is currently being investigated as an AML target in one clinical trial using a bispecific antibody (NCT03038230). Preliminary results show a blast reduction of $\geq$ 50 % in four of 26 evaluable patients while one of these patients achieved a morphological leukemia free state.[105]

### CD135

CD135 is a class III receptor kinase, which is also called FLT3. CD135 is important in hematopoi-esis as it is a key factor for proliferation and differentiation of HSPCs and HSCs into monocytes, dendritic cells (DCs), B and T cells.[106–108] As already mentioned, it is also involved in leukemic cell survival and proliferation.[109]

In our study "Characterization of a novel FLT3 BiTE molecule for the treatment of acute myeloid leukemia", we found that FLT3 expression is negligible on HSPCs and completely absent in HSCs.[69] 78 % AML bulk cells as well as LSCs express CD135 with no difference between initial diagnosis and relapse.[69] That makes FLT3 a promising target antigen for the treatment of AML.

CD135 as target for bispecific antibodies is currently being investigated in a clinical trial (NCT03541369), utilizing the half-life extended AMG427 presented in our work.[69]

A summary of our validation of FLT3 as a target antigen and evaluation of the FLT3xCD3 BiTE antibody for the treatment of AML is given in chapter 1.8.1.

## 1.7   Novel T-cell engaging antibodies

Bispecific T-cell engaging antibodies, as explained in chapter 1.5, show several limitations which can impair their anti-tumor activity.

As already mentioned, these antibodies have a short *in-vivo* half-life, resulting in poor retention times in the target tissue. Additionally, the specificity of the monovalent binding might not be strong enough to target cancer cells, as healthy cells also express the targeted antigens.[73,81–83] Furthermore, monospecific targeting can result in downregulation of the antigen due to escape mutations.[110]

To overcome these limitations, several concepts to improve bispecific antibodies have been designed:

One concept is to fuse a fc domain to the antibody construct to increase its size to about 100 kDa.[84] This results in a slower renal excretion of the antibody.

We used such an half-life extended bispecific antibody construct together with a conventional bispecific antibody construct in our first study "Characterization of a Novel FLT3 BiTE Molecule for the Treatment of Acute Myeloid Leukemia", which will be presented in chapter 1.8.1.

A second concept are tandem diabodies (TandAb, Figure 1D) with bivalent binding for both anti-gens, with a molecular weight of about 114 kDa.[111] With the increased size and bivalent binding, TandAbs show improved half-life and binding affinity compared to conventional single chain anti-body fragments.[112]

Another concept are dual-specific single chain triplebodies as developed by Roskopf et al. in 2014.[73] Triplebodies consist of three scFvs connected via a flexible $(Gly_4Ser)_4$ linker. Due to a size of about 85 - 95 kDa triplebodies show an increased plasma half-life of about 4 hours in mice, which can be translated to approximately 1 day in humans.[73] With these constructs, monospecific bivalent targeting or bispecific bivalent targeting is possible.[113] The bispecific bivalent antibody constructs mediate preferential lysis of double positive over single positive target cells.[113]

We used this antibody concept in our second study "Bifunctional PD-1 × αCD3 × αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia", where we generated a dual function checkpoint inhibitory T cell-engaging (CiTE) antibody construct. This construct combines targeting of AML cells via CD33 with locally restricted PD-1 immune checkpoint blockade to overcome the reported upregulation of PD-1 on T cells and programmed cell death 1 ligand 1 (PD-L1) on primary AML cells after *ex-vivo* coculture with bispecific antibodies [114,115] This study will be presented in detail in chapter 1.8.2.

The progress in the field of bispecific antibody development lets me face the future optimistically. Hopefully, there will soon be a transition in treatment options for AML patients from chemotherapy and allo-SCT to bispecific antibodies with increased response rates and decreases relapse rates.

## 1.8 Summary of publications

### 1.8.1 Publication I:
### Characterization of a Novel FLT3 BiTE Molecule for the Treatment of Acute Myeloid Leukemia[69]

In this first publication, we evaluated FLT3 as a target for the treatment of AML. We characterized two bispecific antibodies, a canonical version and one version with half-life extended properties (AMG427), *in-vitro*, *in-vivo* and *ex-vivo* for the treatment of AML. Expression analyses of FLT3 on 318 AML patient samples at initial diagnosis or relapse and 36 Healthy donors (HDs) were performed. 78 % AML bulk cells and 79 % AML LSCs showed expression of FLT3, with significantly lower expression on HD HSPCs and HD HSCs. FLT3 expression was comparable regardless of disease status (initial diagnosis or relapse) and mutational status (FLT3-ITD mutated or FLT3-ITD wildtype). LSCs of patients with a high allelic frequency (>0.5) for FLT3-ITD showed significantly higher expression of FLT3 than LSCs from patients with a low allelic frequency. Further characterization of healthy human blood cell populations showed absence of FLT3 expression on T, B and NK cells, plasmacytoid DCs, conventional DCs and neutrophils. FLT3 ribonucleic acid (RNA) expression analysis from three publicly available databases (GTEx, Amgen-constructed and XpressWay) revealed low RNA expression of FLT3 in brain, nerve/ganglia, small intestine, kidney, lung, pancreas, spleen, spinal cord, and testis. Immunohistochemistry staining of all tissues (but spinal cord) revealed cytoplasmic expression of FLT3 without surface expression.

After confirming the suitability of FLT3 as a target antigen for the treatment of AML with low risk of on-target off-leukemia toxicities, two different FLT3-BiTE antibody constructs (Figure 2) were tested for their ability to induce T-cell-dependent cellular cytotoxicity (TDCC).
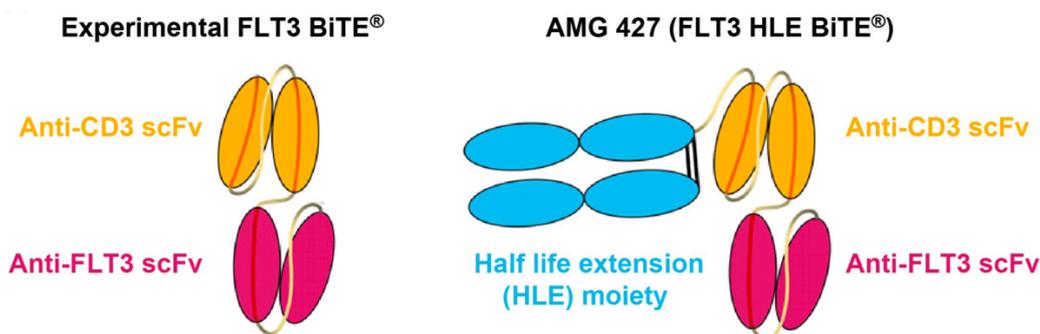


Figure 2: Schematic figure of the canonical FLT3-BiTE (left) and the half-life extended (HLE) FLT3-BiTE (right)[69]

Both antibodies bound human CD3 and CD135 with concentrations in a nanomolar range and induced high cytotoxicity against FLT3 expressing cell lines in coculture with HD T cells. Cytotoxicity was accompanied by upregulation of CD69 and CD25 and secretion of interferon gamma

(IFNγ) and tumor necrosis factor alpha by the T cells. No such effect was observed in cocultures of HD T cells with FLT3 negative cell lines. FLT3 positive cell lines were lysed irrespective of their FLT3-ITD mutational status. The presence of clinically relevant concentrations of soluble FLT3 or soluble FLT3 ligand (FLT3L) reduced the potency of both molecules but not the maximum killing capacity. With these results we conclude that both antibody constructs target FLT3 positive cells specifically and can exert maximum cytotoxicity even in presence of physiological concentrations of soluble FLT3 and soluble FLT3L.

After *in-vitro* evaluation of both antibody constructs, *in-vivo* studies were performed. Both antibodies were tested in mouse models. In an admix model with human T cells and MOLM-13 AML cells, the canonical BiTE molecule inhibited 90 % of tumor growth compared to a control molecule.
AMG427 was evaluated in an orthotopic mouse model with either MOLM-13 or EOL-1 AML cells and human T cells. AMG427 prolonged the survival of the treated mice significantly in both setups.

Next, pharmacokinetic (PK) and pharmacodynamic (PD) studies in cynomolgus monkeys were performed. A 16-day cynomolgus monkey study with the canonical BiTE molecule showed an 85 - 92 % reduction of FLT3 transcript levels in comparison to non-treated animals.
A 8 day PK/PD study with AMG427 revealed a terminal half-life of 33 - 50 hours and showed upregulation of CD69 on T cells and cytokine secretion. In BM, FLT3 transcript levels were reduced by 85 - 97 %. At the end of the study, numbers of peripheral blood (PB) monocytes were reduced. The FLT3 transcript level reduction in BM can be explained by the direct killing of FLT3 expressing progenitor cells in the BM. Reduction of monocytes and FLT3 transcript levels in PB are most likely due to lack of replenishment after elimination of progenitor cells in the BM.

In our *ex-vivo* experiments, 14 AML patient samples were analyzed in an autologous cytotoxicity assay over the time of 9 days with residual patient T cells in presence of the canonical BiTE molecule. The effector to target ratio ranged between 1:2.5 - 1:74. After 9 days, the patients could be classified into 3 groups according to their response. Group one (5 patients) showed continuously increasing cytotoxicity, the second group (4 patients) showed initial cytotoxicity followed by sustained or decreased killing and the remaining 5 patients showed transient or no cytotoxicity. Most patients achieving a response (group 1 and 2) had higher E:T ratios (>1:38) and/or high levels of FLT3 surface expression (MFI ratio >2) compared to non-responders.

As studies with AMG427 in cell lines showed upregulation of PD-1 on T cells leading to a decrease in cytotoxicity, combinatorial studies with AMG427 and PD-1 blockade were performed. The combination of both molecules increased the maximum killing by 12 %. All our findings together warrant the clinical investigation of the FLT3 BiTE molecule which is currently being carried out in a phase I clinical trial with the half-live extended AMG427 (NCT03541369).

## Summary of publications

I performed, analyzed, and evaluated the target antigen expression analysis of the 218 patient samples as well as the *in vitro* and *ex vivo* functional assays with cell lines and patient samples. Rebecca Goldstein, the other co-senior author of this study, performed, analyzed, and evaluated the healthy non-hematopoietic tissue analysis, mouse studies and cynomolgus monkey studies. We both contributed to conceptualization, draft-writing, revision, and editing.

## 1.8.2   Publication II:
## Bifunctional PD-1 × αCD3 × αCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia[115]

In the study entitled "Bifunctional PD-1 x aCD3 x aCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia" published by Herrmann et al in Blood in 2018 we developed and validated a novel bifunctional CiTE antibody for the treatment of AML. Previous studies showed upregulation of the inhibitory checkpoint molecules PD-1 on T cells and PD-L1 on AML cells upon coculture with AMG330.[114,116] Monotherapy with checkpoint inhibitors in hematological malignancies show only limited benefit.[117] We hypothesize that by the generation of a CiTE anti-body combining CD33 targeting with PD-1 checkpoint blockade we can combine the benefits of both treatment strategies. Figure 3 shows schematic drawings of the CiTE molecule and the two control antibodies: single-chain triplebody (sctb) and BiTE-like.
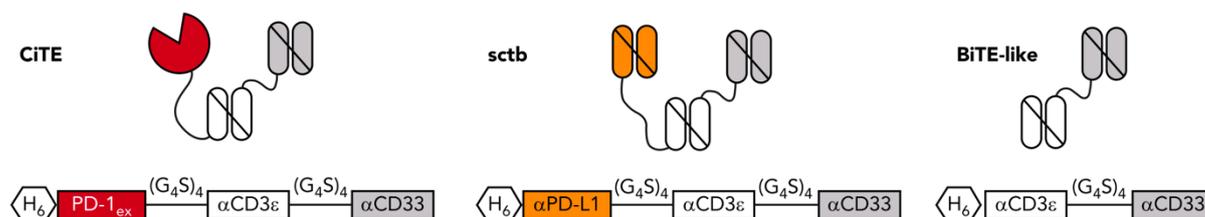


Figure 3: Schematic drawing of CiTE, sctb and BiTE-like molecules

The CiTE molecule consists of a PD-1 extracellular domain with low affinity to PD-1 fused to an $\alpha$CD3x$\alpha$CD33 BiTE-like molecule. In the sctb antibody, the extracellular domain of PD-1 was exchanged with a high affinity $\alpha$PD-L1 scFv.

In a first step, we performed binding studies which show similar binding of the CiTE and sctb molecule to CD33+PD-L1+ AML cell lines and HD T cells. As expected, the $\alpha$PD-L1 scFv showed a higher binding to PD-L1 compared to the PD-1ex domain.

In a next step, we performed T-cell activation assays. HD T cells upregulate the activation markers CD69 and CD25 in presence of PD-L1+-MOLM-13 cells and CiTE, sctb or BiTE-like antibody constructs. In addition, T-cells in coculture with PD-L1+-MOLM-13 cells and CiTE or sctb showed significantly increased secretion of the proinflammatory cytokine IFNγ, and granzyme B compared to T cells in coculture with PD-L1+-MOLM-13 cells and the BiTE-like molecule. In all conditions, T-cell activation was accompanied by PD-1 upregulation.

Cytotoxicity experiments with MOLM-13 or OCI-AML3 cells revealed strong lysis mediated by HD T cells in presence of CiTE and sctb, which could be significantly increased by genetically modi-fying the target cells to express PD-L1.

