Cancer Therapy: Preclinical

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Characterization of CD33/CD3 Tetravalent Bispecific Tandem Diabodies (TandAbs) for the Treatment of Acute Myeloid Leukemia

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Abstract

Purpose: Randomized studies with gemtuzumab ozogamicin have validated CD33 as a target for antigen-specific immunotherapy of acute myelogenous leukemia (AML). Here, we investigated the potential of CD33/CD3-directed tandem diabodies (TandAbs) as novel treatment approach for AML. These tetravalent bispecific antibodies provide two binding sites for each antigen to maintain the avidity of a bivalent antibody and have a molecular weight exceeding the renal clearance threshold, thus offering a longer half-life compared to smaller antibody constructs.

Experimental Design: We constructed a series of TandAbs composed of anti-CD33 and anti-CD3 variable domains of diverse binding affinities and profiled their functional properties in CD33$^+$ human leukemia cell lines, xenograft models, and AML patient samples.

Results: Our studies demonstrated that several CD33/CD3 TandAbs could induce potent, dose-dependent cytolysis of CD33$^+$ AML cell lines. This effect was modulated by the effector-to-target cell ratio and strictly required the presence of T cells. Activation and proliferation of T cells and maximal AML cell cytolysis correlated with high avidity to both CD33 and CD3. High-avidity TandAbs were broadly active in primary specimens from patients with newly diagnosed or relapsed/refractory AML *in vitro*, with cytotoxic properties independent of CD33 receptor density and cytogenetic risk. Tumor growth delay and inhibition were observed in both prophylactic and established HL-60 xenograft models in immunodeficient mice.

Conclusions: Our data show high efficacy of CD33/CD3 TandAbs in various preclinical models of human AML. Together, these findings support further study of CD33/CD3 TandAbs as novel immunotherapeutics for patients with AML. *Clin Cancer Res; 22(23); 5829–38. ©2016 AACR.*

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Introduction

For many years, antigen-specific immunotherapy has been sought as a means to improve the outcomes of patients with acute myelogenous leukemia (AML). For this purpose, an increasing number of cell surface antigens are being evaluated. Among the most extensively studied is CD33 (Siglec-3), a myeloid differentiation antigen that is heterogeneously expressed on a subset of AML cells in almost all patients (1, 2). Several large, randomized trials have demonstrated that the CD33 antibody–drug conjugate, gemtuzumab ozogamicin (GO), improves survival of pediatric and some adult patients with newly diagnosed AML when added to intensive induction chemotherapy (3, 4), validating CD33 as a pharmacologic target. Nonetheless, clinical experience revealed limitations of the antibody–drug conjugate approach to targeting CD33. Recognized challenges for GO include the relatively low abundance of CD33 in some AML patients that limits intracellular delivery of the toxin moiety, and the presence of drug transporter proteins, which extrude the toxin before cellular damage is induced (2). Because GO is insufficiently active in many patients, and given concerns about unwanted toxicity, GO was withdrawn from the market in most countries in 2010.

To improve the efficacy of CD33-directed antibodies, bispecific molecules have been pursued that also recognize an immune effector cell antigen to redirect cytotoxic immune cells toward CD33$^+$ cancer cells (2, 5–7). Recent preclinical data with small bispecific antibodies that combine the variable fragments (Fvs) of the two antibodies on one polypeptide chain, including AMG 330, a CD33/CD3 bivalent bispecific T-cell–engaging (BiTE) antibody, demonstrate that such constructs can bring polyclonal CD3$^+$ T cells in close proximity to CD33$^+$ AML cells, trigger lymphocyte activation, and then lead to efficient cytolysis of tumor cells at low effector-to-target (E:T) cell ratios (8–17). However, because of their low molecular weight of approximately 55 kDa, these and similar antibody constructs are readily excreted by the kidneys, resulting in short half-lives and need for prolonged

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Randomized studies with gemtuzumab ozogamicin have validated CD33 as a target for antibody-specific immunotherapy of acute myelogenous leukemia (AML). Here, we report on the design, engineering, and preclinical characterization of a novel treatment approach for patients with AML that is based on CD33/CD3-directed tandem diabodies (TandAbs). Unlike smaller bispecific antibodies, TandAbs provide two binding sites for each antigen to maintain the avidity of a bivalent antibody and have a molecular weight exceeding the renal clearance threshold, thus offering a longer half-life compared to smaller antibody constructs. Our data show high efficacy of these TandAbs against AML cell lines *in vitro* and in experimental animals as well as against primary AML specimens, supporting their further development for clinical use. In addition to the identification of clinical candidates, our studies also identify important principles of cytolytic activity that are likely relevant for other immunotherapeutic strategies that are based on small bifunctional antibodies.

continuous intravenous administration (18–20). This provides a logistical challenge for drug administration and poses a risk of complications, especially infections, in a vulnerable patient population.

Given these limitations, we have constructed tetravalent tandem diabodies (TandAbs) that recognize both CD33 and CD3. A distinguishing feature of TandAbs is their molecular weight of approximately 106 kDa, which exceeds the first-pass renal clearance threshold, thus offering a longer half-life and hence a pharmacokinetic advantage compared to smaller antibody constructs (21). Furthermore, the tetravalent bispecific TandAb format provides the same avidity for each target as an immunoglobulin G (IgG) antibody (22, 23). Here, we describe the *in vitro* characterization of CD33/CD3 TandAbs for the treatment of human AML in comparative analyses using human AML cell lines, patient-derived AML specimens, and AML xenograft models in immunodeficient mice.

