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## Targeting Human C-Type Lectin-Like Molecule-1 (CLL1) with a Bispecific Antibody for Acute Myeloid Leukemia Immunotherapy\*\*

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**Abstract**

Acute myeloid leukemia (AML), the most common acute adult leukemia and the second most common pediatric leukemia, still has a poor prognosis. Human C-type lectin-like molecule-1 (CLL1) is a recently identified myeloid lineage restricted cell surface marker, which is overexpressed in over 90% of AML patient myeloid blasts and in leukemic stem cells. Here, we describe the synthesis of a novel bispecific antibody,  $\alpha$ CLL1- $\alpha$ CD3, using the genetically encoded unnatural amino acid, p-acetylphenylalanine. The resulting  $\alpha$ CLL1- $\alpha$ CD3 recruits cytotoxic T cells to CLL1 positive cells, and demonstrates potent and selective cytotoxicity against several human AML cell lines and primary AML patient-derived cells in vitro. Moreover,  $\alpha$ CLL1- $\alpha$ CD3 treatment completely eliminates established tumors in an U937 AML cell line xenograft model. These results validate the clinical potential of CLL1 as an AML specific antigen for the generation of a novel immunotherapeutic for AML.

**Keywords**

CLL1; unnatural amino acid; cancer immunotherapy; bispecific antibody; Acute Myeloid Leukemia

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Acute myeloid leukemia (AML), the most common acute adult leukemia and the second most common pediatric leukemia, is a disease characterized by the accumulation of rapidly proliferating and undifferentiated myeloid blasts in the bone marrow and the peripheral blood. Despite decades of clinical research, the five-year survival rate of AML is less than 30% for adult patients regardless of receiving hematopoietic stem cell transplantation (HSCT)<sup>[1]</sup>. A number of surface antigens such as CD33, CD123, CD44, TIM-3, CD47 and CD32 have been explored as target antigens for AML treatment in the last few decades<sup>[2]</sup>. Some of these markers, however, can also be found in the healthy hematopoietic stem cell (HSC) compartment of normal bone marrow<sup>[3]</sup>; indeed, severe hematological toxicity was observed in targeted therapy utilizing an  $\alpha$ CD33 antibody conjugated to calicheamicin (Mylotarg)<sup>[1-2, 4]</sup>. An ideal target for AML should therefore have minimum expression in the HSC compartment and on other normal cells for an improved therapeutic index. Human C-type lectin-like molecule-1 (CLL1, or CLEC12A), a recently identified myeloid lineage

restricted cell surface marker, is an excellent therapeutic target for AML due to its overexpression in blasts and the so-called leukemic stem cells (LSCs)<sup>[5]</sup> in a majority (80–90%) of patients<sup>[3b, 6]</sup>. Importantly, it has been reported that CLL1 expression is absent in the HSC compartment of normal and regenerating bone marrow<sup>[3b, 6d]</sup>. Thus far, a monoclonal antibody targeting CLL1 has demonstrated therapeutic potential against AML cell lines via complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) mechanisms<sup>[6a]</sup>.

Bispecific antibodies that potently induce target cell-dependent T cell activation have recently emerged as a successful immunotherapeutic approach for the treatment of cancer. Bispecific antibodies act through an amplified cytotoxic signal from tumor localized activated effector T cells, making this an attractive approach for killing quiescent cancer stem cells, cancers overexpressing drug pumps, and cancer cells with low levels of surface antigens<sup>[7]</sup>. For AML, a bispecific antibody that targets the CD33 antigen, has previously been evaluated<sup>[8]</sup>. This bispecific antibody is selectively cytotoxic to AML cell lines and primary patient samples, and shows moderate efficacy in mouse xenograft models. However, other AML associated antigens, such as the relatively novel marker CLL1, have yet to be successfully explored as targets for bispecific antibodies.