## Summary of publications

Cytotoxicity experiments with PD-L1$^+$-MOLM-13 and CD33$^+$/PD-L1$^+$-BaF3-cells confirmed the results. Increased target cell lysis was accompanied by increased T-cell proliferation. Mixed target cell assays with CD33$^+$/PD-L1$^+$ and CD33$^-$/PD-L1$^+$ cells showed dose dependent elimination of both cell lines for the sctb and preferential lysis of CD33$^+$/PD-L1$^+$ cells for the CiTE molecule. This demonstrates that the low affinity PD-L1ex domain is not able to redirect T cells to CD33$^-$/PD-L1$^+$ cells.

In the next step, the molecules were evaluated in *ex-vivo* cytotoxicity assays with HD T cells and primary AML cells. The CiTE antibody was able to increase lysis of primary AML cells in 7 of 8 patients compared to the BiTE-like molecule. The sctb showed similar or higher lysis compared to the BiTE-like molecule in all 8 patients. Both molecules led to PD-L1 upregulation on primary AML cells and increased IFN$\gamma$ secretion compared to the BiTE-like molecule. This effect was stronger with the sctb molecule.

To validate the *ex-vivo* findings *in-vivo* we performed mouse studies. PD-L1$^+$-MOLM-13 cells were injected into NOD/SCID mice. After engraftment, *in-vitro* activated HD T cells and antibody constructs were transferred. After 9 days complete eradication of AML cells was seen with the CiTE, sctb and BiTE-like molecules. As human and mouse PD-L1 are cross-reactive, we were able to analyze the potential targeting of non-AML cells. Mice treated with the sctb lost body weight compared to the other groups. This was accompanied by an upregulation of PD-1 on the human CD4$^+$ and CD4$^-$ T cells in BM and spleen. This effect is most likely due to the redirection of T cells to PD-L1$^+$ murine cells by the sctb and is accompanied by irAEs causing weight loss. We conclude that the CiTE antibody effectively targets CD33$^+$ cells without systemic PD-L1 targeting and thereby reducing irAEs and counteracting immune escape based on PD-L1 upregulation.

I performed and analyzed the *ex-vivo* experiments with primary AML cells.

# 2 Publications

## 2.1 Publication I

MOLECULAR CANCER THERAPEUTICS | LARGE MOLECULE THERAPEUTICS

# Characterization of a Novel FLT3 BiTE Molecule for the Treatment of Acute Myeloid Leukemia A C

Bettina Brauchle[1,2], Rebecca L. Goldstein[3], Christine M. Karbowski[4], Anja Henn[5], Chi-Ming Li[3], Veit L. Bücklein[1,2], Christina Krupka[1,2], Michael C. Boyle[4], Priya Koppikar[4], Sascha Haubner[1,2], Joachim Wahl[5], Christoph Dahlhoff[5], Tobias Raum[5], Matthew J. Rardin[3], Christine Sastri[3], Dan A. Rock[3], Michael von Bergwelt-Baildon[1,2,6], Brendon Frank[3], Klaus H. Metzeler[2,6], Ryan Case[3], Matthias Friedrich[5], Mercedesz Balazs[3], Karsten Spiekermann[2,6,7], Angela Coxon[5], Marion Subklewe[1,2,6], and Tara Arvedson[3]

## ABSTRACT

Despite advances in the treatment of acute myeloid leukemia (AML), novel therapies are needed to induce deeper and more durable clinical response. Bispecific T-cell Engager (BiTE) molecules, which redirect patient T cells to lyse tumor cells, are a clinically validated modality for hematologic malignancies. Due to broad AML expression and limited normal tissue expression, fms-related tyrosine kinase 3 (FLT3) is proposed to be an optimal BiTE molecule target. Expression profiling of FLT3 was performed in primary AML patient samples and normal hematopoietic cells and nonhematopoietic tissues. Two novel FLT3 BiTE molecules, one with a half-life extending (HLE) Fc moiety and one without, were assessed for T-cell–dependent cellular cytotoxicity (TDCC) of FLT3-positive cell lines *in vitro, in vivo*, and *ex vivo*. FLT3 protein

was detected on the surface of most primary AML bulk and leukemic stem cells but only a fraction of normal hematopoietic stem and progenitor cells. FLT3 protein detected in nonhematopoietic cells was cytoplasmic. FLT3 BiTE molecules induced TDCC of FLT3-positive cells *in vitro*, reduced tumor growth and increased survival in AML mouse models *in vivo*. Both molecules exhibited reproducible pharmacokinetic and pharmacodynamic profiles in cynomolgus monkeys *in vivo*, including elimination of FLT3-positive cells in blood and bone marrow. In *ex vivo* cultures of primary AML samples, patient T cells induced TDCC of FLT3-positive target cells. Combination with PD-1 blockade increased BiTE activity. These data support the clinical development of an FLT3 targeting BiTE molecule for the treatment of AML.

## Introduction

Newly approved targeted therapies and cytotoxic agents (1) provide opportunities to improve treatment of acute myeloid leukemia (AML), a disease characterized by low survival rates (2). However, these therapeutics are approved for only certain patient subsets, and treatments to benefit broad patient populations are still needed. To date, the most efficacious treatment consists of intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation (HSCT;

refs. 3, 4). The potent antileukemic effect of HSCT is driven by recognition and elimination of allogeneic antigens on chemoresistant leukemic cells by donor T cells. HSCT, as well as donor lymphocyte infusions, which frequently result in durable complete remissions (3), demonstrate the potential for therapies driven by T-cell cytotoxicity (4). However, this regimen may not be an option for all patients due to comorbidities and the high morbidity and mortality rates associated with graft-versus-host disease, highlighting the urgent need for novel therapies (4).

A promising T-cell–based therapeutic approach is to redirect a patient's own T cells to eliminate leukemic cells. This strategy can be accomplished with bispecific T-cell engaging (BiTE) molecules. BiTE molecules consist of a single chain variable fragment (scFv) against a cell surface-expressed tumor-associated antigen (TAA) linked to an scFv against the T-cell coreceptor CD3. Clinical proof of concept for this modality was demonstrated by the CD19-directed BiTE molecule blinatumomab, which is approved for B-cell precursor acute lymphoblastic leukemia. CD19 is an ideal target for a BiTE molecule because it is broadly expressed on B-cell malignancies, its off-tumor expression is limited to normal B cells, and patients can tolerate prolonged B-cell depletion. The successful translation of BiTE molecules to AML therapy requires identification of a suitable cell surface antigen, one that is broadly and selectively expressed by leukemic cells with limited expression on normal tissues.

fms-Related tyrosine kinase 3 (FLT3, CD135) is a lineage-associated growth factor that was previously reported to be expressed on AML blasts and LSCs (5, 6). Expression of FLT3 on normal hematopoietic cells has been reported to be restricted to a subset of hematopoietic stem and progenitor cells (HSPC) in the bone marrow (BM; ref. 7). These data suggest a favorable expression profile for targeting FLT3

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with a BiTE molecule. Mutations in the intracellular portion of FLT3, resulting in constitutive activation, occurring as either internal tandem duplication or point mutations in the tyrosine kinase domain have been identified in approximately 25% or 7% to 10% of patients with AML, respectively. (8–10). Tyrosine kinase inhibitors (TKIs) that target the FLT3 kinase domain were recently approved for patients with mutant *FLT3* and others are undergoing clinical evaluation (11–13). FLT3 TKIs are active primarily in the setting of mutant *FLT3,* whereas BiTE molecules recognize an extracellular protein epitope and bind FLT3 regardless of mutational status.

Here, FLT3 was evaluated as a target for BiTE molecule therapy for the treatment of AML, including expression analysis on disease and normal cells, and two novel FLT3 BiTE molecules were characterized *in vitro, ex vivo,* and *in vivo.* Cell surface FLT3 protein expression was observed on most primary AML (pAML) patient bulk and LSC samples, irrespective of *FLT3* mutational status. Importantly, comparable FLT3 protein expression was observed on patient samples collected at the time of both initial diagnosis and relapse, suggesting a FLT3 BiTE molecule could provide benefit to patients across multiple lines of therapy. FLT3 transcript and protein expression was rigorously evaluated in a panel of normal human tissues, and cell surface FLT3 protein was detected only on a portion of HSPCs and on rare, scattered cells in the tonsil. FLT3 protein was also detected in some non-hematopoietic tissues, including cerebellum and pancreas; however, extensive characterization revealed that the protein was cytoplasmic. Because FLT3 BiTE molecules selectively bind to cells expressing cell surface FLT3, cells expressing cytoplasmic FLT3 protein would not be expected to be depleted.

Two FLT3 BiTE molecules were generated and evaluated: an experimental FLT3 BiTE molecule comprised an anti-CD3 scFv and an anti-FLT3 scFv, and a FLT3 half-life extended (HLE) BiTE molecule (AMG 427) comprised an anti-CD3 scFv fused to an Fc moiety and a unique anti-FLT3 scFv. Because of the size of the experimental FLT3 BiTE molecule, rapid clearance by glomerular filtration is expected to result in a short serum half-life, requiring continuous intravenous (cIV) infusion to maintain an active concentration *in vivo.* The larger AMG 427 was designed to have an extended serum half-life relative to the experimental FLT3 BiTE molecule. Both BiTE molecules induced potent and target-specific T-cell-dependent cellular cytotoxicity (TDCC) against AML cell lines *in vitro,* inhibited tumor growth and provided a survival advantage *in vivo* in xenograft models and exhibited reproducible pharmacokinetic (PK) and pharmacodynamic (PD) profiles in cynomolgus monkeys. The experimental FLT3 BiTE molecule induced TDCC of pAML samples *ex vivo.* Increased *in vitro* TDCC was observed by combining AMG 427 with an anti-PD-1 antibody. These data demonstrate that FLT3 BiTE molecules are capable of inducing TDCC of FLT3-expressing cells *in vitro, in vivo,* and *ex vivo;* moreover, although each FLT3 BiTE molecule was efficacious as a single agent against AML cell lines and pAML samples, combination therapy may provide additional benefit for some patients. AMG 427 is being evaluated patients with relapsed or refractory AML.

## Materials and Methods

### Patient and healthy donor samples

AML and healthy donor (HD) samples were obtained with written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilian University (Supplementary Tables S1 and S2). Human tissue specimens for expression analyses were collected under Institutional Review Board approval with appropriate informed consent. In all cases, materials obtained were surplus to standard clinical practice. Patient identity and protected health information/identifying information were redacted from tissue data and clinical data.

### Key resources

Sources of biological samples, all antibodies and other key reagents are listed in Supplementary Table S1.

### FLT3 protein expression on pAML and HD hematopoietic cells

Cell surface FLT3 protein expression on pAML and HD peripheral blood (PB) or BM samples was assessed by flow cytometry (Navios; Beckman Coulter) using an anti-FLT3 antibody (Supplementary Table S1). Mean fluorescence intensity (MFI) was determined (FlowJo version 10.3) and the MFI ratio (MFI sample/MFI isotype control) was calculated.

### FLT3 transcript expression in AML patient cells and HD nonhematopoietic tissues

*FLT3* transcript expression data were retrieved from The Cancer Genome Atlas [TCGA (14), AML patient samples] in February 2018.

*FLT3* transcript expression data in normal human tissues were retrieved from the Genotype-Tissue Expression project [GTEx (15), HD samples] in April 2018.

5' rapid amplification of cDNA ends (RACE), digital droplet polymerase chain reaction (ddPCR), reverse transcription PCR, immunohistochemistry (IHC), Western analysis, immunoprecipitation, and RNA-seq were conducted using standard techniques. Details in Supplementary Table S1 and Supplementary Materials and Methods.

### AML cell lines: cytotoxicity, T-cell activation, cytokine secretion

Cell lines were initially sourced from DSMZ (MOLM-13, EOL-1, PL-21), ATCC (HL-60, MV4-11 K562, HEL92.1), and ECACC (A2780), and cultured using standard techniques and reagents. In the absence of phenotypic or growth changes, cells were not authenticated or tested for mycoplasma. Cells were used within 2 months of thawing.

Human PBMCs or pan T cells were cultured for 48 hours in the presence or absence of FLT3 expression–positive or FLT3 expression–negative target cells with an effector-to-target (E:T) cell ratio of 10:1 (pan T) or 5:1 (PBMC) and a dose range of FLT3 BiTE molecules. Target cell lysis was measured by loss of luciferase signal (Steady-Glo, Promega; labeled target cell lines express luciferase); or propidium iodide uptake by flow cytometry. T-cell activation markers were assessed by flow cytometry using antibodies against CD4, CD8, CD69, and CD25 labeled with a fluorochrome conjugate (Supplementary Table S1). BiTE-induced cytokine secretion was measured in supernatants using the BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit. Luciferase-based TDCC (pan T, E:T ratio 10:1) was performed with or without 10 ng/mL soluble FLT3 ligand (16) for 48 hours.

### Mouse xenograft models

Animal experimental procedures were conducted in accordance with the German Animal Welfare Law with permission from the responsible local authorities and within the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international standards.

Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice at age 7 weeks were sublethally irradiated prior to tumor cell injection. Mice were injected intravenously via lateral tail vein with $(10^7)$ MOLM-13_Luc or $(5 \times 10^6)$ EOL-1 cells on day 1. After 48 or 72 hours, respectively, mice were injected intraperitoneally with human in vitro–expanded $CD3^+$ T cells (2 or $1.2 \times 10^7$, respectively) and allocated to treatment groups ($n = 10$/group). Five mice allocated to the vehicle group did not receive human T cells. Mice were treated with vehicle or AMG 427 (3, 0.6, or 0.12 mg/kg) every 5 days by intravenous bolus injection into the lateral tail vein starting on day 7, then on days 12, 17, 22, 27, and 34 (MOLM-13_Luc). Mice were treated with vehicle or AMG 427 (1, 0.1, or 0.001 mg/kg) every 7 days by intravenous bolus injection into the lateral tail vein starting on day 9 for a total of six administrations (EOL-1). To block binding of AMG 427 to Fc receptors, a mixture of anti-muFcRII (2.4G2) antibody (8 mg/kg) and human normal immunoglobulin (400 mg/kg of Kiovig) was administered once weekly intraperitoneally throughout the treatment period, starting 1 day prior to the first AMG 427 dose. Mice were monitored daily. PK serum concentrations of AMG 427 were determined by electrochemiluminescence immunoassay (Supplementary Materials and Methods).