Materials and Methods

Construction, expression, and purification of CD33/CD3 TandAbs

For the isolation of human antibody domains specific for CD33, proprietary phage display libraries (AbCheck s.r.o.) with naïve, IgM-derived human single chain Fvs (scFvs) or scFvs with synthetic, randomized CDR3 residues in the VH were screened, using a recombinant CD33-Fc fusion protein containing amino acids 1-243 of human CD33 (24, 25). Five single scFvs were selected, of which three were directly formatted into CD33/CD3 TandAbs (A1, A7, A2). Two scFv domains derived from the naïve library were further affinity matured. One scFv clone was improved by pairwise amino acid walk-through in the CDR3 of the VH. PCR with degenerated codons was used to generate a library with a size of more than 1×10^6 clones with pairwise mutations in the CDR3 of the VH domain. This library was then used for two rounds of panning on human and cynomolgus CD33-Fc fusion proteins. Screening of clones by ELISA using recombinant human and cynomolgus CD33 antigens and flow cytometric analysis with human and cynomolgus $CD33^+$ cell lines yielded affinity-improved scFv domains, of which A3, A4, A5, and A6 were selected. The other scFv clone was improved using the AbAccel approach, a proprietary technology from AbCheck. Clones were screened by ELISA using recombinant CD33 antigen followed by cell binding screens with human and cynomolgus $CD33^+$ cell lines. Among 54 scFv clones with improved affinity for CD33 relative to the parental clone, three scFv domains (A8, A9, A10) were selected for incorporation into TandAbs. These 10 human anti-CD33 scFv domains were used to construct CD33/CD3 TandAbs in combination with four humanized anti-CD3 Fv domains, which were chosen based on their varying affinities to the epsilon chain of CD3. The domain order was VL^{CD3}-VH^{CD33}-VL^{CD33}-VH^{CD3} (Table 1 and Fig. 1). Both outer linkers were composed of six amino acids (Gly-Gly-Ser-Gly-Gly-Ser), while the central peptide linker was one of three sequences: Gly-Gly-Ser-Gly, Gly-Gly-Ser-Gly-Gly, or Gly-Gly-Ser-Gly-Gly-Ser, as shown in Table 1. One TandAb (T151), composed of binding domains for CD3 and an irrelevant antigen (human serum albumin), served as a control. All constructs contained coding sequences for an N-terminal signal peptide and a C-terminal hexahistidine (6xHis)-tag to facilitate antibody secretion, purification, and detection. For TandAb production, suspension-adapted Flp-In CHO cells (Life Technologies) were transfected with the respective gene constructs and selected with Hygromycin B to yield cell pools in which cells possessed a single copy of the product gene. Stable cell pools were typically created within 2 to 3 weeks and subsequently used for the production of secreted recombinant protein in fed batch cultures in shake flasks. Cell culture supernatants were harvested after 10 days, and TandAbs were purified to greater than 90% in a two-step procedure via immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). The purified product forms a single peak with a retention time consistent with an apparent molecular mass of approximately 106 kDa. The integrity of purified TandAbs was assessed by SDS-PAGE. Under denaturing, reducing, and non-reducing conditions, monomeric polypeptides migrated at approximately 50 kDa.

We also attempted to generate BiTE antibodies composed of the CD3 and CD33 binding domains used in our TandAb molecules. However, antibody preparation contained significant amounts of dimers (i.e., flexibodies) with two binding sites for CD33 and two for CD3. We therefore did not conduct comparative studies between TandAb and corresponding BiTE molecules.

Determination of TandAb avidity

The apparent avidity of TandAbs to cells was measured essentially as previously described (26). The $CD33^+$ AML cell line HL-60 or healthy donor human T cells were incubated with serial dilutions of TandAbs in FACS buffer (PBS (Life Technologies) supplemented with 2% FCS (Life Technologies) and 0.1% sodium azide (Roth) for 45 minutes on ice; samples incubated without TandAbs served as negative controls. After repeated washing with FACS buffer, cell-bound TandAbs were detected with 10 μg/mL anti-His antibody (clone 13/45/31-2) followed by FITC-conjugated goat anti-mouse IgG (both Dianova). Cells were washed again and resuspended in FACS buffer containing 2 μg/mL propidium iodide (Sigma) to exclude dead cells. The fluorescence of 5×10^3 living cells was measured with a Guava EasyCyte flow cytometer using the Incyte software (both Merck Millipore). After subtracting the fluorescence intensity values of

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Table 1. Design, binding avidities, and cytotoxic activities of CD33/CD3 TandAbs

TandAb[a]	CD3 domain	CD3 K_D (nmol/L) Human T cells	CD33 domain	CD33 K_D (nmol/L) HL-60 cells	V_H V_L linker sequence (L2)	CD25 induction EC_{50} (pmol/L)[b]	CD69 Induction EC_{50} (pmol/L)[b]	T-cell proliferation in PBMCs EC_{50} (pmol/L)[c]	Cytotoxicity HL-60 cells (% ± SEM)[d]	Cytotoxicity KG-1a cells (% ± SEM)[d]
T563	64	1.3	A5	0.4	GGSG	6	7	7	82.9 ± 3.7	80.2 ± 1.9
T550	64	1.5	A4	0.3	GGSG	6	3	2	84.7 ± 2.3	85.6 ± 1.6
T547	06	1.9	A5	0.5	GGSG	10	6	6	48.0 ± 2.4	78.6 ± 2.3
T562	64	2.1	A3	0.3	GGSG	10	7	6	86.0 ± 0.4	69.8 ± 5.7
T597	64	2.1	A8	9.7	GGSG	ND	225	500	12.4 ± 1.0	0.0 ± 0.2
T613	64	2.3	A10	5.6	GGSG	ND	57	264	24.5 ± 1.9	1.1 ± 0.2
T546	06	2.4	A3	0.5	GGSG	11	7	9	43.2 ± 15.8	74.6 ± 3.2
T548	06	2.6	A6	0.3	GGSG	11	5	6	52.7 ± 8.1	84.7 ± 1.4
T581	64	3.3	A7	5.0	GGSG	30	114	30	4.2 ± 0.2	0.7 ± 0.4
T605	64	4.1	A9	0.7	GGSG	10	4	7	74.2 ± 7.4	44.4 ± 5.3
T564	64	5.1	A6	0.3	GGSG	1	2	3	86.0 ± 1.4	81.3 ± 1.5
T589	64	6.3	A2	2.8	GGSG	9	5	6	79.4 ± 3.5	83.8 ± 2.9
T522	64	49.7	A1	13.7	GGSGGS	134	65	50	6.3 ± 3.3	2.1 ± 0.7
T553	89	55.7	A4	0.2	GGSG	30	22	23	70.4 ± 2.5	1.3 ± 0.4
T497	11	69.5	A5	1.0	GGSGGS	116	74	74	23.8 ± 6.9	0.3 ± 0.3
T479	11	69.8	A4	0.2	GGSG	42	27	4	80.9 ± 3.6	4.6 ± 2.1
T481	11	79.3	A6	0.5	GGSG	94	62	44	24.1 ± 4.0	0.7 ± 0.8
T480	11	81.9	A5	1.1	GGSG	117	87	63	13.1 ± 3.6	0.0 ± 0.5
T498	11	86.3	A6	0.4	GGSGG	39	21	48	45.7 ± 6.4	1.4 ± 0.2
T478	11	94.2	A3	0.6	GGSG	92	91	89	8.0 ± 1.6	0.4 ± 0.4
T609	89	97.2	A9	0.4	GGSG	41	17	37	73.7 ± 2.6	1.5 ± 0.3
T593	89	143.8	A2	4.1	GGSG	98	75	38	31.2 ± 3.9	1.1 ± 0.3

Abbreviation: ND, no CD25 activation detectable.
[a]TandAbs are listed in order of increasing CD3 avidity.
[b]CD25 and CD69 induction was measured after 24 hours in unfractionated PBMC cultures.
[c]T-cell proliferation induced by CD33/CD3 TandAbs in unfractionated PBMCs with $CD33^+$ cells present.
[d]Cytotoxicity (%) after 48 hours of $DAPI^+$ cells at a TandAb concentration of 25 pmol/L in the presence of healthy donor T cells at an E:T cell ratio of 5:1 from three independent experiments performed in duplicate wells.

the cells stained with the secondary and tertiary reagents alone, the values were used for analysis with Prism (version 6.00; GraphPad). For the calculation of K_d, the equation for one-site-binding (hyperbolic) was used (26).