Previously, we reported an efficient method for generating bispecific antibodies (BiFabs) with defined geometries by conjugating two antigen-binding fragments (Fab) of antibodies that are site-specifically modified with bio-orthogonal chemical linkers<sup>[9]</sup>. Using this same method, we now report the synthesis of a novel CLL-1 targeting BiFab,  $\alpha$ CLL1- $\alpha$ CD3, and a comparison of its *in vitro* and *in vivo* activity to a similarly constructed CD33 targeting BiFab,  $\alpha$ CD33- $\alpha$ CD3. We show that although both BiFabs are cytotoxic toward AML cell lines and patient-derived cells, the  $\alpha$ CLL1- $\alpha$ CD3 bispecific antibody has increased potency *in vitro*, and in contrast to  $\alpha$ CD33- $\alpha$ CD3, completely eliminates established tumors in a subcutaneous xenograft mouse model using the human AML cell line U937.

To synthesize  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3, we first expressed *p*-acetylphenylalanine (*p*AcF) mutants of  $\alpha$ CD33 (clone hM195)<sup>[10]</sup>,  $\alpha$ CLL1 (clone 1075.7)<sup>[6a]</sup> and  $\alpha$ CD3 (clone UCHT1) Fabs in *Escherichia coli* (*E. coli*)<sup>[11]</sup>. Briefly, a *Methanococcus jannaschii*-derived orthogonal amber suppressor tRNA and aminoacyl-tRNA synthetase (aaRS) pair, which was previously evolved to selectively incorporate *p*AcF into proteins, was coexpressed with Fab genes containing a TAG codon at position S206 (LC S206-*p*AcF  $\alpha$ CD33) or S203 (LC S203-*p*AcF  $\alpha$ CLL1) on the light chain, or K138 on the heavy chain (HC K138-*p*AcF  $\alpha$ CD3) in the presence of *p*AcF. The molecular weight of each Fab mutant was verified by SDS-PAGE and ESI-MS (Table S1). Notably, the  $\alpha$ CD33 and  $\alpha$ CLL1 Fab mutants showed comparable binding affinities to their corresponding antigens as revealed by ELISA analysis- IC<sub>50</sub> values of the  $\alpha$ CD33 and  $\alpha$ CLL1 Fab mutants are ~ 7.0 and 6.1 nM, respectively (Fig. S1).  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3 were generated according to a previously reported protocol<sup>[9b]</sup> (Fig. 1A, Table. S1), purified by size exclusion chromatography (GE Healthcare), and removed of endotoxin with Mustang Q membrane (Pall Corp.). The overall recovery yields of purified BiFabs were ~65%. The size of the BiFabs was confirmed by SDS-PAGE gel (Fig. 1B) and the binding affinities of BiFabs to purified CD33 or CLL1 antigens were comparable to the  $\alpha$ CD33 and  $\alpha$ CLL1 Fab mutants as

measured by ELISA, indicating that the conjugation reactions do not interfere with antibody-antigen interactions (Fig. S1). Moreover, the  $\alpha$ CD33 and  $\alpha$ CLL1 Fabs selectively bind to the human AML cancer cell line U937 (CD33<sup>+</sup>, CLL1<sup>+</sup> and CD3<sup>-</sup>);  $\alpha$ CD3 Fab binds only to human T cells (CD33<sup>-</sup>, CLL1<sup>-</sup> and CD3<sup>+</sup>), and  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3 bind both U937 and T cells with comparable affinity as determined by flow cytometry (Fig. 1C and 1D).