### Cynomolgus monkey studies

Cynomolgus monkeys were cared for in accordance to the Guide for the Care and Use of Laboratory Animals, Eighth Edition (17). Animal care is detailed in Supplementary Materials and Methods.

Cell surface FLT3 protein expression on hematopoietic cells from cynomolgus monkeys was assessed as described in the Supplementary Materials and Methods.

The experimental FLT3 BiTE molecule was evaluated in a 16-day cIV study ($n = 3$) and was administered at step doses increasing every 3 days, intended to achieve $C_{ss}$ 0.05, 0.2, 0.5, and 2 nmol/L for 3 days each. Methods for PK and PD assessment are detailed in Supplementary Materials and Methods. AMG 427 was evaluated in an 8-day, repeated-dose study with three dose levels ($n = 3$/group). AMG 427 was administered intravenously on days 1, 2, and 5.

### Cytotoxicity against pAML cells

AML patient samples were cultured (Supplementary Materials and Methods) with experimental FLT3 BiTE molecule or control BiTE molecule at 5 ng/mL (92 pmol/L) and replenished at 3-day intervals. Viable $CD33^+$/$CD2^-$ cells (Supplementary Table S1) were determined by flow cytometry, and total cell count was used to determine AML cell count.

### Combination with PD-1–blocking antibody

Human pan T cells were stimulated 1:1 with CD3/CD28 Dynabeads (Thermo Fisher Scientific) for 48 hours, then cocultured 1:1 with PD-L1–transfected MOLM-13 cells (MOLM-13_PD-L1) and dose range of AMG 427 in the absence or presence of 10 μg of a PD-1–blocking antibody (Supplementary Table S1). After 24 hours, MOLM-13_PD-L1 cell viability was determined by TO-PRO-3 uptake by flow cytometry.

## Results

### AML patient sample cell surface FLT3 protein expression

Leukemic bulk cells from BM or PB from 318 newly diagnosed or relapsed AML patients were evaluated for cell surface FLT3 protein expression. Of the analyzed samples, 78% (248/318) were positive for FLT3 protein expression (MFI ratio >1.5; **Fig. 1A**, gating strategy,

Supplementary Fig. S1A, top; MFI ratio calculation, Supplementary Fig. S1B). Interpatient heterogeneity in FLT3 protein expression was observed (MFI ratio range 0.1–32.7; **Fig. 1A**), similar to what has been reported for other AML-associated antigens (18). The FLT3 protein expression profile was similar, regardless of FLT3-ITD mutational status (**Fig. 1B**), time of sample collection (initial diagnosis versus relapse, **Fig. 1C**), or FLT3-ITD allelic ratio (**Fig. 1D**). Cell surface FLT3 protein expression was detected on leukemic stem cells (LSC; $CD34^+$/$CD38^-$) in 79% (122/155) of AML patient samples (**Fig. 1E**). As observed for bulk cells, FLT3 protein expression on LSCs was similar, regardless of FLT3-ITD mutational status (**Fig. 1F**) or initial diagnosis versus relapse (**Fig. 1G**). Higher FLT3 expression was detected on samples with high FLT3-ITD allelic ratio (**Fig. 1H**, $P < 0.0098$).

No clear correlation was observed in an analysis of FLT3 protein expression intensity on AML patient bulk cells at initial diagnosis with different disease characteristics, including French American British group, core binding factor abnormalities [i.e., translocation t(8;21) and inversion inv(16)], nucleophosmin 1 (NPM1) and FLT3-ITD mutations, Medical Research Council cytogenetic-based risk classification (19), and 2010 European Leukemia Net classification (Supplementary Fig. S1C–S1F; Supplementary Table S2; ref. 20).

### FLT3 transcript and protein expression in normal human hematopoietic cells

In hematopoietic cell samples derived from HDs, the FLT3 protein MFI ratio was consistently low and less than that of pAML samples. The MFI ratio on HD $CD34^+CD38^-$ cells ($n = 18$), comprising hematopoietic stem cells and multipotent progenitors, was 0.58 ± 0.26 and the MFI ratio on $CD34^+CD38^+$ cells ($n = 36$), comprising the oligopotent progenitors, was 1.6 ± 0.5 (**Fig. 1I**; gating strategy Supplementary Fig. S1A, bottom; MFI ratio calculation, Supplementary Fig. S1B). In comparison, the MFI ratio for pAML samples was significantly higher than either of the HD samples ($P < 0.0001$ comparison to either HD $CD34^+CD38^-$ cells or HD $CD34^+CD38^+$ cells) at 3.6 ± 3.6 for the bulk samples ($n = 318$) and 2.9 ± 2.3 for the LSC samples ($n = 155$). Paired analysis of $CD34^+CD38^-$ and $CD34^+CD38^+$ cells from 13 HDs showed that FLT3 protein expression was statistically lower on $CD34^+CD38^-$ than $CD34^+CD38^+$ cells (**Fig. 1J**).

FLT3 protein expression was subsequently evaluated on individual stem cell and oligopotent progenitor subsets from two HD. Expression was variable, and no subset was uniformly positive or negative (**Fig. 1K**, gating strategy; Supplementary Fig. S2A). The rank order of FLT3 protein expression was: granulocyte/macrophage progenitor > common lymphoid progenitor > common myeloid progenitor > HSC and MPP > megakaryocyte/erythrocyte progenitor (MEP), most of the latter population falling below detection.

On mature hematopoietic cells isolated from blood from two HD, there was no detectable cell surface FLT3 protein on T or B lymphocytes, natural killer cells, plasmacytoid or conventional dendritic cells, monocytes, or neutrophils (Supplementary Fig. S2B). Collectively, these data demonstrate that there are differences in expression between disease and normal cells, and within hematopoietic stem and progenitor populations, which may translate to differences in susceptibility to FLT3 BiTE-mediated killing.

### FLT3 transcript and protein expression in normal human nonhematopoietic tissues

The presence of FLT3 transcript in nonhematopoietic tissues was assessed in three different datasets including GTEx RNA-seq database (15), an Amgen-constructed RNA-seq database, and XpressWay
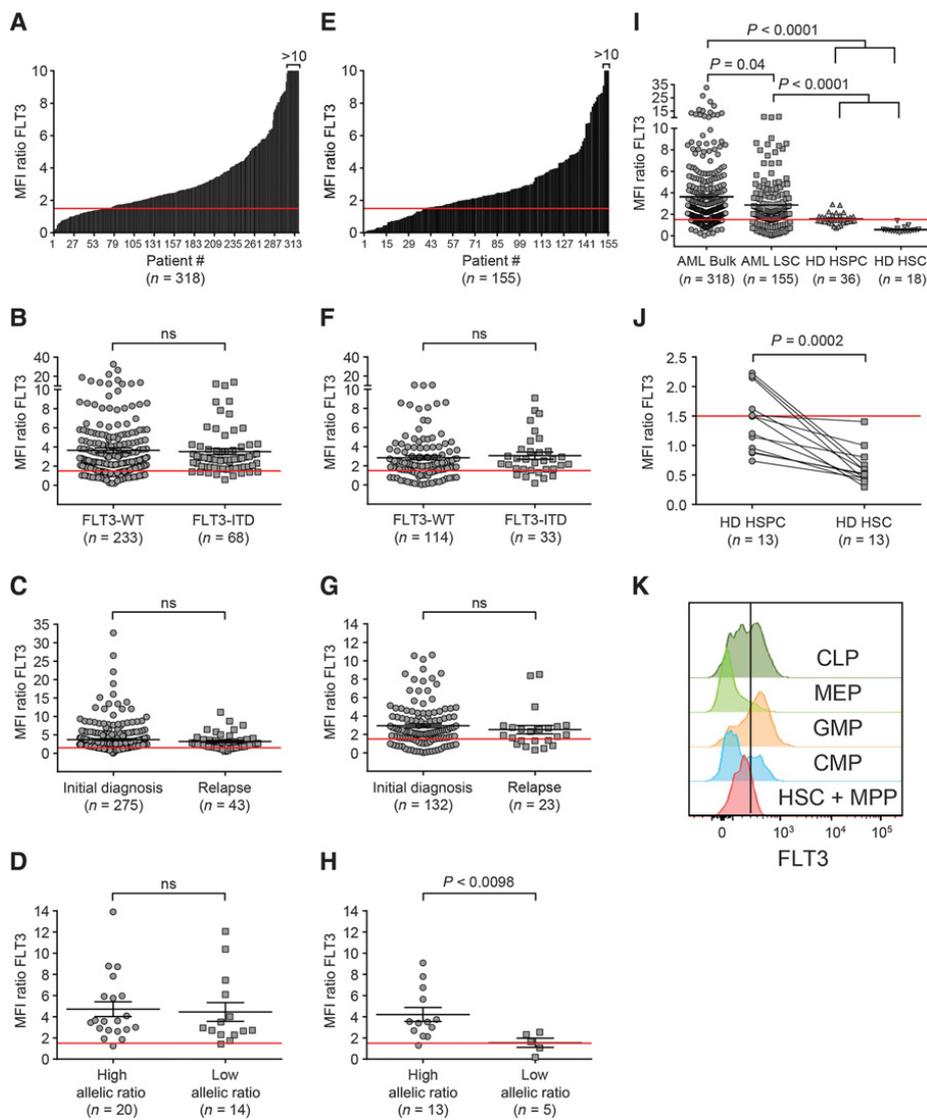
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**Figure 1.**
Cell surface FLT3 protein expression on AML patient bulk cells (**A–D**), AML LSCs (**E–H**), and HD cells (**I–K**). Red line indicates FLT3 positivity (MFI ratio $\geq 1.5$). **A,** FLT3 protein expression on pAML bulk cells (CD45$^{DIM}$/SSC$^{LOW}$) at initial diagnosis or relapse ($n = 318$). Comparison of FLT3 protein expression on (**B**) AML bulk cells expressing wild-type *FLT3* ($n = 233$) vs. mutant *FLT3* (ITD mutation; $n = 68$; $P = 0.22$), (**C**) AML bulk cells expressing high ($\geq 0.5$) *FLT3-ITD* allelic ratio ($n = 20$) vs. low (<0.5) *FLT3-ITD* allelic ratio ($n = 14$; $P = 0.655$), or samples collected at (**D**) initial diagnosis ($n = 275$) vs. relapse ($n = 43$; $P = 0.99$). **E,** FLT3 protein expression on pAML CD34$^+$/CD38$^-$ LSCs at initial diagnosis or relapse ($n = 155$). Comparison of FLT3 expression on AML LSCs of patients with (**F**) wild-type *FLT3* ($n = 114$) vs. mutant *FLT3* (ITD mutation; $n = 33$; $P = 0.43$), (**G**) LSCs expressing high ($\geq 0.5$) *FLT3* allelic ratio ($n = 13$) vs. low (<0.5) *FLT3* allelic ratio ($n = 5$; $P = 0.0098$), or samples collected at the time of (**H**) initial diagnosis ($n = 132$) vs. relapse ($n = 23$; $P = 0.37$). **I,** Surface FLT3 expression on HD CD34$^+$CD38$^+$ ($n = 36$) vs. patient leukemic bulk cells ($n = 318$; $P < 0.0001$) and HD CD34$^+$CD38$^-$ ($n = 18$) vs. patient LSCs ($n = 155$; $P < 0.0001$). **A–I,** Mann–Whitney $U$ test (mean $\pm$ SEM); ns, not significant. **J,** Paired analysis of FLT3 expression on HD samples ($n = 13$; $P = 0.0002$; Wilcoxon matched-pairs signed rank test). **K,** Analysis of FLT3 protein expression on progenitor subsets. Black vertical line represents the FLT3 protein expression cutoff determined from an unstained negative control.
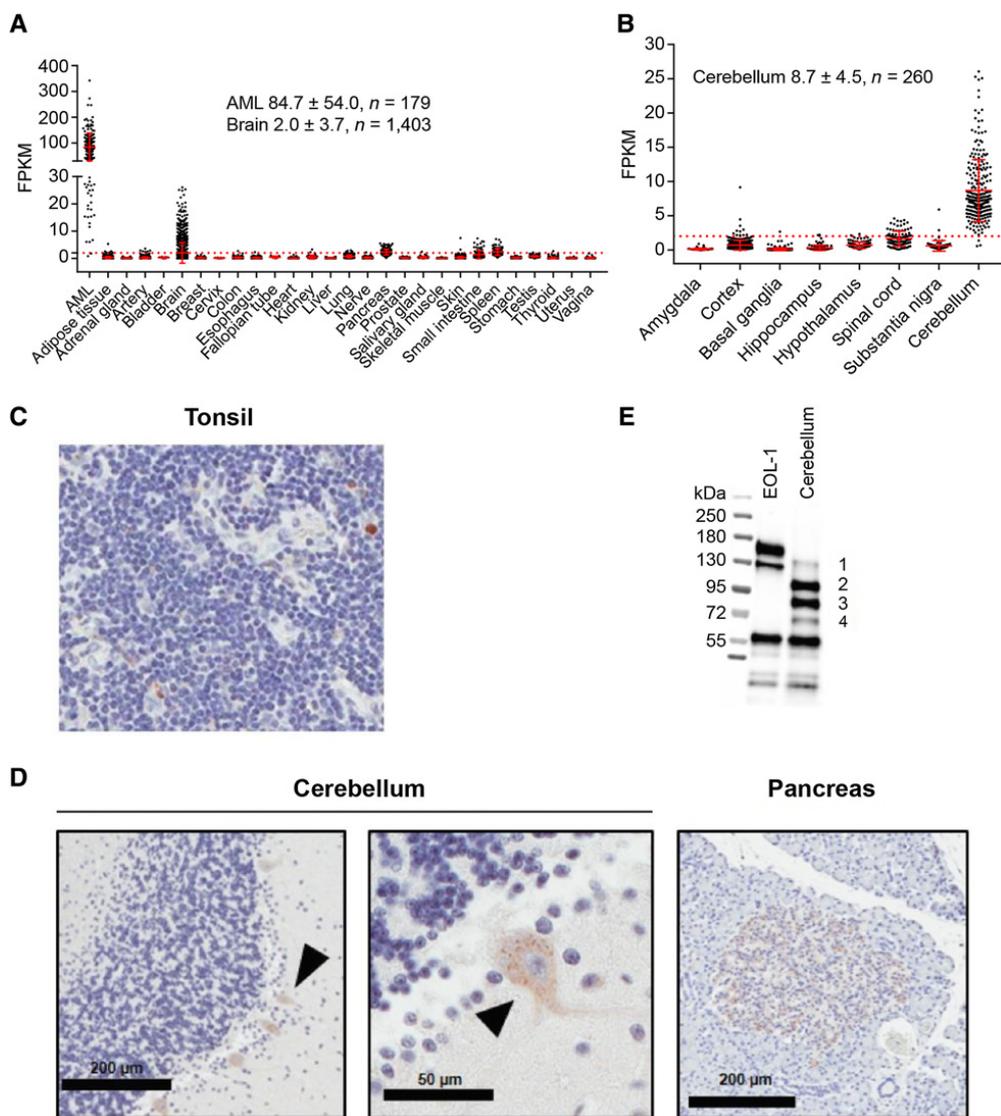
Novel FLT3 BiTE Molecule for AML Treatment