Preparation of healthy donor peripheral blood mononuclear cells or T cells

For the quantification of TandAb-induced induction of CD25 and CD69 expression and the determination of CD3 avidities of bispecific antibodies, buffy coats were commercially obtained from healthy donors (Red Cross Germany, Mannheim, Germany), and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation after overnight storage at room temperature. T cells were subsequently enriched by negative selection using immunomagnetic beads (Stem Cell Technologies). For cytotoxicity assays, unstimulated PBMCs were collected from healthy adult volunteers via leukapheresis by the Fred Hutchinson Cancer Research Center (FHCRC) Hematopoietic Cell Processing Core under research protocols approved by the FHCRC Institutional Review Board (IRB) after written informed consent was obtained. T cells were enriched through magnetic cell sorting either via CD3 Microbeads ("positive enrichment") or the Pan T-Cell Isolation Kit ("negative selection," both from Miltenyi Biotec), and then frozen in aliquots and stored in liquid nitrogen. Thawed cell aliquots were labeled with 3 μmol/L CellVue Burgundy (eBioscience) according to the manufacturer's instructions (13). Although antibody-mediated cytotoxicity was greater with negatively selected T cells than positively enriched T cells, possibly due to competition between CD3 microbeads and the CD33/CD3 TandAbs for binding to CD3, the relative cytotoxic activities of individual TandAbs were unaffected by the method of T-cell selection (data not shown). All cytotoxicity assays were performed with positively enriched healthy donor T cells.

Human AML cell lines and primary human AML cells

Mycoplasma-free human myeloid HL-60 and KG-1a cancer cell lines were obtained and maintained as described previously (27, 28). Specifically, HL-60 (kindly provided by Dr. Irwin D. Bernstein, Fred Hutchinson Cancer Research Center) was maintained in RPMI-1640 medium (Gibco Invitrogen) supplemented with 5% heat-inactivated bovine calf serum (BCS; HyClone). KG-1a cells (kindly provided by Dr. D.E. Banker, Fred Hutchinson Cancer Research Center) were maintained in RPMI medium 1640 with 25 mmol/L HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; GIBCO Invitrogen) supplemented with 10% heat-inactivated FBS (HyClone), 1 mmol/L minimum essential media (MEM) sodium pyruvate, and 0.1 mmol/L MEM nonessential amino acids (both GIBCO Invitrogen). Neither cell line was recently authenticated. Frozen aliquots of Ficoll-isolated mononuclear cells from pretreatment ("diagnostic") peripheral blood or bone marrow specimens from adults with AML were obtained from AML cell repositories at FHCRC, with a sample size chosen to span the entire cytogenetic spectrum of human AML. We used the 2008 WHO criteria to define AML (29) and the refined United Kingdom Medical Research Council (MRC) criteria to assign cytogenetic risk (30). Patients provided written informed consent for the collection and use of their specimens for research purposes under protocols approved by the FHCRC IRB. Clinical data were de-identified in compliance with the Health Insurance Portability and Accountability Act.

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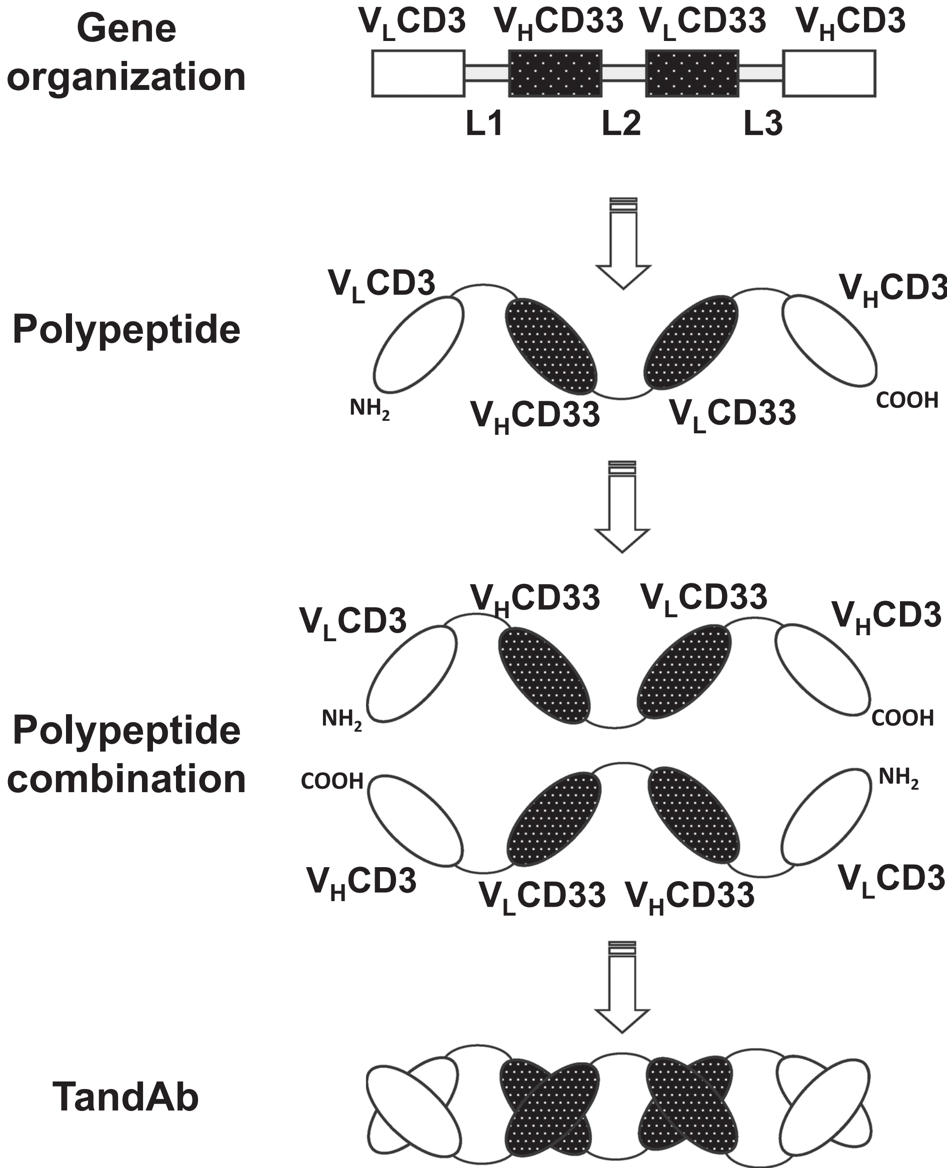

Figure 1.
CD33/CD3 TandAb design. Schematic of the gene organization and domain order of the bispecific, tetravalent CD33/CD3 TandAbs. Sequence of L1 and L3 linker was GGSGGS for all TandAbs studied here. L2 linker varied as listed in Table 1.