To test the ability of  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3 to recruit effector cells and lyse target AML cancer cells, we developed a flow cytometry based cell viability assay (detailed protocol available in SI) to quantify the absolute numbers of viable AML cells in the presence of human peripheral blood mononuclear cells (PBMC). As shown in Fig. 2A, at an effector/target cell (E/T) ratio of 10/1, both  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3 potently lysed more than 90% of U937 cells after 24h of incubation, with EC<sub>50</sub> values of ~445 pM and ~41 pM, respectively; after 48h incubation the EC<sub>50</sub> values were approximately 25 pM ( $\alpha$ CD33- $\alpha$ CD3) and 2.1 pM ( $\alpha$ CLL1- $\alpha$ CD3), respectively. Similar potencies were observed with the HL-60 AML cell line (CD33<sup>+</sup> and CLL1<sup>+</sup>) after 24h of incubation (Fig. 2B). In contrast, neither BiFab displayed any significant cytotoxicity to the CD33<sup>-</sup> and CLL1<sup>-</sup> human B-lymphoblast cell line, RS4;11 (Fig. S2). Moreover, a negative control using a non-conjugated mixture of the corresponding antibodies, failed to show any significant toxicity at concentrations up to 25 nM. To determine whether the cytotoxicity of the BiFabs is associated with antigen abundance on target cells, three human AML cell lines with different CD33 and CLL1 expression levels (U937: CD33 and CLL1 high; KASUMI-3: CD33 and CLL1 medium; and KG-1A: CD33 and CLL1 low; see Fig. S3) were used to compare the cytotoxicities of BiFabs after a 24h incubation period. Differential toxicities and potencies of both BiFabs were observed and correlated with the antigen expression levels of each cell line (Fig. 2C–2D). Mechanistic studies indicated antigen-specific T cell activation and proliferation (Fig. S4–S5). Taken together, our data confirm that the BiFabs potently and selectively lyse target cells in an antigen-dependent manner. Notably,  $\alpha$ CLL1- $\alpha$ CD3 is 5-fold more potent on average than  $\alpha$ CD33- $\alpha$ CD3 against all cell lines tested, underscoring the potential advantage of  $\alpha$ CLL1- $\alpha$ CD3 as a therapeutic for AML.

Next, to provide more clinically relevant evidence for the therapeutic potential of the BiFabs, we tested the *ex vivo* toxicity of  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3 against primary AML patient derived samples. PBMCs from seven AML patients (denoted as AML1-7, Table S2) were isolated using Ficoll density gradient centrifugation, and analyzed for subgroups of leukemic blasts<sup>[12]</sup>, T cells, and CD33<sup>+</sup>/CLL1<sup>+</sup> cells (Table S2) by flow cytometry. Fig. S6 depicts a representative gating scheme to identify blasts from a primary specimen (7-AAD<sup>-</sup>/CD34<sup>+</sup>/CD45<sup>dim</sup> or 7-AAD<sup>-</sup>/SSC<sup>low</sup>/CD45<sup>dim</sup>). Flow cytometric analysis revealed that blasts in primary patient samples have differential expression levels of the CD33 and CLL1 antigens<sup>[13]</sup>, as determined by mean fluorescence intensity (MFI) values (see Table S2). Interestingly, among the seven primary samples, one (AML1) is CD33<sup>-</sup>/CLL1<sup>+</sup>, one (AML6) is CD33<sup>+</sup>/CLL1<sup>-</sup>, and the remaining five samples are CD33<sup>+</sup>/CLL1<sup>+</sup>.

Patient PBMCs were incubated in specialized serum-free medium (SFM) for a maximum of 6 days<sup>[14]</sup> with varying concentrations of BiFabs and monitored for cytotoxicity at different

time points by flow cytometry.  $\alpha$ CLL1- $\alpha$ CD3 induced satisfactory target cell lysis of AML1 (CD33<sup>+</sup>/CLL1<sup>+</sup>) blast cells within 24h at 3.2 pM and reached a plateau of ~72% blast killing at 80 pM (Fig. 3A and Fig. S7). However,  $\alpha$ CD33- $\alpha$ CD3 showed poor cytotoxicity (EC<sub>50</sub> ~601 pM) against AML1 blast cells, likely a consequence of the different CLL1 and CD33 expression levels (Table S2). In contrast, AML6 (CD33<sup>+</sup>/CLL1<sup>-</sup>) blast cells did not respond to a high concentration (25 nM) of  $\alpha$ CLL1- $\alpha$ CD3 after 6 days of incubation (Fig. S8), but showed modest cytotoxicity with  $\alpha$ CD33- $\alpha$ CD3, confirming the target selectivity of BiFabs in primary patient samples. As for the five samples that are double-positive (CD33<sup>+</sup>/CLL1<sup>+</sup>), modest to excellent cytotoxicity (EC<sub>50</sub> values ranging from 37–5170 pM, Table S2) was observed after 3–6 days of incubation with either  $\alpha$ CLL1- $\alpha$ CD3 or  $\alpha$ CD33- $\alpha$ CD3 (Fig. S9–S11). Of note, although AML7 blast cells express both CD33 and CLL1 at high levels, this primary sample failed to respond to both BiFab treatments under our assay conditions (Fig. 3D). The onset of blast cell death also differed amongst the samples. For instance, unlike the AML1 blasts which rapidly (~24h) responded to  $\alpha$ CLL1- $\alpha$ CD3 (Fig. 3A), the AML5 blasts only showed detectable cytotoxicity after 24h, and reached a plateau after 72h incubation (Fig. 3B) with a maximum blast killing of 85% (EC<sub>50</sub> ~513 pM) and 73% (EC<sub>50</sub> ~37 pM) for  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3, respectively (Fig. S11 and Table S2). In all instances, delayed or lack of responsiveness to BiFab treatment may potentially be attributed to the heterogeneity and/or suppressed T cell activity of primary samples [13].