**Figure 2.**
*FLT3* transcript expression in normal human solid tissues. *FLT3* transcript expression in AML and normal solid tissue (**A**) and nervous system regions (**B**) shown as fragments per kilobase of transcript per million mapped reads (FPKM). Data are represented as mean ± SD for the indicated tissues. IHC of FLT3 protein in human tonsil (**C**), cerebellum, and pancreas (**D**). Low (left) and high (middle) magnification cerebellum and low magnification pancreas (right) images demonstrate punctate cytoplasmic immunostaining and lack of membranous staining. **E,** Immunoblot of FLT3 protein immunoprecipitated from EOL-1 and human cerebellum protein lysates. Bands between 130 and 180 kDa for EOL-1 and numbered bands shown in the gel for cerebellum were isolated and analyzed by mass spectrometry (see Supplementary Fig. S3D; Supplementary Table S11).

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Profile Report (Asterand UK Acquisition Limited). Low levels of *FLT3* transcript were detected in brain, nerve/ganglia, small intestine, kidney, lung, pancreas, spleen, spinal cord, and testis (**Fig. 2A**; Supplementary Table S3). Within the brain, *FLT3* transcripts localized to the cerebellum (**Fig. 2B**). Although *FLT3* transcript was not consistently detected in all tissues listed above, all tissue types identified as transcript-positive in any dataset were subsequently evaluated for FLT3 protein expression by IHC (spinal cord being the only exception). Of these tissues, the only example of cell surface-localized FLT3 protein was on rare, scattered cells in the tonsil (**Fig. 2C**). In all other tissues evaluated, including brain stem, cerebrum, cerebellum, kidney, pancreas, pituitary, prostate, skeletal muscle, stomach, testis, and thyroid, FLT3 protein staining was cytoplasmic (**Fig. 2D**). Within the cerebrum and cerebellum, FLT3 protein staining consisted of cytoplasmic staining of multifocal neurons, and this staining pattern was consistent in multiple sections of brain, with no membranous staining observed in neurons. Diffuse cytoplasmic staining was observed in alveolar macrophages, indicating the likely source of the transcript signal in lung (Supplementary Table S3). Taken together, these data suggest that although FLT3 transcript and protein are present in peripheral tissues, including the brain, FLT3 protein is cytoplasmic and therefore not anticipated to be targeted by an anti-FLT3 BiTE molecule.

Additional analysis of *FLT3* transcript and FLT3 protein expression in the cerebellum revealed that the majority of *FLT3* transcripts isolated from the cerebellum were shorter than those isolated from a control AML cell line. Transcript sequencing revealed these truncations were due to frequent intron insertion/retention or exon skipping. Quantification of alternatively-spliced *FLT3* transcripts using digital droplet PCR (ddPCR) indicated that in this study at least 70% to 85% of cerebellum *FLT3* transcripts lacked exonic regions or retained intronic sequences, suggesting that only a small portion of *FLT3* transcripts in cerebellum samples analyzed would be intact (Supplementary Fig. S3). Assessment of FLT3 protein from human cerebellum lysate by immunoprecipitation-western analysis identified only FLT3 protein bands that were lower in molecular weight than full-length FLT3 protein from a positive control AML cell line lysate (**Fig. 2E**). FLT3 protein bands from a cerebellum sample were characterized by mass spectrometry, revealing only peptides from the extracellular domain of FLT3; by contrast, bands from the control AML cell line lysate contained multiple peptides from both the intracellular and extracellular regions of FLT3 (Supplementary Fig. S3D; Supplementary Table S11). In sum, the transcript and peptide data suggest that most transcripts from the cerebellum encode FLT3 peptides that are not full-length and may explain why FLT3 is not detectable on the cell surface of cells in the cerebellum.

### FLT3 BiTE molecules induced TDCC of FLT3-expressing AML cell lines

Two different FLT3 BiTE molecules (Supplementary Fig. S4A) were evaluated. Each BiTE molecule comprised a distinct anti-FLT3 scFv that bound FLT3 within a 51 amino acid region, associated with an anti-CD3 scFv. The compact size of BiTE molecules (MW ∼55 kDa) has been reported to be important for the generation of a productive immunologic synapse (21); however, proteins this size are generally rapidly eliminated by the kidneys. To increase the serum half-life, an Fc moiety was added to produce AMG 427. To ensure that the presence of the Fc would not impact *in vitro* or *in vivo* activity, the two BiTE molecules were evaluated in similar assay panels. Both molecules bound human FLT3 and CD3 protein with sub- or single-digit nanomolar affinities (Supplementary Table S5). A panel of cell lines exhibiting a range of FLT3 protein expression (MFI ratio: 2.6–23.8;

Supplementary Fig. S4B) similar to that observed on pAML samples (**Fig. 1A**) was selected to evaluate FLT3 BiTE molecule *in vitro* potency. Both molecules similarly induced TDCC against five FLT3 protein-expressing cell lines with single digit picomolar potency (**Fig. 3A**; Supplementary Table S6). A relationship between FLT3 expression level and potency was not apparent, likely due to the high E:T ratio. TDCC was similar for both BiTE molecules in cell lines homozygous or heterozygous for wild type (wt) or ITD mutant (mut) *FLT3*, and selectivity was demonstrated as cell lines lacking FLT3 protein expression were not lysed (**Fig. 3A**; Supplementary Table S6). TDCC was accompanied by upregulation of the T-cell activation markers CD69 and CD25 and secretion of T-cell-derived effector cytokines IFNγ and TNFα in the presence of FLT3 protein-expressing cells, but not in the presence of FLT3 protein-negative cells (**Fig. 3B–E**; Supplementary Table S6).

Soluble FLT3 (sFLT3) can be detected in AML patient serum at concentrations up to 141 ng/mL (22). In TDCC assays, clinically relevant concentrations of sFLT3 reduced AMG 427 potency 6-44-fold, but maximum killing was still achieved (**Fig. 3F**). Soluble FLT3 ligand (sFLT3L) can be detected in AML patient serum at concentrations up to 9 ng/mL (23). Although neither the experimental FLT3 BiTE molecule nor AMG 427 binds the ligand-binding domain of FLT3, sFLT3L binding to FLT3 induces internalization of FLT3 (24), and could alter BiTE-mediated TDCC. In the presence of 10 ng/mL sFLT3L, the potency of AMG 427-mediated TDCC was reduced two-to six-fold (**Fig. 3F**); however, maximum killing was still achieved in all three cell lines tested. These data demonstrate that FLT3 BiTE molecules induce target-specific TDCC equivalently, and that complete killing occurs in the presence of disease-relevant concentrations of sFLT3 and sFLT3L.

### Experimental FLT3 BiTE molecule and AMG 427 inhibited tumor growth and increased survival in mouse xenograft models

Both the experimental FLT3 BiTE molecule and AMG 427 were evaluated in mouse tumor models. As neither BiTE molecule bound mouse Flt3, immunocompromised mice administered with human tumor cells and T cells were used. The experimental FLT3 BiTE molecule was evaluated in an admix model in which athymic nude mice were injected with MOLM-13 AML cells and *in vitro*-expanded human CD3+ T cells in Matrigel. Animals were dosed intraperitoneally with experimental FLT3 BiTE molecule or control BiTE molecule daily for 10 days. Tumor growth was inhibited by 90% in mice treated with the experimental FLT3 BiTE molecule relative to the control BiTE molecule (*n* = 10, *P* < 0.0001; Supplementary Fig. S5).

AMG 427 was evaluated in two orthotopic mouse xenograft models in which either EOL-1 or MOLM-13 AML cells were injected on day 1 and after 72 or 48 hours (EOL-1 and MOLM-13, respectively), mice were injected with *in vitro*-expanded human CD3+ T cells. Mice were treated with vehicle or AMG 427 every 7 days starting on day 9 (EOL-1) or every 5 days starting on day 7 (MOLM-13). In the EOL-1 model, all animals from the control groups developed leukemic disease and were euthanized between days 27 and 52 following AML cell injection with median survival of 36 and 37 days (**Fig. 4A**). Weekly treatment with AMG 427 prolonged survival at all doses tested, with 17 of 30 animals surviving until study end on day 108. As ≥50% of animals were alive at study end, the median survival could not be calculated; however, compared with vehicle, AMG 427 significantly extended survival (*n* = 10, *P* < 0.001; **Fig. 4A**). In the more aggressive MOLM-13 model, all mice in the control groups died within 20 days after injection of AML cells, with median survival of 18 days.
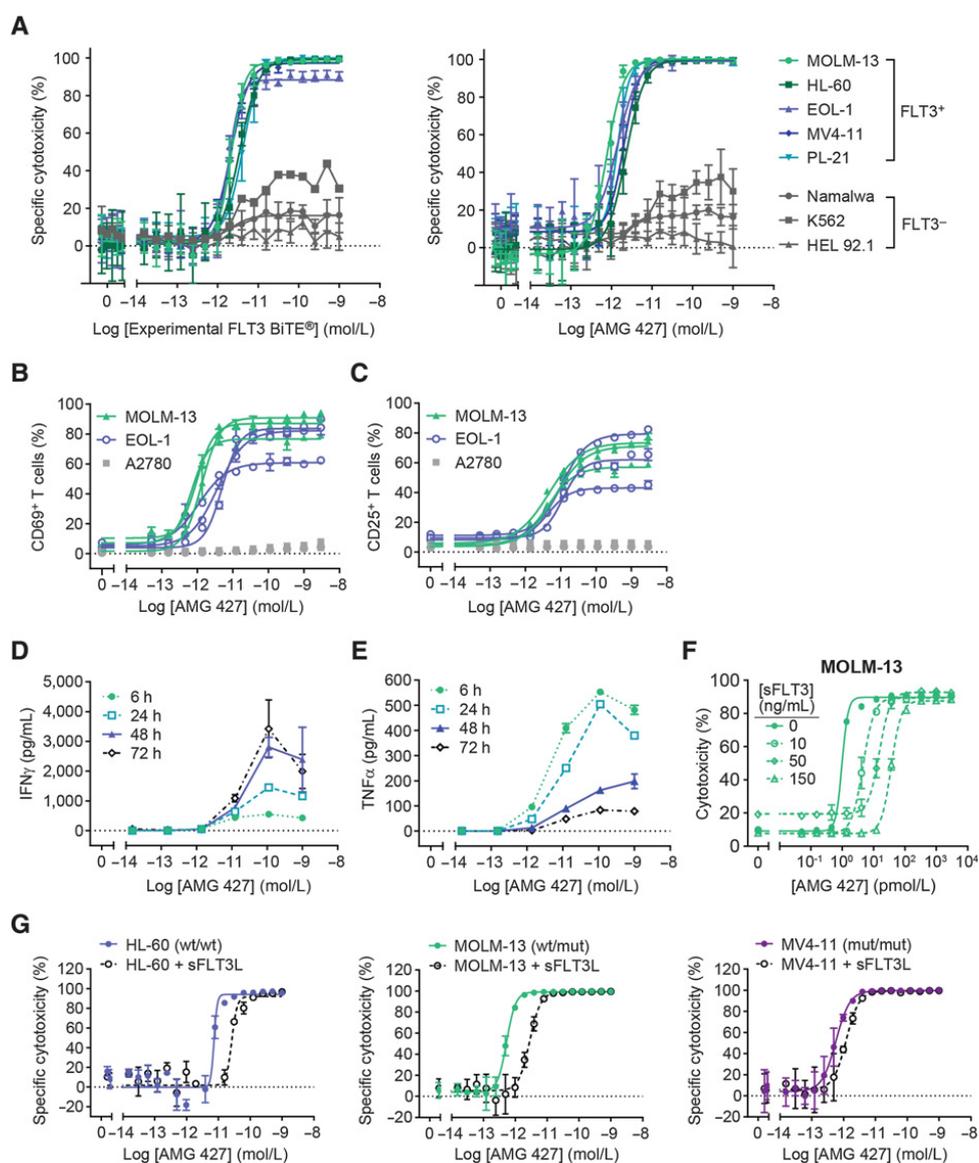
**Novel FLT3 BiTE Molecule for AML Treatment**