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Immunophenotypic characterization of primary AML specimens

After thawing, cells were stained with directly labeled antibodies recognizing CD33 (clone P67.6, PE-Cy7-conjugated), CD3 (clone SK7, PerCP-conjugated), CD34 (clone 8G12, APC-conjugated, all from BD Biosciences), and CD45 (clone HI30, APC-eFluor780-conjugated, eBioscience). To identify nonviable cells, samples were stained with 4′,6-diamidino-2-phenylindole (DAPI). At least 10,000 events were acquired on a Canto II flow cytometer (BD Biosciences), and DAPI cells analyzed using FlowJo (Tree Star; refs. 29, 30).

Quantification of TandAb-induced T-cell activation in unfractionated PBMC cultures

Purified PBMCs were cultured in the presence of various concentrations of TandAbs. After 24 hours, T-cell activation was assessed by flow cytometry after staining of cells with fluorescently labeled antibodies recognizing CD4/CD8, CD25, and CD69. EC_{50} values for induction of CD25 and CD69 expression, and correlations between EC_{50} values and avidity were calculated by nonlinear regression/sigmoidal dose response using Prism.

Quantification of T-cell proliferation

PBMCs or enriched human T cells were cultured in the presence of 11 serial 1:5-dilutions starting at 1 μg/mL of the TandAbs in triplicates. CD3/CD28 activator beads (Dynal Life Technologies) were used as a positive control. After incubation for 4 days, cultures were pulsed overnight with bromodeoxyuridine (BrdUrd) and the incorporation of BrdUrd in proliferating cells quantified using a BrdUrd ELISA (Roche Diagnostics, Mannheim, Germany). EC_{50} values and correlations between EC_{50} values and binding avidities were calculated by nonlinear regression/sigmoidal dose response using Prism.

Quantification of CD33 expression

CD33 expression on AML cell lines and primary AML cells was quantified as described previously (13, 28).

Quantification of TandAb-induced cytotoxicity

To quantify antibody-induced cytotoxicity, AML cells were incubated at 37°C (in 5% CO_2 and air) in 96-well round bottom plates at 5 to 10×10^3 cells per well in culture medium containing various concentrations of the TandAbs as well as CellVue-labeled T cells at different E:T cell ratios, similar to studies described previously (see Supplementary Fig. S1 for analytic strategy; refs. 13, 16, 17). After 48 hours, cell numbers and viability, using DAPI to detect non-viable cells, were determined using a LSRII cytometer (BD Biosciences) and analyzed with FlowJo. Results from cytotoxicity assays are presented as the mean percentage of specific cytotoxicity ± SEM (13, 16, 17).

Activity in xenograft models

In a prophylactic model, 8-week-old NOD/MrkBomTac-Prkdcscid female mice (obtained from Taconic, Denmark, n = 9/group), weighing 20.7 ± 1.48 g, were subcutaneously inoculated with HL-60 cells on day 0. HL-60 cells (CLS Cell Lines Service GmbH were cultivated in RPMI-1640 medium (PAN-Biotech GmbH) and mixed with purified human T cells. One control group did not receive T cells (n = 4). Mice were treated intravenously on days 0, 1, 2, 3, and 4 with either vehicle or CD33/CD3 TandAb T564 (0.1, 1, or 10 μg). The experiment concluded at 29 days. Mean tumor volumes (mm^3) were measured. In a treatment model, HL-60 tumors were established subcutaneously in sublethally irradiated 8-week-old NOD/MrkBomTac-Prkdcscid female mice, weighing 20.6 ± 1.4 g. On day 10 after inoculation, when tumor volumes were 50 to 150 mm^3 (mean 73 ± 11 mm^3), mice (n = 8/group) were injected with 1.5×10^7 activated human T cells intraperitoneally. From days 13 to 21, animals received TandAb T564 (50 μg/animal/d) or vehicle intravenously mean tumor volumes (mm^3) were measured.

Results

Characterization of CD33/CD3 TandAbs

A series of CD33/CD3 TandAbs were constructed through various combinations of 10 human anti-CD33 variable domains and four human anti-CD3 Fvs. On basis of expression titers, homodimer content, and CD33 avidity, 18 candidates were selected for further testing. Four TandAbs with lower CD33 avidity (T522, T581, T597, and T613) were included in the study to assess the effect of differences in CD33 avidity. For all 22 candidate molecules, purity was >90% as measured by SEC. Properties are summarized in Table 1.

TandAb-induced T-cell activation and proliferation

As a first functional characterization of our candidate TandAbs, we measured antibody-induced activation of T cells in unfractionated PBMCs from healthy volunteers that contained $CD33^+$ cells such as mature monocytes (Supplementary Fig. S2). T-cell activation was detected as induction of cell surface expression of CD25 and CD69 24 hours after exposure to TandAbs, with EC_{50} values for individual TandAbs ranging from undetectable to 134 pmol/L for CD25 and from 1 to 225 pmol/L for CD69 (Table 1). EC_{50} values for both activation markers correlated with the avidity of the TandAbs for CD3 (for CD25: $r = 0.787$, $P < 0.0001$, for CD69: $r = 0.482$, $P = 0.023$). In 4-day assays that assessed the ability of the candidate TandAbs to induce T-cell proliferation in unfractionated PBMCs, EC_{50} values for individual TandAbs ranged from 2 to 500 pmol/L and correlated with avidity for CD3 ($r = 0.764$, $P < 0.0001$) as well as CD33 ($r = 0.622$, $P = 0.002$; Supplementary Fig. S3). Induction of CD25 and CD69 was not observed in enriched T-cell cultures, that is, cultures that did not contain $CD33^+$ cells (data not shown). This observation indicated that $CD33^+$ cells were strictly required for stimulation of T cells, that is, that bivalent binding of CD3 by CD33/CD3 TandAbs alone was not sufficient to trigger T-cell activation and subsequent proliferation in the absence of $CD33^+$ cells.