Considering the significantly lower proportion of T cells relative to blasts in AML patient samples (Table S2), and the high potential for suppressed T cell activity in these patients, we next tested whether BiFabs can redirect *ex vivo* expanded autologous T cell activity in the less responsive primary patient samples (i.e., AML2, AML3, AML4, AML6 and AML7). Briefly, one vial of frozen patient PBMCs were thawed, activated by  $\alpha$ CD3/ $\alpha$ CD28 antibody conjugated beads, and maintained with recombinant human IL-2 (rhIL-2) to enrich for mature T cells; 1–2 days prior to experimentation, cells were rested for 24 to 48h in SFM medium without rhIL-2. The *ex vivo* expanded T cells were then mixed with freshly thawed PBMCs from the same patient and seeded into a 96-well plate at indicated E/T ratios. In the case of AML3 blast cells at an E/T ratio of 1/1, cell lysis was observed in 24h, and reached a plateau of 85% (EC<sub>50</sub> ~37 pM) and 73% (EC<sub>50</sub> ~5.8 pM) blast killing after 48h with  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3, respectively (Fig. 3C and Table S2). These results demonstrate an overall improvement in EC<sub>50</sub> and induction time compared to the cytotoxicity of non-activated autologous T cells induced by BiFabs. Similar results were observed with activated T cells from AML2, AML4, AML6 and AML7 blasts (Fig. S9, S10, S8, 3D and Table S2). Interestingly, AML7 blast cells, which previously failed to respond to both BiFabs under native conditions, displayed excellent response when activated autologous T cells were used as effector cells with EC<sub>50</sub> values of 77 and 12 pM at day 2 for  $\alpha$ CD33- $\alpha$ CD3 and  $\alpha$ CLL1- $\alpha$ CD3, respectively. However, even in the presence of expanded T cells,  $\alpha$ CLL1- $\alpha$ CD3 treatment was ineffective against AML6 (CLL1<sup>-</sup>) blast cells, confirming the excellent selectivity of  $\alpha$ CLL1- $\alpha$ CD3 (Fig. S8 and Table S2). Of note, nonspecific killing of blasts cells was not observed at an E/T ratio as high as 4/1 with the same expanded autologous T cells in the absence of BiFabs (Fig. S12). Collectively, these results highlight the significance of functional T cells in this bispecific antibody approach (Fig. 3D and Table S2). Finally, it should be noted that among the 5 AML patients that are

both CD33<sup>+</sup> and CLL1<sup>+</sup> (AML2, AML3, AML4, AML5, and AML7), αCLL1-αCD3 showed higher potency than αCD33-αCD3 in 4 samples (AML3, AML4, AML5, and AML7, Table S2), which is in excellent agreement with previous *in vitro* killing results in AML cell lines.