**Figure 3.**
FLT3 BiTE molecules have potent cytotoxic activity against FLT3-expressing AML cell lines. **A**, Specific cytotoxicity of FLT3 BiTE molecules (experimental FLT3 BiTE molecule, left; AMG 427, right) in TDCC assay with FLT3-positive and FLT3-negative cell lines cultured at a 1:10 ratio with human pan T cells for 48 hours (mean ± SD, $n = 6$ technical replicates, representative curves for 1 of ≥3 T-cell donors). Expression of CD69 (**B**) and CD25 (**C**) on T cells from TDCC assays of FLT3-positive cell lines MOLM-13 and EOL-1 or FLT3-negative cell line A2780 co-cultured with human PBMCs at a 1:5 ratio with AMG 427 for 48 hours (mean ± SD, $n = 2$ technical replicates; each curve represents a different PBMC donor). Concentration of IFNγ (**D**) and TNFα (**E**) in supernatants of TDCC assay of human PBMCs and EOL-1 cells (5:1 ratio) at time points indicated (mean ± SD, $n = 3$ technical replicates of one representative donor). **F,** TDCC of AMG 427 in the presence of sFLT3 (mean ± SD, $n = 2$ technical replicates, one representative donor of 2). **G,** Specific cytotoxicity of AMG 427 in the presence or absence of 10 ng/mL sFLT3L (mean ± SD, $n = 3$ technical replicates).

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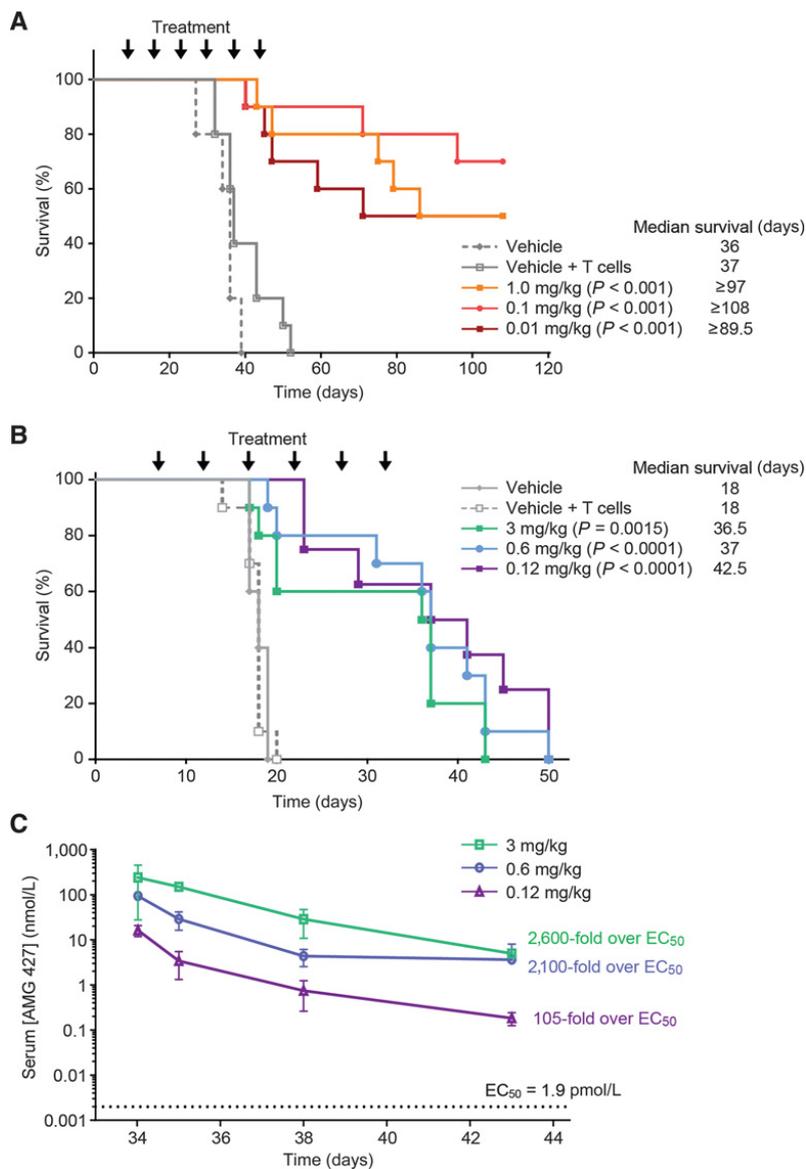


**Figure 4.**
AMG 427 extends survival in mouse xenograft models. Survival analysis of EOL-1 (**A**) and MOLM-13 (**B**) orthotopic mouse models treated with AMG 427 or vehicle. Arrows indicate days of treatment ($n = 5$, vehicle group; $n = 10$, all other groups). Statistical significance was determined using Kaplan–Meier estimator with Mantel–Cox log rank to compare treated groups with the vehicle + T cells control group. **C,** PK profile of AMG 427 in mouse serum at times indicated after last administered dose in **B**.

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Compared with vehicle, treatment with AMG 427 significantly extended survival at all doses tested ($n = 10$, $P \leq 0.0015$, **Fig. 4B**). No significant difference in overall survival was observed between different dose levels. Comparable PK profiles were observed within each cohort for all dose levels, and serum concentrations remained above the TDCC assay-determined $EC_{50}$ for at least 9 days following the final administration (days 34–43, **Fig. 4C**). Serum half-life of AMG 427 ranged from 33 to 47 hours. These data demonstrate that both FLT3 BiTE molecules were active *in vivo* in mouse tumor models.

### PK and PD profile of FLT3 BiTE molecules in cynomolgus monkeys

The experimental FLT3 BiTE molecule and AMG 427 bound human and cynomolgus monkey FLT3 and CD3 protein with comparable affinity (Supplementary Table S5). BiTE-induced TDCC was similar for both constructs using either cynomolgus monkey or human effector cells (**Fig. 3**; Supplementary Fig. S6). To assess the PK/PD relationship, both FLT3 BiTE molecules were evaluated *in vivo* in cynomolgus monkeys. Both molecules were well tolerated. PD endpoints included *FLT3* transcript levels (primer and probes in Supplementary Table S9) in BM and blood and circulating sFLT3L levels. Reduction of *FLT3* transcript levels in BM was likely due to direct killing of *FLT3* transcript-expressing hematopoietic progenitor cells, and reduction in the blood was likely due to lack of replenishment of *FLT3* transcript-expressing cells from the BM. This hypothesis is supported by data showing that there are cells in the BM that express both *FLT3* transcript (**Fig. 5B** and **F**) and surface-localized FLT3 protein (Supplementary Fig. S7B), making them recognizable by FLT3 BiTE molecules, whereas none of the *FLT3* transcript-expressing cells in blood express detectable surface-localized FLT3 protein (Supplementary Fig. S7A), and are therefore not recognizable by FLT3 BiTE molecules.

The experimental FLT3 BiTE molecule was evaluated in a 16-day study (**Fig. 5A–D**) in cynomolgus monkeys, with intra-animal ($n = 3$) dose escalations every 3 days intended to achieve steady-state concentrations ($C_{ss}$) of 0.05, 0.2, 0.5, and 2 nmol/L (**Fig. 5A**; Supplementary Table S8). *FLT3* transcript levels were reduced in BM at day 17 (the only time point evaluated) relative to non-treated animals (**Fig. 5B**) and in blood on days 4, 7, 10, and 17, by an average of 85% to 92%, relative to levels measured before treatment (**Fig. 5C**). Soluble FLT3L levels increased dose-dependently over the course of the study, reaching maximum levels of 13,000 to 15,500 pg/mL at the end of the study (**Fig. 5D**). Ligand accumulation is likely due to depletion of FLT3 protein-expressing cells. The fold-over-$EC_{50}$ (*in vitro* TDCC data; Supplementary Fig. S6) for each of the four dose levels ($C_{ss} \sim 0.05$, 0.2, 0.5, and 2 nmol/L) was 25-, 64-, 165-, and 780-fold. The percent reduction in *FLT3* transcript level in blood did not deepen once drug concentration was above $C_{ss}$ 0.2 nmol/L (64-fold-over-$EC_{50}$), suggesting that the concentration required to achieve maximal target cell elimination from blood was somewhere between $C_{ss}$ 0.05 and 0.2 nmol/L (25- and 64-fold-over-$EC_{50}$, TDCC data; Supplementary Fig. S6; Supplementary Table S7).

AMG 427 was evaluated in an 8-day multiple dose study (**Fig. 5E–H**) in cynomolgus monkeys. All animals were treated on days 1, 2, and 5 with doses intended to achieve a maximal serum concentration ($C_{max}$) of 1 nmol/L (Group 1), 5 nmol/L (Group 2), and 10 nmol/L (Group 3; $n = 3$/group). The study duration was limited to 8 days to minimize loss of exposure due to antidrug antibody formation, and multiple doses were administered to ensure target coverage for the entire study. Exposures of AMG 427 over 7 days were

reproducible within each of three dose groups (**Fig. 5E**), and exposure, $C_{max}$, and $C_{min}$ all increased in an approximately dose-proportional manner (Supplementary Table S8). The terminal half-life ranged from 33 to 50 hours (Supplementary Table S9). Hallmarks of BiTE molecule activity including upregulation of CD69 on T cells and cytokine secretion were observed (Fig. 8A and B). Because FLT3 is not expressed on the surface of cells in the blood (Supplementary Fig. S7), AMG 427-mediated upregulation of CD69 on T cells likely resulted from cells expressing FLT3 surface protein in the BM. Increases in serum concentrations of IFNγ, IL6, MCP-1, and TNFα were observed in response to the first dose but were attenuated in response to subsequent doses (Supplementary Fig. S8B). The fold-over-$EC_{50}$ levels at $C_{min}$ (*in vitro* TDCC data; Supplementary Fig. S6; Supplementary Table S9) for the respective groups was 45-, 158-, and 396-fold. Within the BM, *FLT3* transcript levels were reduced by 85% to 95% on day 4 and by 93% to 97% on day 8 (**Fig. 5F**). Within the blood, the *FLT3* transcript levels were reduced to a nearly undetectable level (≥97%) at the lowest dose level and earliest time point, and a similar level of depletion was maintained across all higher exposures and time points (**Fig. 5G**). Monocytes were reduced at the end of the study (Supplementary Fig. S8C), which may reflect lack of replenishment due to direct killing of BM progenitors. Minor decreases in plasmacytoid dendritic cells (pDC; Supplementary Fig. S8C) are challenging to interpret as the number of circulating dendritic cells was low and enumeration of rare cells is prone to error. Soluble FLT3L levels increased dose-dependently over the course of the study, reaching maximum levels of 12,000 to 23,000 pg/mL in each of the three groups (**Fig. 5H**). Time-dependent improvements in PD were observed for those endpoints which had not already reached maximal levels when first analyzed, as demonstrated by the increase in *FLT3* transcript reduction in BM from group 1 on day 4 (85%) to day 8 (93%, **Fig. 5F**) and changes in sFLT3L (**Fig. 5H**; Supplementary Table S9). This demonstrates that greater efficacy at a given dose level may be observed with longer duration of exposure.

The *in vivo* activity of the experimental FLT3 BiTE molecule and AMG 427 was most directly comparable using the PD endpoint of *FLT3* transcript in blood. For the experimental FLT3 BiTE molecule, the greatest activity occurred between days 4 and 7 at a $C_{ss}$ of 0.05 to 0.2 (25- to 64-fold-over-$EC_{50}$; Supplementary Table S8). For AMG 427, the greatest activity was observed on day 3 at a $C_{min}$ of 0.24 nmol/L in Group 1 (≤45-fold-over-$EC_{50}$; Supplementary Table S9). Although the time points of data collection differed, these results suggest that both FLT3 BiTE molecules are active at similar fold-over-$EC_{50}$ values *in vivo*.