TandAb-induced cytotoxicity against human $CD33^+$ AML cell lines

To screen our 22 CD33/CD3 TandAbs for their cytotoxic potency against $CD33^+$ target cells, we selected two human leukemia cell lines as well-defined AML model systems. Specifically, we chose HL-60 cells as a model with relatively high levels of CD33 expression [MFI (mean ± SEM), 3,133 ± 215, n = 3; ref. 28], whereas KG-1a cells served as model with relatively limited CD33 cell surface display (MFI, 277 ± 11, n = 3; ref. 28). None of the CD33/CD3 TandAbs exerted any noticeable cytotoxic effect on AML cell lines in the absence of T cells, confirming that T cells are required for TandAbs to exert their cytotoxic effects (data not shown). In the presence of T cells, the extent of TandAb-induced specific cytotoxicity depended on the concentration of the TandAb as well as the E:T cell ratio. Direct comparison of the 22 CD33/CD3 TandAbs and the negative control TandAb that did not contain a CD33 binding domain (T151) reproducibly demonstrated considerable differences in antibody-induced cytotoxicity in both HL-60 cells and KG-1a cells (Table 1). Several TandAbs were highly active against both HL-60 and KG-1a cells (T550, T562, T564, and T589). Other TandAbs had high activity against HL-60 but low or no activity against KG-1a cells (T479, T605, and T609) or lower activity on HL-60 compared with KG-1a (T546, T547, and T548). Finally, a number of the TandAbs had limited cytotoxicity on both cell lines (T478, T480, T481, T497, T498, T522, T553, T581, T593, T597, and T613). On basis of these results, nine TandAbs with diverse anti-CD3 avidity were selected for further study: T479, T546, T548, T550, T562, T564, T589, T605, and T609.

Cytotoxic activity in AML patient specimens

To further characterize the cytotoxic properties of selected TandAbs, we obtained pretreatment specimens from 29 AML patients. Upon thaw, all specimens had >58% AML blasts, as determined by flow cytometry based on CD45/side-scatter properties. Twenty-seven of these 29 specimens had >50% viable cells upon thaw and >50% viable cells after 48 hours in cytokine-containing liquid culture and were included in our analyses. AML specimen characteristics are presented in Table 2. Median age of the patients was 58.1 (range, 23.9–76.2) years. Cytogenetic disease risk was favorable in 2, intermediate in 18, and adverse in 7. Information on the mutation status of *NPM1*, *FLT3*, and *CEBPA* was incomplete, however, one sample was known to be $CEBPA^{double\text{-}mutant}$, and one sample was $NPM1^{pos}$/$FLT3\text{-}ITD^{neg}$. The median percentage of myeloid blasts and $CD3^+$ T cells in the studied specimens was 86.1% (range, 58.4%–97.0%) and 2.0% (range: 0-11.9%), respectively, and the median sample viability after 48 hours in culture was 80.1% (range, 53.6%–93.6%).

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Table 2. Characteristics of primary AML specimens

	All patients (n = 27)[a]	Newly diagnosed AML (n = 15)	Relapsed/refractory AML (n = 12)
Median age (range), years	58.1 (23.9–76.2)	64.0 (40.2–76.2)	44.4 (23.9–67.4)
Cytogenetic/molecular risk			
Favorable	2	2	—
Intermediate	18	10	8
CEBPA$^{double\text{-}mutant}$	*1*	*1*	—
NPM1^{pos}/FLT3-ITD^{neg}	*1*	-	*1*
NPM1^{pos}/FLT3-ITD^{pos} or NPM1^{neg}/FLT3-ITD^{pos}	*10*	*5*	*5*
Adverse	7	3	4
Specimen source			
Bone marrow	11	4	7
Peripheral blood	16	11	5
Median % blasts (range)	86.1 (58.4–97.0)	86.1 (66.7–95.5)	86.7 (58.4–97.0)
Median CD33 expression on blasts (range)[b]	849 (5–5,356)	849 (5–5,356)	788 (7–2,242)
Median % T cells (range)	2.0 (0–11.9)	1.6 (0–11.9)	2.1 (0.7–8.7)
Median % viability at 48 hours (range)	80.1 (53.6–93.6)	76.0 (53.6–93.6)	83.5 (63.9–93.1)

[a]Only specimens with >50% viability at completion of experiments are described.
[b]Mean fluorescence intensities

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Fifteen of the patients had newly diagnosed AML, whereas 12 either had relapsed ($n = 7$) or refractory ($n = 5$) disease at the time of specimen collection. Characteristics of the specimens from patients with newly diagnosed AML were similar to those with relapsed/refractory disease with regard to CD33 expression on myeloid blasts, amount of autologous T cells, proportion of myeloid blasts, and culture viability.

The addition of CD33/CD3 TandAbs only (no added T cells) to AML patient specimens resulted in modest, dose-dependent cytotoxicity (Fig. 2), demonstrating that autologous T cells present in the specimens from patients with active AML can be engaged to lyse leukemic cells. With addition of healthy donor T cells, the cytotoxic activity of individual TandAbs was dependent on the antibody concentration and the E:T cell ratio (Fig. 2). Cytotoxic activity was independent of whether the patient had newly diagnosed or relapsed/refractory disease (Fig. 2, Fig. 3A and B). Potent cytotoxicity was observed in patient samples with poor-risk characteristics. There was no correlation between TandAb-induced specific cytotoxicity and CD33 expression for the two most potent TandAbs, as shown in Figs. 3C and4D. Antibodies T609 < T479 < T605 displayed the lowest cytotoxicity of those tested. Both T609 and T479 have relatively low avidity for CD3 (Table 1). Among the nine candidate TandAbs, six had similar cytotoxic activity in primary AML cells at 25 pmol/L: T546, T548, T550, T562, T564, and T589, although T550 appeared slightly more potent, as indicated by more pronounced cytotoxic effects relative to other TandAbs at lower TandAb concentrations (2.5 and 10 pmol/L).

Xenograft models

One of the CD33/CD3 TandAbs (T564) was selected for *in vivo* studies based on cytotoxicity, high target avidity, stimulation of T-cell activation and proliferation in the presence of $CD33^+$ target cells. T564 demonstrated dose-dependent tumor growth delay in a prophylactic HL-60 graft NOD/scid mouse model (Fig. 4A). T564 also substantially inhibited tumor growth in an established HL-60 xenograft NOD/scid mouse model (Fig. 4B).