The *in vivo* anti-tumor activity of the αCLL1-αCD3 was assessed in immunodeficient mice bearing tumors derived from human AML cell line U937. U937 cells were inoculated into the subcutaneous flank of NOD/SCID/IL-2Rγ<sup>-/-</sup> (NSG) mice and human PBMCs isolated from a healthy donor were injected intravenously (I.V.) into the mice on the same day. Xenografts were allowed to establish to an average size of 100–150 mm<sup>3</sup>, after which human T-cells from the same healthy donor that had been expanded *in vitro* were injected I.V. into the tumor bearing mice. One day after T-cell injection, mice were injected I.V. with either the BiFabs or PBS daily for 10 days; the daily dosing schedule was dependent upon the half-life (~5h) of similar BiFabs in mice (unpublished data). Tumors in PBS treated mice showed rapid growth with a doubling time of ~2 days. In contrast, a significant reduction in tumor growth was observed in mice treated with BiFabs (Figure 4A). Moreover, the tumors in αCLL1-αCD3 treated mice steadily decreased in size and were barely detectable after 10 daily injections, demonstrating the ability of αCLL1-αCD3 to eradicate U937 tumor xenografts (Figure 3A). Single-cell preparations of residual tumors were analyzed by flow cytometry and revealed the presence of intra-tumoral T-cells in all BiFab-treated mice, whereas no significant T-cell infiltration was observed in tumors from PBS-treated animals (Figure 4B). Additionally, no negative effects on the general health or body weights of the mice were observed in the BiFab-treated groups (Figure S13).

AML is a heterogeneous cancer with hierarchical cell populations that vary between individual patients, making it difficult to completely remove all malignant cells by a therapeutic agent that targets a single antigen. Indeed, analysis of AML CD33 and CLL1 expression in patients reveals simultaneous expression in most patients, but cases of single antigen expression (CD33<sup>+</sup>/CLL1<sup>-</sup> or CD33<sup>-</sup>/CLL1<sup>+</sup>) have also been reported in the literature [6b] and were observed in our AML patient samples. We, therefore, decided to generate a αCLL1-αCD3 bispecific antibody and compare it to a similarly generated αCD33-αCD3. αCLL1-αCD3 demonstrated potent and selective *in vitro* and *in vivo* cytotoxicity against various AML cell lines as well as *ex vivo* cytotoxicity against primary patient samples. Interestingly, αCLL1-αCD3 outperformed αCD33-αCD3 (despite similar apparent target binding affinities to their respective antigens) in all the AML cell lines we tested. However, it is worth pointing out that, given the heterogeneity of primary patient specimens, the efficacies of our BiFabs (and other T cell engagers) are determined by a multitude of parameters including E/T ratio, antigen expression level, cytogenetics, and treatment history of patients. Nevertheless, this study demonstrates the potential of CLL1 as an AML target antigen, and our αCLL1-αCD3 as a promising T cell engaging bispecific agent. We are currently assessing the efficacy of αCLL1-αCD3 in additional primary samples and its ability to discriminate LSC and HSC populations in bone marrow samples from healthy donors and AML patients.

## Experimental Section

Experimental Details are available in the supporting information.

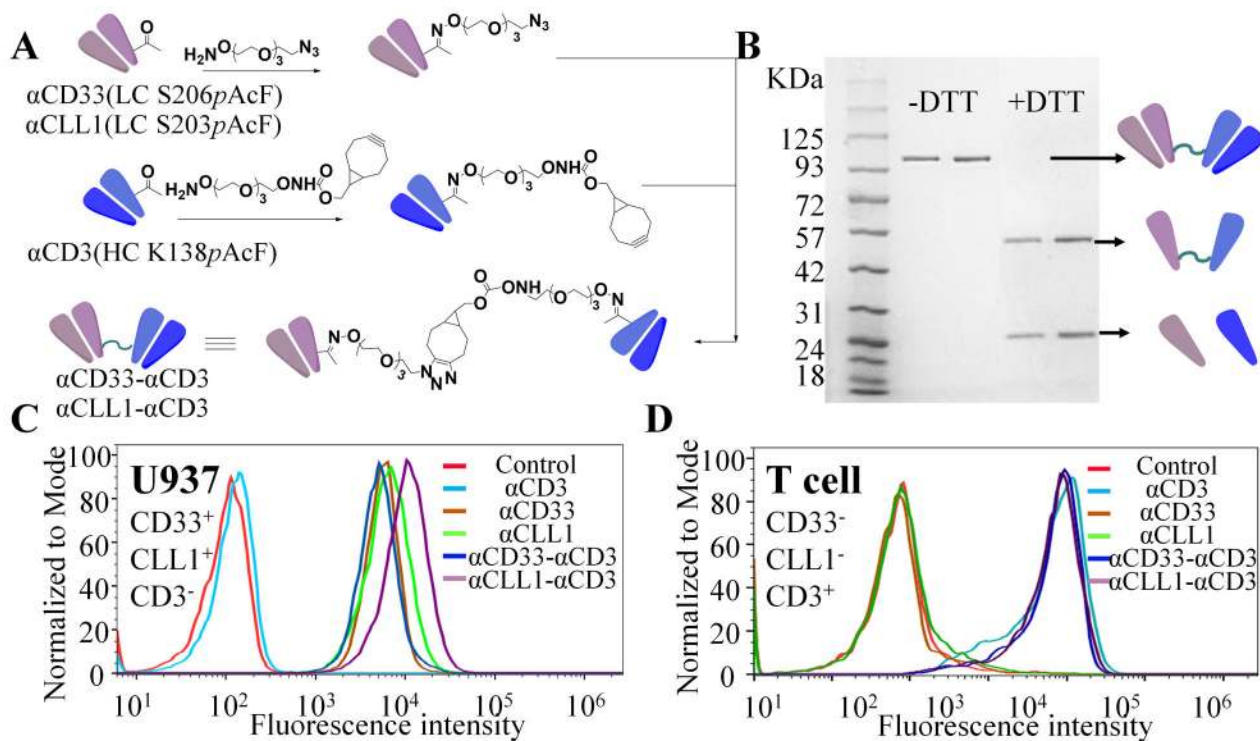
## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