### Experimental FLT3 BiTE molecule–induced TDCC of patient samples *ex vivo*

A long-term culture system (25) was used to evaluate experimental FLT3 BiTE molecule-mediated cytotoxicity in 14 pAML samples (Supplementary Table S10) over 9 days. The autologous E:T ratio was calculated from the number of T cells and pAML cells in each sample at the beginning of the experiment and ranged from 1:2.5 to 1:74. Three patterns of cytotoxicity were observed: (1) continuously-increasing cytotoxicity (**Fig. 6A**, left, representative sample Supplementary Fig. S9A); (2) initial cytotoxicity followed by sustained or decreased killing (**Fig. 6A**, middle); (3) transient or no cytotoxicity over the 9 days (**Fig. 6A**, right). Analysis of FLT3 surface protein expression of the pAML cells and E:T ratio revealed that most of the samples in groups 1 and 2 contained FLT3 protein-positive pAML cells (MFI Ratio > 1.5) and a higher E:T ratio (>1:38, 75th
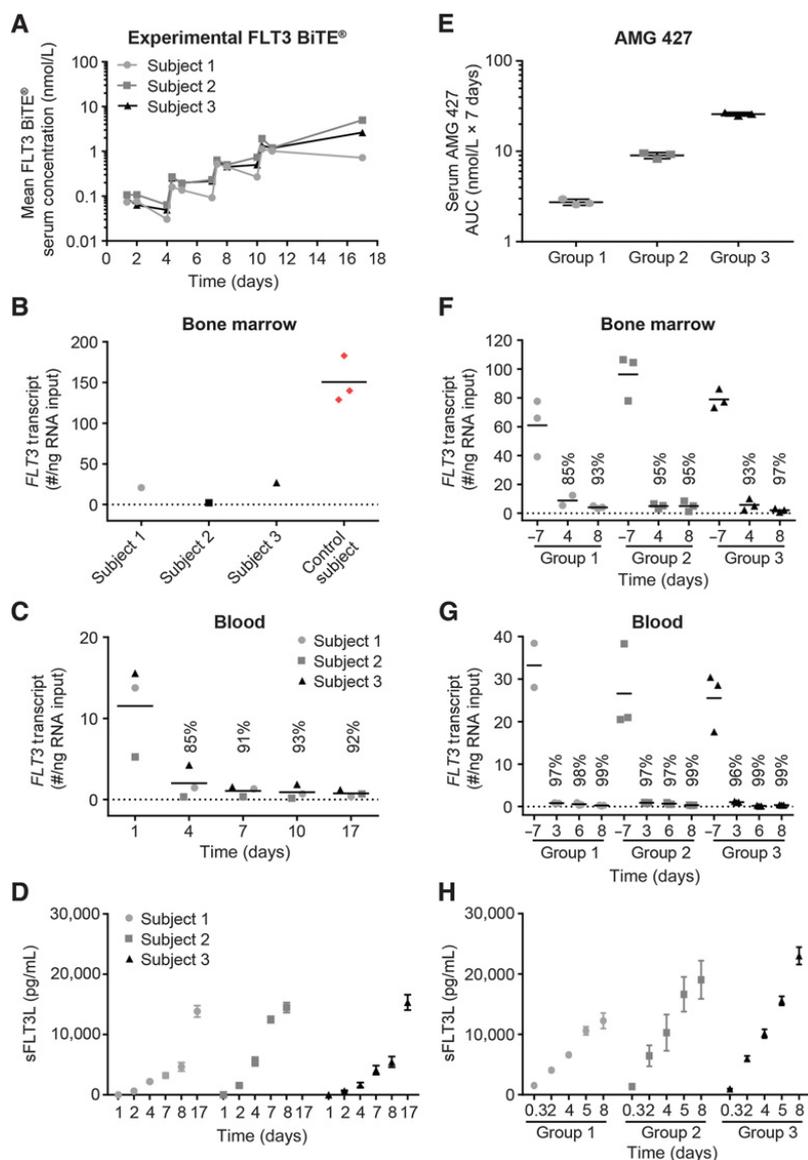
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**Figure 5.**
Preclinical PK and PD profile of FLT3 BiTE molecules (experimental, **A–D**; HLE, **E–G**) in cynomolgus monkeys. **A,** Serum concentration of experimental FLT3 BiTE molecule in cynomolgus monkeys dosed by cIV infusion. Data for each animal plotted separately ($n = 3$). Abundance of *FLT3* transcript in bone marrow (**B**) at day 17 and blood (**C**) measured by ddPCR at the times indicated dosed as in **A**; percent decrease in *FLT3* transcript shown for each time point (each animal plotted separately, lines represent mean, $n = 3$ technical replicates). **D,** Serum concentration of sFLT3L for three different animals dosed as in **A**; mean $\pm$ SD, $n = 1$–3 technical replicates. **E,** Mean exposure of AMG 427 $\pm$ SD ($n = 3$/group) represented as AUC in nanomolar times 7 days. Abundance of *FLT3* transcript measured by ddPCR at the times indicated in bone marrow (**F**) and blood (**G**) in animals dosed as in **A**. Lines represent mean, $n = 3$/group; percent decrease in *FLT3* transcript shown for each time point. **H,** Serum concentration of sFLT3L in cynomolgus monkeys dosed as in **A**; mean $\pm$ SD, $n = 3$/group.

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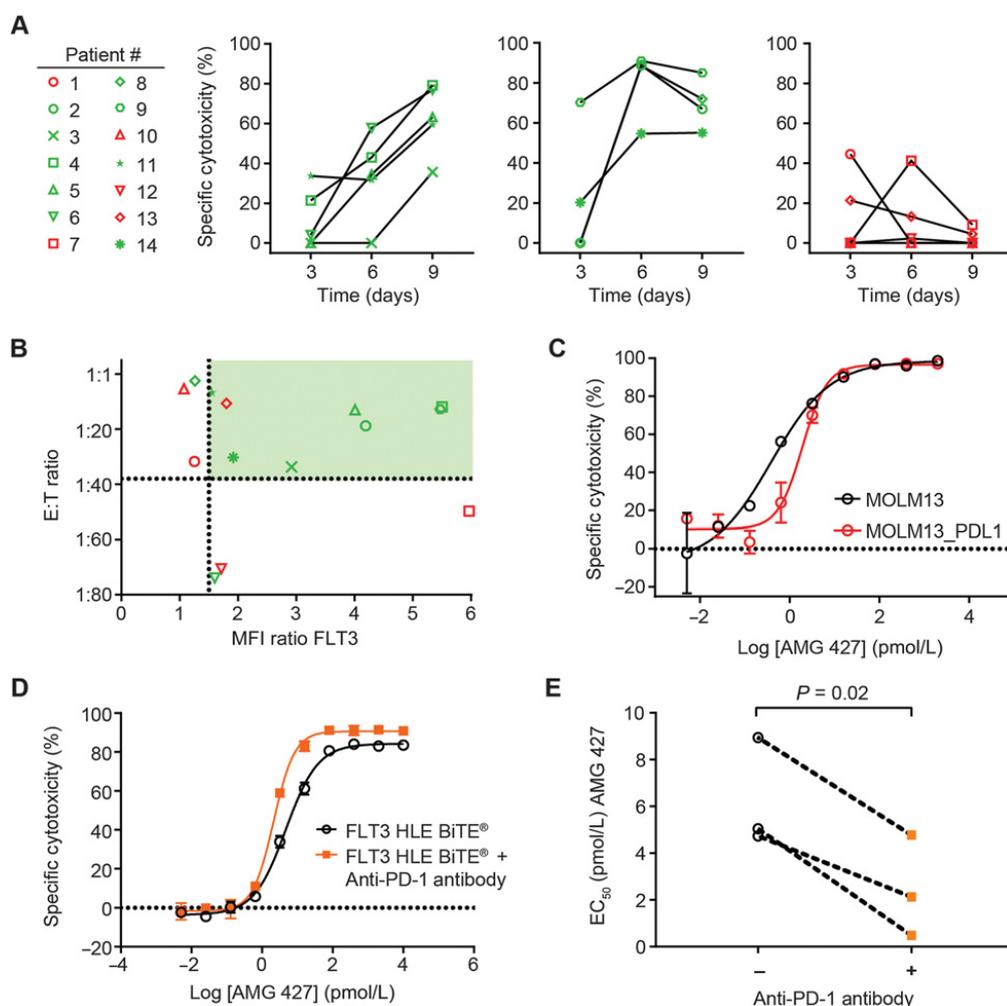


**Figure 6.**
FLT3 BiTE molecule cytotoxicity in AML patient samples *ex vivo* as a single agent and in combination with a PD-1–blocking antibody. **A,** Specific cytotoxicity relative to control BiTE molecule for 14 pAML patient samples cultured with experimental FLT3 BiTE molecule for 9 days. Patients were grouped according to their responses: continued responders (green symbols, left), partial responders (green symbols, center), and nonresponders (red symbols, right). **B,** Initial MFI ratio vs. autologous E:T cell ratio of 14 patients with pAML evaluated in **A**. Vertical dotted line represents FLT3 positivity (MFI ≥ 1.5). Horizontal dotted line represents high E:T (75th percentile, 1:38). Green shading represents E:T ratio and MFI with higher probability of showing response. **C,** Specific cytotoxicity of parental (black circles) or PD-L1–transfected (red circles) MOLM-13 cells cultured for 24 hours 1:1 with CD3/CD28-activated human pan T cells and a dose range of AMG 427. Data are shown as mean ± SD ($n = 2$ technical replicates, one of two representative T-cell donors. **D,** Specific cytotoxicity of PD-L1–transfected MOLM-13 cells cultured for 24 hours 1:1 with CD3/CD28-activated human pan T cells and AMG 427 with (orange squares) or without (black circles) 10 µg of an anti-PD-1 blocking antibody (mean ± SD, $n = 2$ technical replicates, one of three representative T-cell donors). **E,** AMG 427 $EC_{50}$ ± anti-PD-1 blocking antibody as in **D**, $n = 3$ T-cell donors, $P = 0.02$, paired $t$ test.

percentile; **Fig. 6B**), whereas most of the samples in group 3 expressed low levels of FLT3 protein (MFI ratio <2) and/or had a low E:T ratio (<1:38). These data demonstrate that both target expression and T-cell abundance are important factors for FLT3 BiTE-mediated target cell killing.

**AMG 427 potency was increased in combination with a PD-1–blocking antibody**

T-cell activation induces PD-1 expression, and reports show that PD-1 engagement by ligands PD-L1 or PD-L2 decreases T-cell activity (26). Co-culture of pAML specimens with a CD33-targeting BiTE

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molecule induces PD-1 expression on T cells and PD-L1 expression on AML blasts (27). Similarly, AMG 427-mediated T-cell activation induced a dose-dependent increase in PD-1 expression (Supplementary Fig. S9B), and potency in TDCC assays was reduced five-fold if the target cells expressed PD-L1 (relative to target cells lacking PD-L1; **Fig. 6C**). Combination of a PD-1–blocking antibody with AMG 427 restored TDCC potency, decreasing the $EC_{50}$ by an average of 2.5-fold ($n = 3$ T-cell donors, $P = 0.02$) and increasing maximum killing by 12% (**Fig. 6D** and **E**). These data demonstrate that AMG 427-mediated target cell killing may be enhanced by combination with PD-1 blockade, as has been demonstrated for other BiTE molecules (27, 28).

## Discussion

Blinatumomab demonstrates that a BiTE molecule can engage patient T cells to eliminate CD19-expressing disease cells, and this activity can provide clinical benefit for patients with acute lymphoblastic leukemia and non-Hodgkin lymphoma (29–31). Here FLT3 BiTE molecules for the treatment of patients with AML are characterized. FLT3 meets the requirements of a BiTE molecule target as cell surface protein is broadly expressed on disease samples, with limited expression on normal tissues. In disease samples, cell surface FLT3 protein was detected on the majority of 318 pAML samples. The level of expression was comparable between bulk samples and LSCs, suggesting that both subsets could be targeted at similar therapeutic exposures. In addition, mean FLT3 protein expression on pAML samples was comparable, regardless of *FLT3* mutational status, *FLT3*-ITD allelic ratio, or initial diagnosis versus relapse, suggesting that a FLT3 BiTE molecule would benefit a broad patient population. In normal hematopoietic cells, cell surface FLT3 protein was detected on subsets of BM stem and progenitor cells (excluding MEPs). Within each subpopulation, a portion of cells were cell surface FLT3-positive (MFI ratio ≥ 1.5), so as to suggest their elimination by a FLT3-targeting BiTE molecule. However, a portion of these cells were FLT3 protein-negative, consistent with literature reports of FLT3 protein- and transcript-negative cells within HD hematopoietic progenitor populations and HSCs (GEO accession code GSE75478; refs. 7, 32).These results suggest that although some HSPCs would be eliminated by a FLT3 BiTE molecule, there is a FLT3-negative population that could potentially repopulate the BM following cessation of treatment. FLT3 transcript and protein expression were also evaluated in normal non-hematopoietic tissues. To ensure a thorough assessment, *FLT3* transcript expression was evaluated in several databases and further characterized by qPCR-based analysis of a panel of tissues. Protein expression was subsequently evaluated in tissues shown to contain *FLT3* transcript in any dataset. Although FLT3 transcript and protein were detected in some solid tissues, no membranous protein staining was observed, indicating that these cells would not be targeted by a FLT3 BiTE molecule. Additional analysis of FLT3 transcript and protein in the cerebellum demonstrated that most transcripts were not full-length due to alternative splicing, and similarly, the FLT3 protein was also not full-length. By evaluating both FLT3 transcript and protein expression using multiple sources and orthogonal methods, it was possible to build a detailed understanding of the normal tissue expression and based on these results, FLT3 BiTE treatment is not anticipated to target normal non-hematopoietic tissues.

Given the favorable expression profile of FLT3 as an AML target, two potent and specific BiTE molecules were generated: one experimental FLT3 BiTE molecule that has a short serum half-life and the other, AMG 427, which contains an Fc-moiety to extend serum half-life. *In vitro*, these molecules demonstrated TDCC against human

FLT3-positive cancer cell lines with similar picomolar potency ($EC_{50}$) and this TDCC was associated with T-cell activation and cytokine secretion and was not affected by the presence of sFLT3 or sFLT3L at concentrations found in patients with AML. These data demonstrate it is possible to generate a BiTE molecule capable of eliminating FLT3-expressing cells, and that despite incorporation of the Fc moiety, the larger size does not impact the ability of AMG 427 to effectively form an immunologic synapse and induce T-cell–mediated target cell killing.