Discussion

Antigen-specific immunotherapy presents a promising strategy for the treatment of many human cancers, including AML. Although an increasing number of antigens have been considered as target for such therapies in AML, most exploited thus far is CD33, particularly with the CD33 antibody-drug conjugate, GO. However, only subsets of patients have shown improvements in survival with GO, and several other unconjugated or conjugated CD33 antibodies have failed in the clinic (2). Bispecific antibodies that harness cytotoxic effector cells in the elimination of cancer cells are an attractive and long-pursued means of increasing the efficacy of therapeutic antibodies. Although initial efforts were largely unsuccessful, recent clinical data with the CD19/CD3 BiTE antibody blinatumomab in acute lymphoblastic leukemia support the validity of this strategy (18, 20), but important limitations with regard to pharmacokinetics have been recognized. Therefore, the approach we have taken here relies on T-cell–recruiting TandAbs to potentially overcome such limitations. Specifically, properties of TandAbs relative to other fragment antibody formats, such as BiTE antibodies, are avidity and a higher molecular weight that avoids first-pass renal clearance (21). The CD33/CD3 TandAbs have not yet undergone pharmacokinetic testing in relevant preclinical animal models. However, the T-cell recruiting anti-CD19/CD3 TandAb AFM11 displayed $t_{1/2}$ of 18 to 22 hours in mouse, a non-crossreactive species (26). The NK cell recruiting anti-CD30/CD16 TandAb AFM13 demonstrated terminal half-lives ($t_{1/2}$) in patients with Hodgkin lymphoma that increased with dose, ranging from 8.72 to 19.2 hours (21). In contrast, the reported $t_{1/2}$ of blinatumomab in humans is approximately 1.25 hours (31). Taken together, these results suggest that the $t_{1/2}$ of the anti-CD33/CD3 TandAbs should exceed that of the BiTE antibodies although target-mediated drug disposition may influence the pharmacokinetics of the molecules (32).

Using a series of anti-CD33 and anti-CD3 Fvs, we were able to generate a large number of candidate CD33/CD3 TandAbs that could be stably produced in transfected cell lines. Our comprehensive *in vitro* evaluation of constructs that featured a broad range of avidities for CD3 or CD33 revealed important mechanistic principles of these antibodies and identified promising candidates for further investigation. Together, our findings support three main conclusions. First, CD33/CD3 TandAbs can induce potent cytolysis of $CD33^+$ cell lines and primary AML cells in the presence of T cells in a dose-dependent

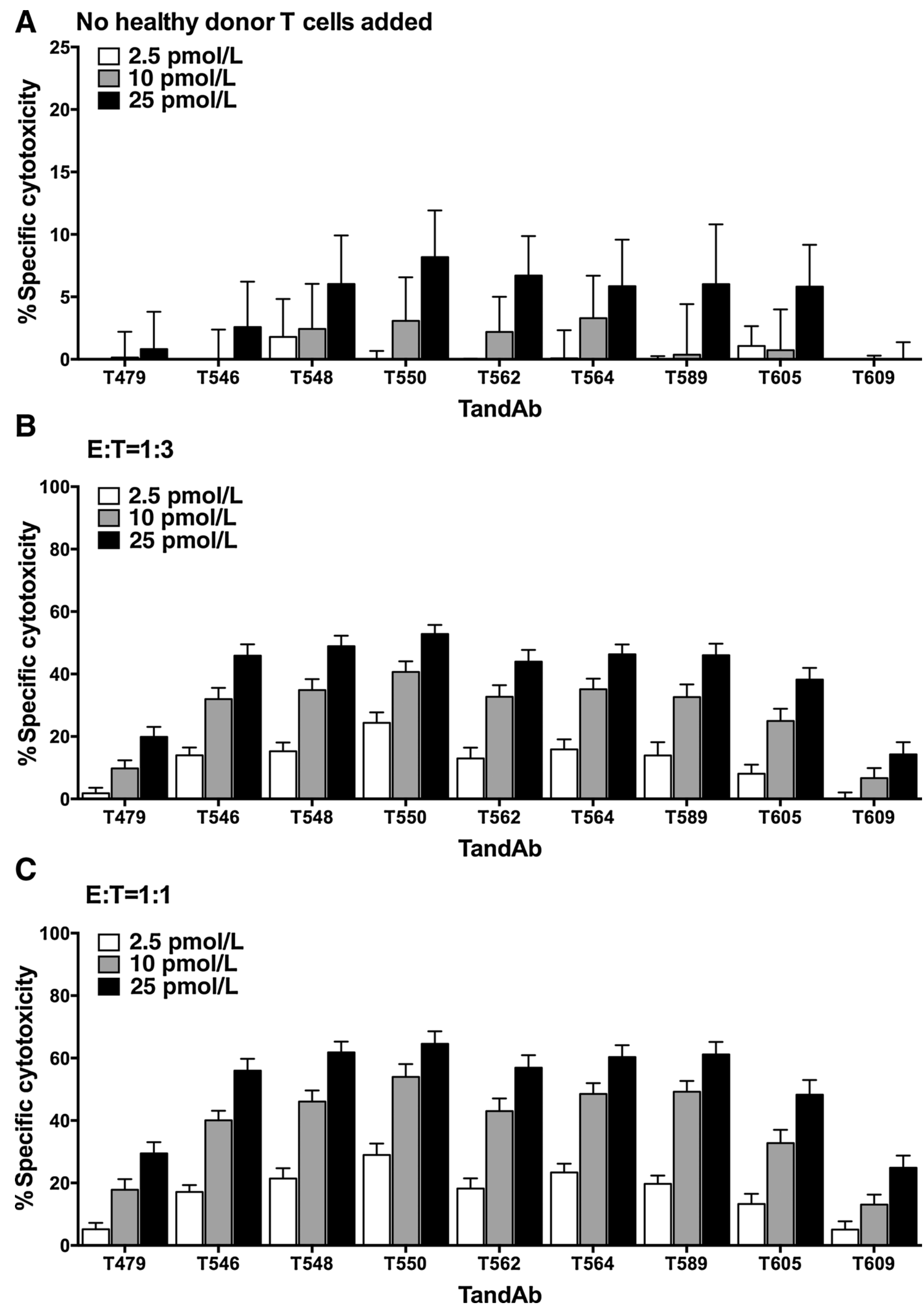

Figure 2.
Cytotoxicity of selected TandAbs in primary AML specimens. Blood or bone marrow specimens from patients with AML ($n = 27$) were incubated with increasing concentrations of one of nine TandAbs (**A**) with antibody and healthy donor T cells at an E:T cell ratio of either 1:3 (**B**) or 1:1 (**C**) as indicated. After 48 hours, cell counts were determined and cytotoxicity was assessed with DAPI staining to quantify drug-specific cytotoxicity. Results are shown as mean ± SEM for the percentage of specific cytotoxicity from experiments performed in duplicate wells. Note the difference in the scale of panels.