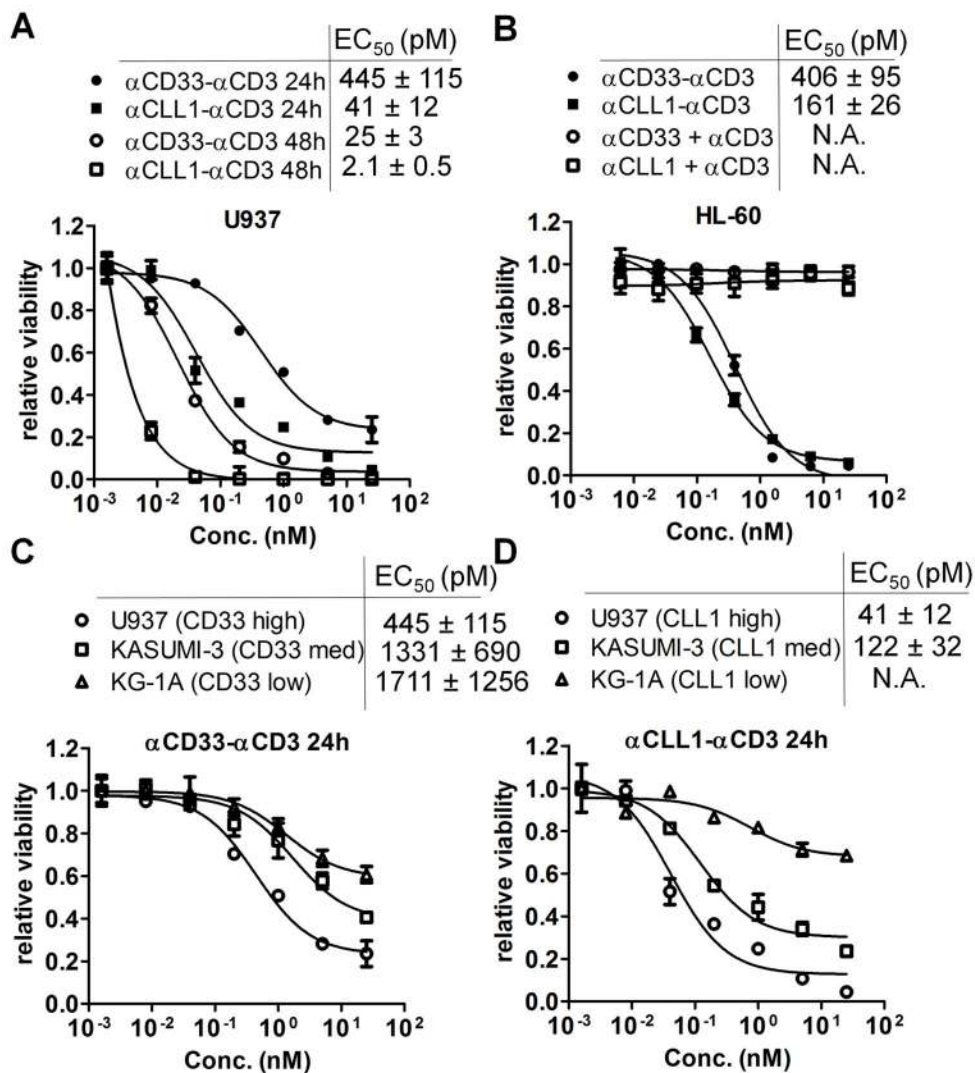
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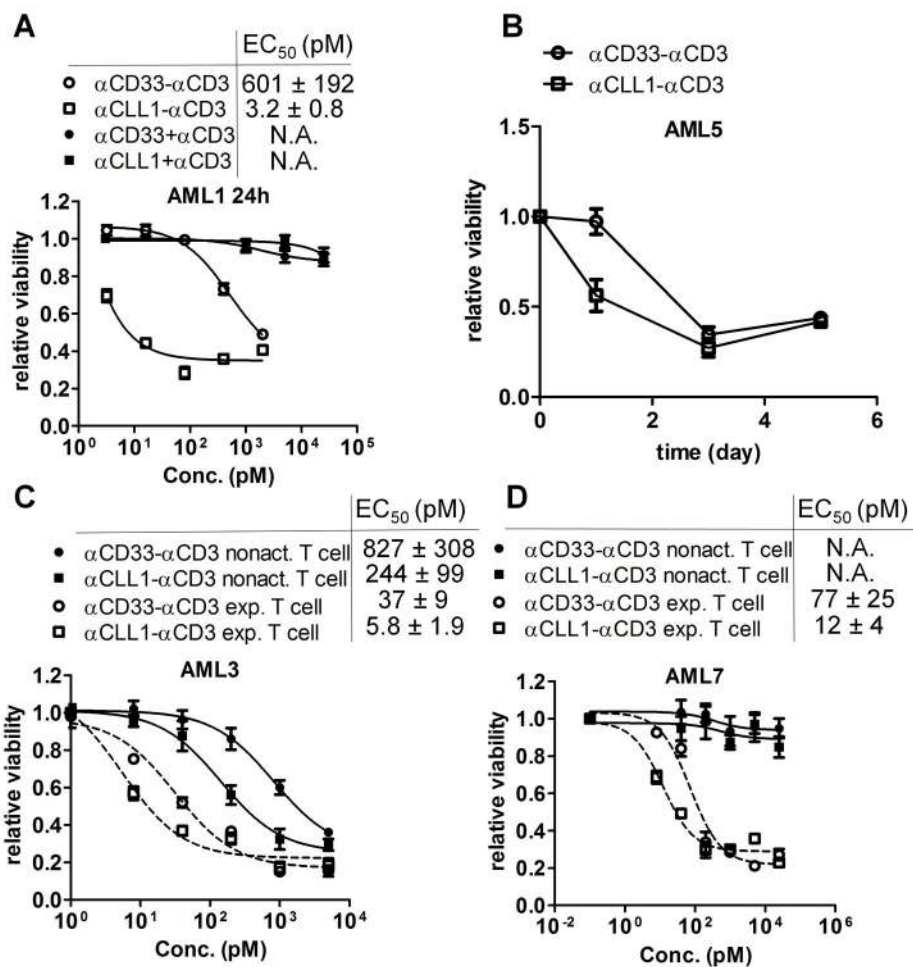
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**Figure 1.**