In cynomolgus monkeys, both FLT3 BiTE molecules mediated depletion of cell surface FLT3-expressing target cells as demonstrated by decreases in *FLT3* transcript in the blood and BM. Although cell surface FLT3 protein was not detected on human or cynomolgus monkey peripheral immune cells, *FLT3* transcript can be detected in pDCs and monocytes. These cell types have short half-lives *in vivo* (33, 34), and administration of a FLT3 BiTE molecule is expected to eliminate a portion of the precursor cells that give rise to them, which may explain the decreases in *FLT3* transcript observed in the blood of cynomolgus monkeys treated with a FLT3 BiTE molecule. Within the BM, *FLT3* transcript was reduced by ≥85% at all doses by the first timepoint tested (day 4), demonstrating that BiTE-mediated target cell killing can occur rapidly. At this same timepoint, the degree of depletion increased as the dose increased [85% (low dose) vs. 95% (medium dose), and 93% (high dose), respectively], suggesting that increased exposure can lead to deeper responses. Within the low-dose group, the reduction in *FLT3* transcript levels increased from 85% to 93% between days 4 and 8, suggesting that deeper responses may also be achieved by maintaining the same exposure for longer. This hypothesis is supported by the sFLT3L endpoint, which improved with either higher exposure or increased time of exposure.

BiTE molecule-mediated lysis of AML blasts within patient samples was evaluated in long-term culture assays using autologous T cells. The degree of anti-AML activity was associated with FLT3 expression on the target cells and the E:T ratio, with improved activity seen in AML samples with a higher FLT3 protein expression and an E:T ratio >1:38. The impact of the E:T ratio highlights the importance of T-cell fitness to enable successful responsiveness to BiTE molecule therapy. One well-established mechanism of reducing T-cell activity is induction of PD-1 expression. BiTE molecule–mediated T-cell activation is accompanied by expression of PD-1 on corresponding T cells and this expression has been associated with resistance to blinatumomab treatment (28, 35). PD-1 is expressed on 20% to 30% of AML patient T cells (36, 37) and has been shown to increase to 50% to 60% at relapse (38). *PD-L1* mRNA expression is upregulated in patients with AML (39) and correlates with cell surface protein expression (40). Although not usually detected at diagnosis (41), PD-L1 protein is upregulated on AML blasts during therapy, after HSCT, and at relapse (39, 42). Upregulation of PD-L1 on AML blasts is reported to be induced by cytokines such as IFNγ (43, 44), which may be the mechanism of PD-L1 upregulation on pAML blasts treated *ex vivo* with a CD33-targeting BiTE molecule (27). In a mouse model engineered to express human CD3, combination studies of BiTE molecules with checkpoint inhibitors exhibit additive effect (45). Herein, AMG 427-mediated activation of T cells was associated with rapid induction of PD-1 expression and subsequent reduced killing of PD-L1–expressing target cells, suggesting that combination with PD-1 blockade may improve BiTE-mediated activity. Indeed, the combination of BiTE molecule and a PD-1–blocking antibody in a TDCC assay resulted in decreased $EC_{50}$ and increased maximum killing in all donors tested. As expression of checkpoint

molecules, including PD-1, has been observed in patients with AML (39), and may be increased following chemotherapy (43), this combination therapy warrants clinical evaluation.

## Disclosure of Potential Conflicts of Interest

## Authors' Contributions

**B. Brauchle:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **R.L. Goldstein:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **C.M. Karbowski:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **A. Henn:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **C.-M. Li:** Methodology, data curation, formal analysis, writing-original draft, writing-review and editing, project administration. **V.L. Bücklein:** Data curation, formal analysis, writing-original draft, writing-review and editing. **C. Krupka:** Conceptualization. **M.C. Boyle:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **P. Koppikar:** Conceptualization, data curation, formal analysis, writing-original draft, writing-review and editing. **S. Haubner:** Formal analysis. **J. Wahl:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **C. Dahlhoff:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **T. Raum:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **M.J. Rardin:** Formal analysis, writing-original draft, writing-review and editing, project administration. **C. Sastri:** Methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **D.A. Rock:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **M. von Bergwelt-Baildon:** Writing-original draft, writing-review and editing. **B. Frank:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **K.H. Metzeler:** Data curation, formal analysis, writing-original draft, writing-review and editing. **R. Case:** Methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **M. Friedrich:** Conceptualization, formal analysis, writing-original draft, writing-review and editing. **M. Balazs:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing. **K. Spiekermann:** Formal analysis, writing-original draft, writing-review and editing. **A. Coxon:** Conceptualization, writing-original draft, writing-review and editing, study supervision. **M. Subklewe:** Conceptualization, methodology, data curation, formal analysis, writing-original draft, writing-review and editing, project administration, study supervision. **T. Arvedson:** Conceptualization, methodology, formal analysis, writing-original draft, writing-review and editing, study supervision.

## Data Sharing Statement

## Acknowledgments

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## 2.2 Publication II

**Regular Article**

MYELOID NEOPLASIA

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

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**KEY POINTS**

- Characterization of an αPD-L1 × αCD3 × 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.

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 programmed cell death-ligand 1 (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 interferon-γ) and leads to efficient and highly selective cytotoxicity against CD33$^+$PD-L1$^+$ cell lines (50% effective concentration = 2.3-26.9 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 anticancer 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. (*Blood*. 2018;132(23):2484-2494)

## Introduction

The treatment of acute myeloid leukemia (AML) remains challenging in 2018. Only one-half of the patients are eligible for curative intensive induction chemotherapy, and the majority will relapse because of the persistence of chemoresistant leukemic stem cells. Allogeneic hematopoietic stem cell transplantation as postremission 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 have 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 (Mylotarg) has proven to be safe and led to reapproval 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, in which 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 1B/2 study with the αPD-1 mAB nivolumab in combination with azacytidine 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 1 polypeptide chain. Additionally, we attached the extracellular domain (amino acid 33-149) 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 with a BiTE-like molecule, and induces efficient cancer cell eradication. Notably, in vitro the CiTE demonstrates a high selectivity for CD33$^+$PD-L1$^+$ cells, whereas PD-L1$^+$ cells are not affected. This is further supported in a murine model system, where no indication for the development of irAEs because of 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-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 a 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

PD-1$_{ex}$ was amplified from human muscle complementary DNA (cDNA; *PDCD1* gene). The αPD-L1 scFv was published before (YW243.55.S70, atezolizumab-derived) with variable light and variable heavy chains connected by a (G$_4$S$_4$)$_4$ 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) 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 *Escherichia coli* BL21 cells (NEB, Ipswich, MA). 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 and 300 mM NaCl (pH 6.5). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analytical SEC (Superdex 200 increase 5/150, GE Healthcare). For mouse studies, proteins were prepared in 1× DPBS (Thermo Fisher Scientific) and endotoxin levels were confirmed to be <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).[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 (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 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 supplemental Table 2, available on the *Blood* Web site.

### Flow cytometry

Flow cytometry measurements were performed on a Guava easyCyte 6HT instrument (Merck Millipore, Burlington, MA) and analyzed using GuavaSoft, version 3.1.1 (Merck Millipore) or on an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data were evaluated using FlowJo, version 9.6 (Tree Star Inc., Ashland, OR). Commercial antibodies are listed in the supplemental Methods. Surface antigen density of cell lines was evaluated with QIFIKIT (Agilent Dako, Santa Clara, CA). Apparent dissociation constants were determined by calibrated flow cytometry as
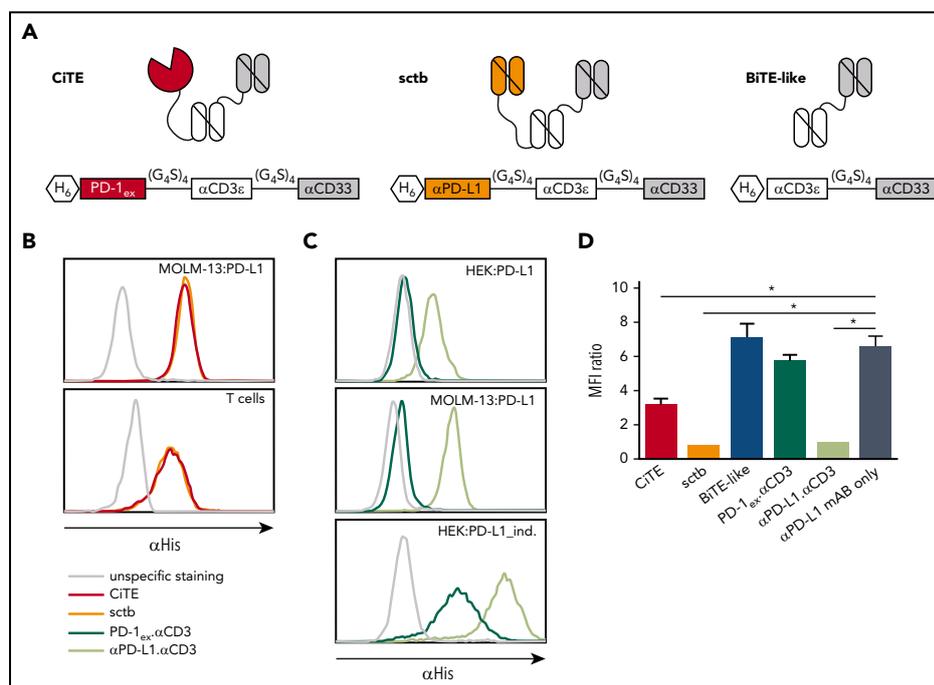
**Figure 1. Schematic drawing and binding characteristics of CiTE, sctb, and control molecules.** (A) Modular composition of CiTE, sctb, and BiTE-like molecule. (B) Binding analysis of CiTE and sctb to MOLM-13:PD-L1 cells and HD T cells at 15 ng/µL concentration by flow cytometry. (C) Binding analysis 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. Gray line indicates unspecific staining by the secondary antibody. Histograms show 1 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 *t* test with Welch correction was applied. *$P < .05$, **$P < .01$, ***$P < .001$. MFI, mean fluorescence intensity.

described.[38] A total of 3.0 to 3.4 µm Rainbow Calibration particles (BioLegend, San Diego, CA) served as calibration control. Data points were normalized to the maximum mean fluorescence intensity and fitted to a 1-site specific binding model.

### T-cell activation and cytotoxicity assays

HD T cells were incubated with target cell lines at an effector to target cell (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 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 hours, T-cell activation was assessed by flow cytometry quantifying the CD2$^+$CD69$^+$, CD2$^+$CD25$^+$, or CD2$^+$PD-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). After 72 hours, total target cell numbers were assessed by flow cytometry as live CD2$^-$CD33$^+$ or CD2$^-$PKH67$^+$ population, respectively, and normalized to negative control. Data were transformed with a 4-parameter nonlinear fit

model. Interferon-γ (IFN-γ) and Granzyme B release were determined after 72 hours 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). Preactivated T cells derived from an 18-day ex vivo peripheral blood mononuclear cell 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] The 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 nonlinear fit model.

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

### Ex vivo redirected lysis assay of cocultured AML patient cells

Redirected lysis assays of AML patient samples were performed in α-MEM (Thermo Fisher Scientific) supplemented with 12.5%
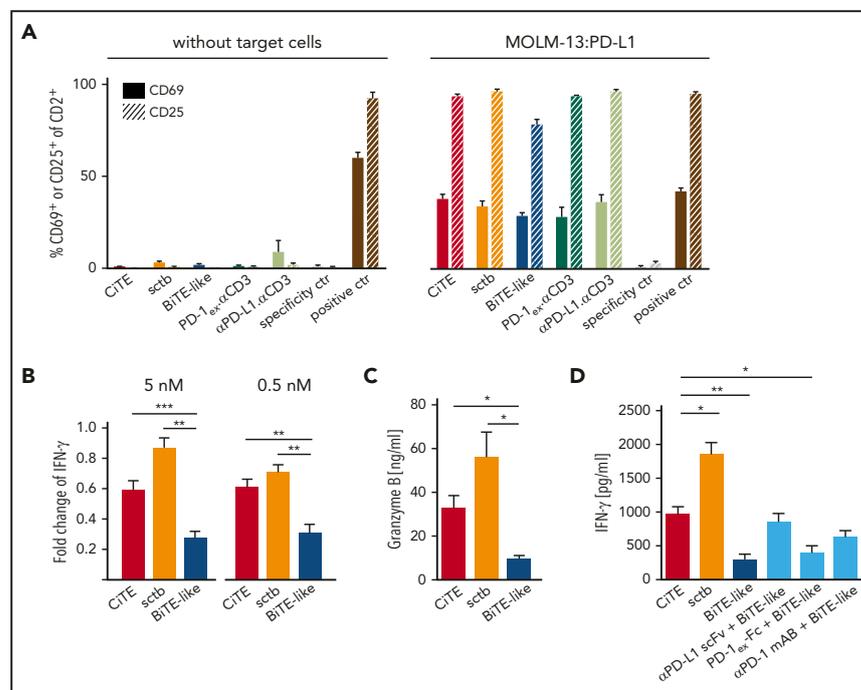
**Figure 2. CiTE-mediated T-cell activation depends on crosslink to target cells and is enhanced compared with BiTE-like molecule.** (A) CiTE- and sctb-induced upregulation of CD69 and CD25 on HD T cells in comparison with controls as analyzed by flow cytometry in the absence or presence of MOLM-13:PD-L1 after 96 hours 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 and 0.5 nM protein concentrations. (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 hours at an E:T ratio of 2:1 using nonstimulated HD T cells. Bar charts represent mean values of (B,D) n = 4-5 or (C) n = 4-6 independent experiments with SEM as error bars. For statistical analysis, unpaired Student t test with Welch correction was applied. *P < .05, **P < .01, ***P < .001.

fetal calf serum, 12.5% horse serum, 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), and a distinct cytokine cocktail on irradiated MS-5 cells as described elsewhere.[10,13,40-42] HD T 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 previously.[10,13]

### Murine AML xenograft studies

Female non-obese diabetic severe combined immunodeficiency γ (NSG) mice 170 to 265 days of age were housed under pathogen-free conditions at the research animal facility of the Helmholtz Zentrum München, Munich, Germany. Animal experiments were approved by regional regulating authorities (Regierung von Oberbayern) and conducted as described in a published protocol.[43] At day 0, $2 \times 10^4$ MOLM-13:PD-L1 cells were injected IV. At day 3, $10^7$ in vitro preactivated T cells were transferred intraperitoneally and mice were randomized into 5 groups: 3 treatment groups containing 6 mice each, a specificity control group

of 4 mice, and a 1× DPBS control group of 5 mice. At day 4, 1.7 pmol of molecules/g body weight or 1× DPBS were daily IV injected until day 12. At day 13, mice were euthanized, 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) applying unpaired Student 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 PD-1$_{ex}$ to an αCD3.αCD33 BiTE-like molecule. The CiTE was compared with 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; supplemental Figure 1A). Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analytical SEC (supplemental Figure 1B-C) and protein stability was assessed by fluorescence-based thermal shift assay (supplemental Figure 1D).