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fashion. Second, for optimal activation of T cells and maximum cytotoxicity in AML cell lines and patient specimens *in vitro*, high avidity for CD3 is required, consistent with CD3-binding being crucial for the mechanism of action. And third, TandAbs with high avidity for CD33 and CD3 were highly and broadly active in primary AML patient specimens regardless of cytogenetic risk, stage of the disease, and CD33 expression levels. Such breadth of activity may be clinically significant.

Small bispecific antibodies that recruit $CD3^+$ T cells to an antigen-expressing tumor can trigger T-cell activation and proliferation and facilitate the destruction of tumor cells through perforin/granzyme–mediated apoptosis (33). Consistent with this general mechanism, T cells were activated by CD33/CD3 TandAbs, as shown by induction of CD25 and CD69, and subsequently stimulated to proliferate. Of note, EC_{50} values for both activation markers correlated with the K_D value of the TandAbs for CD3, identifying the avidity for CD3 as critical determinant for efficient T-cell activation. Furthermore, TandAb-induced T-cell proliferation correlated with K_D values for CD3 as well as CD33, indicating that high avidity to both CD3 and CD33 is required for maximum T-cell engagement. However, even for molecules with high avidity to CD3, activation of T cells and subsequent

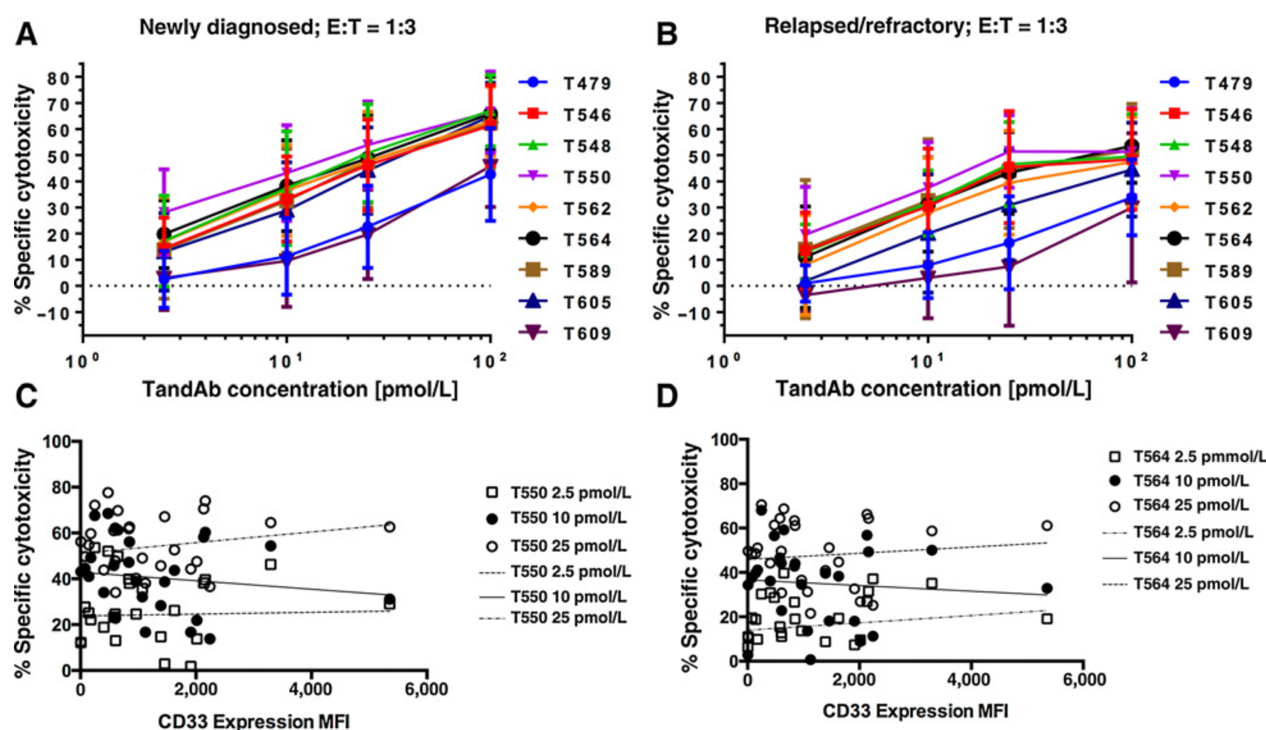

Figure 3.

Cytotoxicity of selected TandAbs in primary AML specimens and correlation with CD33 expression levels. Blood or bone marrow specimens from patients with AML ($n = 27$) were incubated with increasing concentrations of one of nine TandAbs. **A** and **B,** Cytotoxic activity of TandAbs was measured in newly diagnosed (**A**) and relapsed/refractory AML patient samples (**B**) over a range of CD33 expression. The percentage of specific cytotoxicity is plotted as a function of increasing concentration of antibody. **C** and **D,** Specific cytotoxicity plotted as a function of CD33 expression in primary AML patient samples at 2.5, 10, and 25 pmol/L for TandAbs T550 (**C**) and T564 (**D**) with an E:T ratio of 1:3.

proliferation requires the presence of CD33$^+$ target cells, arguing against the concern of non-specific T-cell activation as a result of efficient binding to T cells.

To characterize the cytotoxic properties of the CD33/CD3 TandAbs, we first conducted cytotoxicity screening studies in AML cell lines. Selected TandAbs were then analyzed using primary AML specimens. These analyses demonstrated that the TandAbs not only activated T-lymphocytes but also re-directed polyclonal T cells from healthy donors as well as autologous T cells from patients with AML to effectively lyse CD33$^+$ AML cells even at low E:T cell ratios. The anti-AML effect of these TandAbs was dependent on antibody concentration and E:T cell ratio, and required the presence of both T cells and CD33$^+$ target cells—mechanistic determinants that are similar to those identified recently for small CD33/CD3-directed bispecific antibodies (8–17). Importantly the TandAbs' avidity of CD3 and CD33 strongly influenced the degree of cytotoxic activity in that, for high antileukemia potency, high avidity to both CD33 and CD3 was required.

TandAbs with high avidity to CD33 and CD3 were highly active in a large number of primary AML specimens. In our studies, the cytotoxic activity was independent of whether the patient had newly diagnosed or relapsed/refractory disease, and potent cytolytic effects were observed in samples with poor-risk cytogenetic abnormalities. For the most potent constructs in our series, we did not find any correlation between TandAb-induced specific cytotoxicity and CD33 expression. These findings suggest that high-avidity CD33/CD3 TandAbs could be active against human AML across the entire cytogenetic/molecular disease spectrum, even in cases of minimal CD33 expression. Of note, drug-specific cytotoxicity was also seen in the presence of residual autologous T cells but was significantly augmented by the addition of controlled amounts of healthy donor T cells as anticipated under the assay conditions used here. CD33/CD3 TandAb-mediated cytotoxicity in the autologous setting is under investigation. In prophylactic and established AML xenograft models, T564 significantly inhibited tumor growth when administered with human T cells. Importantly, the antitumor activity observed in the established model suggests that T564 successfully recruited human T cells to the tumor.