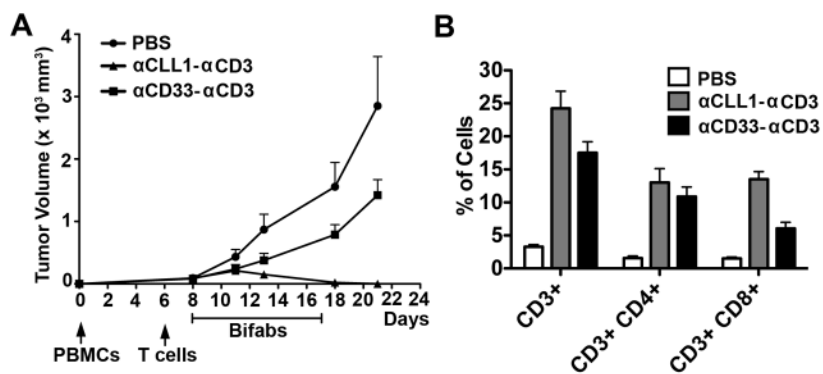
Synthesis and characterization of  $\alpha\text{CD33}-\alpha\text{CD3}$  and  $\alpha\text{CD33}-\alpha\text{CD3}$ . Synthetic scheme for the BiFabs (A); SDS-PAGE gel of BiFabs: lane 1 protein ladder; lanes 2–3  $\alpha\text{CD33}-\alpha\text{CD3}$  and  $\alpha\text{CD33}-\alpha\text{CD3}$  under non-reducing condition, respectively; lanes 4–5  $\alpha\text{CD33}-\alpha\text{CD3}$  and  $\alpha\text{CD33}-\alpha\text{CD3}$  under reducing condition, respectively (B); Binding of Fab mutants and BiFabs to human AML cell line U937(C) and to human T cells (D); goat anti-human Kappa-RPE (Southern Biotech Associate) was used as the secondary antibody for cell labeling.