The binding properties and apparent dissociation constants of CiTE and sctb to antigen-presenting cells were analyzed by flow cytometry (supplemental Figure 2). When investigating CiTE and sctb as whole molecules, both bound similarly to CD33$^+$PD-L1$^+$ AML cell lines and HD T cells (Figure 1B). Because 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 (supplemental Table 1A). As expected,

our results showed weak physiological binding of PD-1$_{ex}$ (described in the low micromolar 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 with the αPD-L1 scFv, the CiTE was able to block subsequent binding of the αPD-L1 mAB on CD33$^+$PD-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-L1+ cells, both CiTE and sctb led to a significant increase in IFN-γ and Granzyme B levels compared with the BiTE-like molecule. We also observed an upregulation of PD-1 upon T-cell activation (supplemental Figure 3).

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 with high-affinity blocking agents plus BiTE-like molecule, whereas the equimolar addition of PD-1$_{ex}$-Fc (low-affinity blocking module) triggered a weaker IFN-γ release (Figure 2D). We conclude that the fusion of PD-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 because of the low affinity of PD-1$_{ex}$. Furthermore, we expect the CiTE to address CD33+PD-L1+ cells more efficiently than CD33+PD-L1− cells because of avidity-dependent binding of the αCD33 scFv and PD-1$_{ex}$. To test this hypothesis, the molecules were incubated with nonstimulated HD T cells and MOLM-13 or MOLM-13:PD-L1 cells, expressing high levels of CD33 (Figure 3; supplemental Figure 5; supplemental Table 1). 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-L1+ target cells (supplemental Figure 4). Interestingly, both molecules revealed similar 50% effective concentration (EC$_{50}$) values despite their different affinities for PD-L1. Consistent with the previous characterization, the low-affinity PD-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. Because CD33 levels on AML cells exhibit a high inter- and intrapatient heterogeneity,[10] the results were confirmed with OCI-AML3 and OCI-AML3:PD-L1 cells, which express low CD33 levels (Figure 3B,D; supplemental Table 1). The advantage of the bifunctional CiTE and sctb in comparison with 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-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.
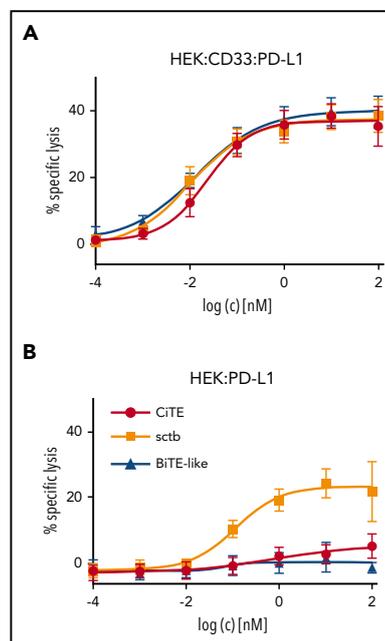
**Figure 4. CiTE induces preferential lysis of CD33+PD-L1+ cells and has no activity against CD33−PD-L1+ cells.** Dose-dependent preferential lysis of (A) HEK:CD33:PD-L1 over (B) HEK:PD-L1 cells induced by CiTE, sctb, or BiTE-like molecule and vice versa. Preactivated HD T cells were incubated with target cells and CiTE, sctb, or BiTE-like molecule for 4 hours at a total E:T ratio of 2:1. EC$_{50}$ values for CiTE, sctb, and BiTE-like molecule were 22.8, 10.4, 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.

We next evaluated whether the CiTE molecule is able to induce elimination of CD33+PD-L1+ cells selectively in the presence of PD-L1+ cells. To this end, preferential lysis was analyzed in a mixed target cell population (Figure 4; supplemental Figure 6). Although the CiTE triggered preferential lysis of CD33+PD-L1+ cells, molecules with high affinity to PD-L1 revealed dose-dependent elimination of both CD33+PD-L1+ and PD-L1+ cell lines. This indicates that the low-affinity PD-1$_{ex}$ module is not sufficient to redirect T cells to PD-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
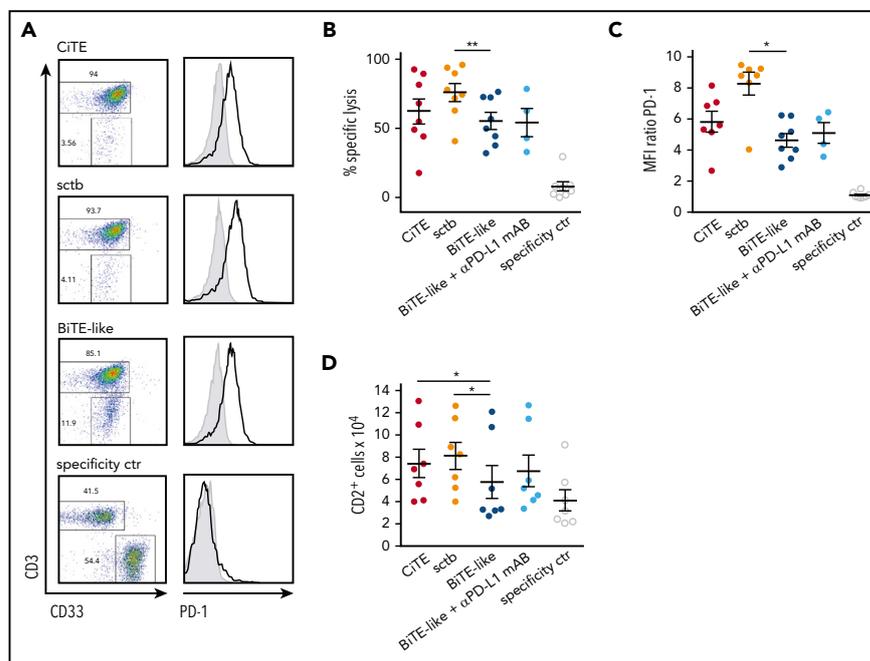
**Figure 5. CiTE and sctb enhance lysis of patient-derived primary AML cells and increase T-cell proliferation.** (A) Cytotoxic lysis of primary AML cells from an exemplary patient and PD-1 expression on T cells after 3 to 4 days of cocultivation. (B) Mean cytotoxic lysis of primary AML cells induced by respective molecules after 3 to 4 days. (C) PD-1 expression on T cells after 3 to 4 days. (D) T-cell proliferation after 6 to 7 days measured by flow cytometry. CiTE, sctb, BiTE-like molecule, and specificity control were applied at 10 nM; 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. *$P < .05$, **$P < .01$, ***$P < .001$.

of PD-L1 on primary AML patient cells (supplemental Figure 7A). In 7 of 8 patients, the CiTE was able to induce equal or enhanced redirected lysis of target cells compared with the BiTE-like molecule (62 ± 9% compared with 55 ± 6%), and the sctb triggered similar or higher lysis in all patients (76 ± 6%) (Figure 5A-B; supplemental Figure 7B). An increase in T-cell proliferation was induced by the CiTE and sctb in contrast to the BiTE-like molecule through prolongation of coculture time to 6 to 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; supplemental Figure 7C). 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

Because T cell–based immunotherapies such as BiTEs, chimeric antigen receptor T cells and hematopoietic stem cell transplantation rely on T-cell activation, the induced proinflammatory response will consistently evoke PD-L1 upregulation on AML cells.[13,49-52] 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 3 treatment groups (Figure 6B). In contrast, the control cohort showed 1% to 3% AML cells in the BM, which resembles minimal residual disease criteria of <5% myeloblasts in humans (supplemental Figure 8A).[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-L1 checkpoint blocking modules to murine PD-L1, which bound murine PD-L1 with comparable affinities than human PD-L1 (supplemental Figure 8B). Mice treated with the high-affinity sctb lost body weight compared with the other treatment groups (Figure 6D; supplemental Figure 8C). PD-1 was significantly upregulated on CD4⁺ and CD4⁻ T cells in the BM (Figure 6C); 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.
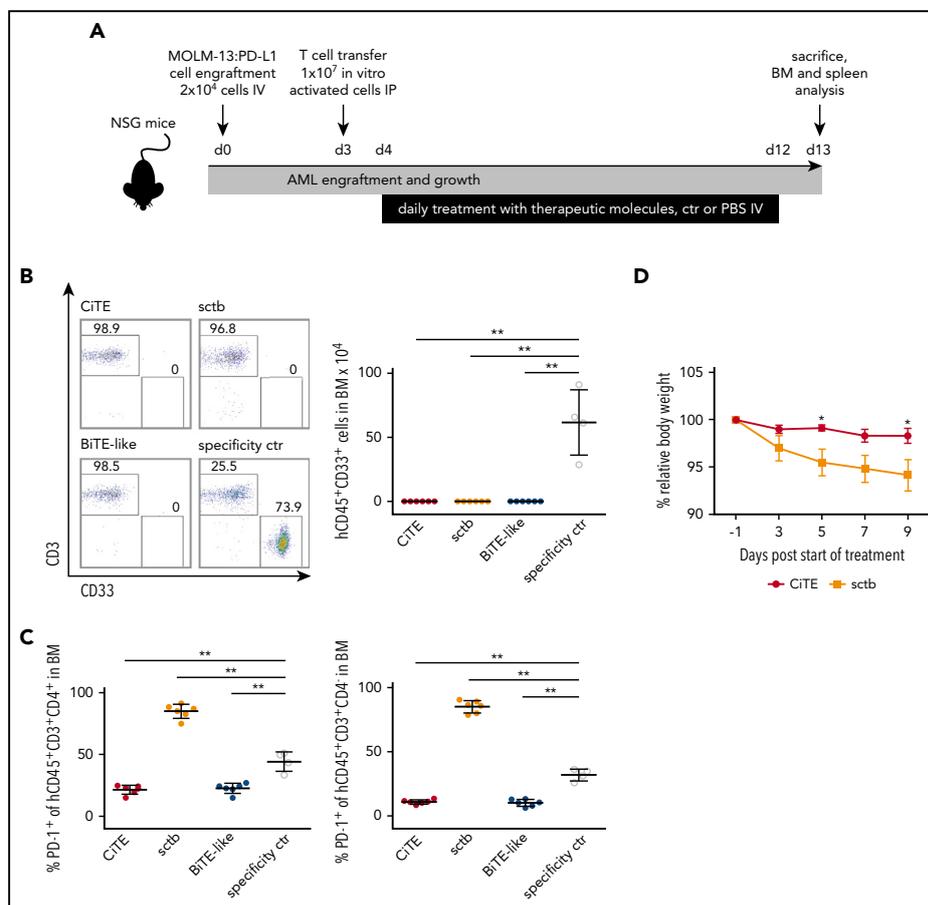
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**Figure 6. CiTE leads to eradication of AML cells in a murine NSG xenograft model without indication of enhanced PD-1 upregulation or body weight loss.** (A) Experimental design of mouse studies. (B) Remaining engrafted MOLM-13:PD-L1 (live hCD45+CD33+) 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 to 6 mice. Error bars in (B-C) indicate standard deviation, in (D) SEM. For statistical analysis, Mann-Whitney $U$ test was applied. *$P$ < .05, **$P$ < .01, ***$P$ < .001. IP, intraperitoneally.

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 postremission 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.

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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-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-L1$^+$ cells in vitro, whereas PD-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 with high-affinity PD-1 and PD-L1 blocking agents.

Because CD3-addressing approaches by T-cell–engaging molecules are effective at very low protein concentrations (picomolar or even subpicomolar),[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, T-cell receptor 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 T-cell receptor 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 muromonab (Orthoclone OKT3) frequently experience cytokine release syndrome.[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 cytokine release syndrome 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. Because of 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 because 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 gemtuzumab ozogamicin consequentially results in neutropenia.[10,61-66]

Because 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 mAB are under intensive investigation.[16,47,48,67] We were able to demonstrate that the CiTE molecule, despite the low-affinity PD-1$_{ex}$ domain, induces similar IFN-γ levels in comparison with 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-L1$^+$ double-positive target cells. This is expected to translate into a decreased incidence of irAEs, 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-L1$^+$ target cells in vitro and does not show adverse events in vivo. Because of its beneficial performance compared with the BiTE format, we consider the CiTE 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.

## Authorship

Contribution: 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 acute myeloid leukemia patient samples and analyzed the results. A.O.W. 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 the 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.

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