Our data with PBMC co-cultures highlight the fact that CD33 on normal hematopoietic cells, in particular monocytes and other maturing and mature myeloid cells, serves as a target for CD33/CD3 TandAbs and leads to activation of T cells, an observation that is consistent with findings obtained with bivalent CD33/CD3 bispecific antibodies such AMG 330 (11, 14). This "on-target, off-AML" effect is expected to result in significant cytopenias and may pose a clinical challenge for the care support of patients with AML when potent CD33/CD3-directed small bispecific antibodies are used.

In summary, the data presented here show that CD33/CD3-directed TandAb molecules can induce potent cytolysis of CD33$^+$ leukemic cells *in vitro* and inhibit tumor growth *in vivo*. Our detailed preclinical analyses provide support for the concept that

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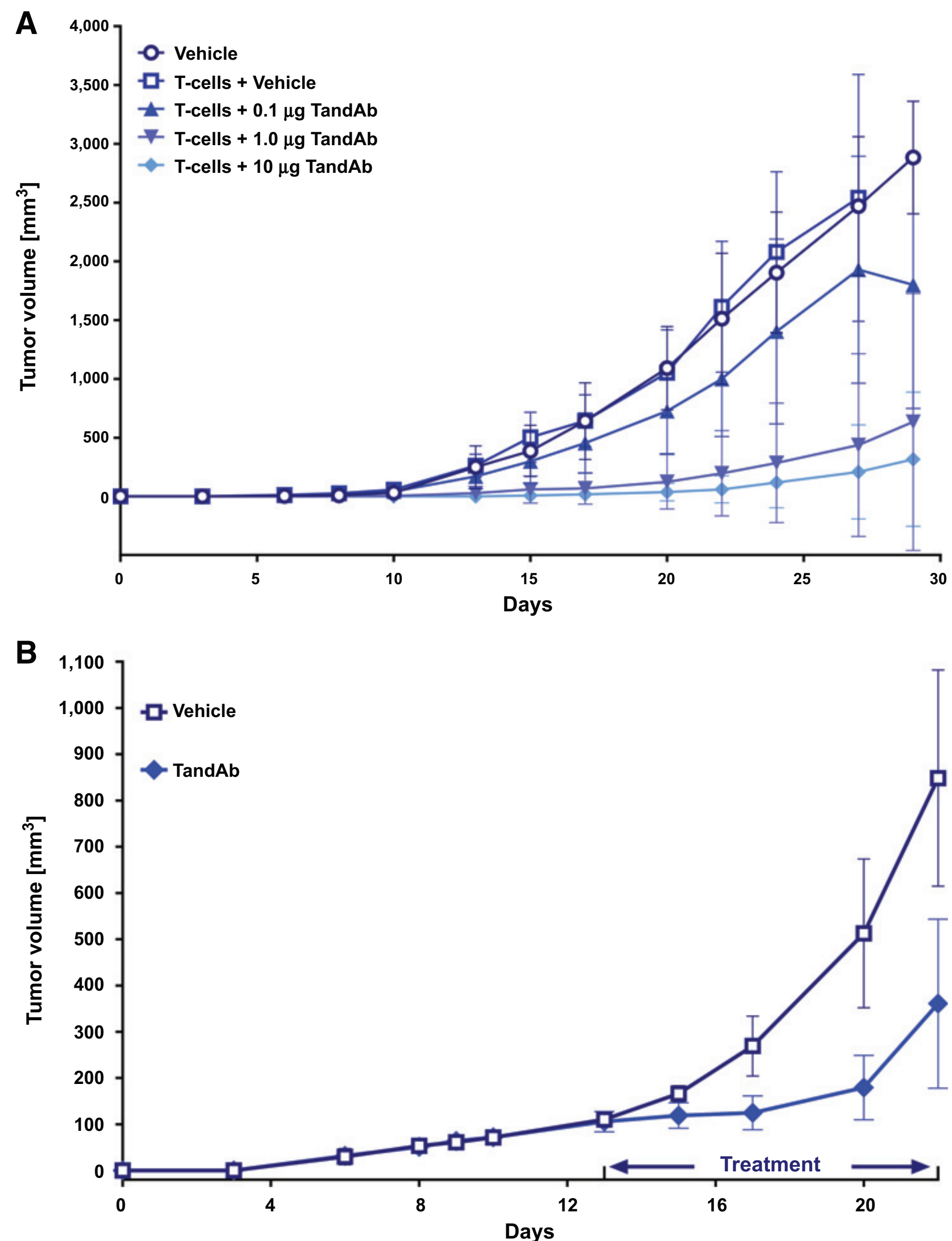

Figure 4.
Activity of TandAb T564 in xenograft models. **A,** A prophylactic model. On day 0, NOD/scid mice (n = 9/group) were inoculated subcutaneously with HL-60 cells. HL-60 cells were mixed with purified human T cells. The control group did not receive T cells. Mice were treated intravenously on days 0, 1, 2, 3, and 4 with either vehicle or CD33/CD3 TandAb T564 (0.1, 1, or 10 µg). Mean tumor volumes (mm^3) ± SD are presented. **B,** An established model. HL-60 tumors were established subcutaneously in sublethally irradiated NOD/scid mice. On day 10, when tumor volumes were 50 to 150 (mean of 73 ± 11 mm^3), mice (n = 8 per group) were intraperitoneally injected with 1.5×10^7 activated human T cells. From days 13 to 21, animals received TandAb T564 (50 µg/animal/d) or vehicle intravenously. Mean tumor volumes ± SD are presented.

high avidity to both CD33 and CD3 is required to maximize TandAb-induced T-cell activation and anti-AML efficacy. As high-avidity CD33/CD3-directed TandAbs are broadly active in primary AML *in vitro* and can escape renal clearance, our data suggest that such molecules should be further explored as novel option for the treatment of patients with AML.

Disclosure of Potential Conflicts of Interest

E.A. Zhukovsky has ownership interest (including patents) in Affimed Therapeutics. J.A. Fox is an employee of Sunesis Pharmaceuticals. J. Guenot has ownership interest (including patents) in Amphivena Therapeutics. R.B. Walter reports receiving commercial research grants from Amgen, Amphivena Therapeutics, and Seattle Genetics, has ownership interest (including patents) in Amphivena Therapeutics, and is a consultant/advisory board member for Amphivena Therapeutics, Covagen, Emergent Biosolutions, Pfizer, and Seattle Genetics. No potential conflicts of interest were disclosed by the other authors.

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