**Figure 2.**

*In vitro* cytotoxicity of  $\alpha$ CLL1- $\alpha$ CD3 and  $\alpha$ CD33- $\alpha$ CD3 redirecting healthy PBMCs against various human AML cell lines - U937 (A) and HL60 (B) after 24h or 48h incubations. Cytotoxicity curves of  $\alpha$ CD33- $\alpha$ CD3 against U937, Kasumi-3 and KG-1A (C), and of  $\alpha$ CLL1- $\alpha$ CD3 against U937, Kasumi-3 and KG-1A (D) after a 24h incubation period. In all experiments, target cells were stained by either PKH26 or CellVue Claret Far Red (Sigma-Aldrich) and incubated with PBMCs at a 1/10 ratio. Each data point represents a mean of triplicate samples. Error bars are representative of standard deviation.

**Figure 3.**

*Ex vivo* cytotoxicity of  $\alpha$ CLL1- $\alpha$ CD3 and  $\alpha$ CD33- $\alpha$ CD3 against primary AML patient samples. A) Relative viability of AML1 (CD33<sup>-</sup>/CLL1<sup>+</sup>) blasts treated with  $\alpha$ CD33- $\alpha$ CD3,  $\alpha$ CLL1- $\alpha$ CD3, non-conjugated Fab mixture of  $\alpha$ CD33 +  $\alpha$ CD3, or non-conjugated Fab mixture of  $\alpha$ CLL1 +  $\alpha$ CD3 for 24hr; B) time-dependent *ex vivo* viability of AML5 (CD33<sup>+</sup>/CLL1<sup>+</sup>) blasts treated with 1 nM  $\alpha$ CD33- $\alpha$ CD3 or  $\alpha$ CLL1- $\alpha$ CD3; relative viability of (C) AML3 (CD33<sup>+</sup>/CLL1<sup>+</sup>) and (D) AML7 (CD33<sup>+</sup>/CLL1<sup>+</sup>) blasts treated with  $\alpha$ CD33- $\alpha$ CD3 or  $\alpha$ CLL1- $\alpha$ CD3 with non-activated autologous T cells in PBMCs for 96hr (filled) or with *ex vivo* expanded autologous T cells for 48hr (open).

**Figure 4.**

*In vivo* anti-tumor activity of αCLL1-αCD3 and αCD33-αCD3. (A) U937 cells and PBMCs were injected in NSG mice on Day 0. Upon observation of a palpable tumor, *ex vivo* expanded T cells were injected, and 24h later, animals were dosed daily with αCLL1-αCD3 (1mg/kg), αCD33-αCD3 (1mg/kg), or PBS control for 10 days. (B) T cell infiltrates in U937 tumors treated with αCLL1-αCD3 (1mg/kg), αCD33-αCD3 (1mg/kg), or PBS.