

Targeting Natural Killer Cells to Acute Myeloid Leukemia *In Vitro* with a CD16×33 Bispecific Killer Cell Engager and ADAM17 Inhibition

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Abstract

Purpose: The graft versus leukemia effect by natural killer (NK) cells prevents relapse following hematopoietic stem cell transplantation. We determined whether a novel bispecific killer cell engager (BiKE) signaling through CD16 and targeting CD33 could activate NK cells at high potency against acute myelogenous leukemia (AML) targets.

Experimental Design: We investigated the ability of our fully humanized CD16 × CD33 (CD16×33) BiKE to trigger *in vitro* NK cell activation against HL60 (CD33⁺), RAJI (CD33⁻), and primary AML targets (*de novo* and refractory) to determine whether treatment with CD16×33 BiKE in combination with an ADAM17 inhibitor could prevent CD16 shedding (a novel inhibitory mechanism induced by NK cell activation) and overcome inhibition of class I MHC recognizing inhibitory receptors.

Results: NK cell cytotoxicity and cytokine release were specifically triggered by the CD16×33 BiKE when cells were cultured with HL60 targets, CD33⁺ *de novo* and refractory AML targets. Combination treatment with CD16×33 BiKE and ADAM17 inhibitor resulted in inhibition of CD16 shedding in NK cells, and enhanced NK cell activation. Treatment of NK cells from double umbilical cord blood transplant (UCBT) recipients with the CD16×33 BiKE resulted in activation, especially in those recipients with cytomegalovirus reactivation.

Conclusion: CD16×33 BiKE can overcome self-inhibitory signals and effectively elicit NK cell effector activity against AML. These *in vitro* studies highlight the potential of CD16×33 BiKE ± ADAM17 inhibition to enhance NK cell activation and specificity against CD33⁺ AML, which optimally could be applied in patients with relapsed AML or for adjuvant antileukemic therapy posttransplantation. *Clin Cancer Res*; 19(14); 3844–55. ©2013 AACR.

Introduction

Patients with intermediate and poor-risk acute myelogenous leukemia (AML) who achieve complete remission after induction chemotherapy benefit from allogeneic hematopoietic cell transplantation (HCT; ref. 1). The success of HCT is due, at least in part, to T cells and

natural killer (NK) cells exerting a graft versus leukemia (GVL) effect capable of eradicating remaining AML cells. We have shown that haploidentical NK cell infusions can be used safely to improve outcomes in patients with refractory AML in a nontransplantation setting (2, 3). However, unlike T cells, which generate antigen-specific receptors to drive their effector functions, NK cells rely on surface receptors to bind a potential target cell. Interactions between NK cell-activating and -inhibitory receptors with target cell ligands determine whether or not an NK cell will elicit an immune response. According to the missing-self hypothesis, the lack of expression of inhibitory ligands (usually MHC class I molecules) and the overexpression of activating ligands by target cells determines whether NK cell killing occurs (4, 5). Thus, the ability of NK cells to mount an immune response against AML target cells is largely dependent on the target surface expression of ligands. This limitation is a major obstacle to NK cell therapy as responses may lack potency and specificity for leukemic targets.

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Translational Relevance

Natural killer (NK) cells are innate immune effectors capable of cytotoxicity and cytokine production. CD16 is an Fc receptor that signals potent activation and is downregulated by ADAM17, a transmembrane CD16 sheddase. This article reports on a novel bispecific killer cell engager (BiKE) signaling through CD16 and targeting the myeloid antigen CD33 [CD16 × CD33 (CD16×33) BiKE], which activates NK cells specificity against *de novo* and refractory acute myelogenous leukemia (AML) targets. Moreover, MHC class I expression by AML can ligate inhibitory NK cell receptors as a mechanism of immune escape. Combination therapy with a CD16×33 BiKE and ADAM17 inhibitor can overcome inhibitory signals mediated by class I MHC recognition and CD16 clipping, resulting in potent NK cell killing of CD33⁺ AML targets. Application of these strategies into settings that induce minimal residual disease such as allogeneic transplantation, especially in those with enhanced NK cell activity induced by cytomegalovirus reactivation, is warranted.

In a normal physiologic setting, antibody binding triggers antibody-dependent cell cytotoxicity (ADCC), a process involving NK cell activation, the release of cytokines and cytolytic granules, and induction of target cell apoptosis. ADCC is primarily mediated by the low-affinity Fc receptor CD16 (FcγRIIIA or CD16A). CD16 is a potent NK cell-activating receptor that, upon signaling, induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM), triggering the release of lytic granules and cytokines such as IFN-γ and TNF-α (6–8). In addition, CD16 downregulation in NK cells occurs when these cells interact with target cells (9), an effect that we have shown to be a result of ectodomain shedding of CD16 upon NK cell activation. CD16 shedding by NK cells is mediated by the transmembrane glycoprotein, a disintegrin and metalloprotease-17 or ADAM17 (10). These data show the central importance of CD16 in triggering NK cell-mediated ADCC.

One approach to improving NK cell therapy may be the use of antibodies that are designed to specifically bind to both target antigens and NK cell-activating receptors. We have recently shown that CD16-directed bispecific (CD16×19 BiKE) and trispecific (CD16×29×22 TriKE) single-chain fragment variable (bscFv and tscFv) recombinant agents directly trigger NK cell activation through CD16, significantly increasing NK cell cytolytic activity and cytokine production against lymphoid tumor targets (11, 12). In the present study, we designed a novel bispecific killer cell engager antibody (BiKE), developed specifically to signal through CD16 and target the myeloid differentiation antigen CD33 [named CD16 × CD33 (CD16×33) BiKE]. CD33 is a myeloid surface antigen expressed on the AML

blasts of approximately 85% of patients (13). We tested whether CD16×33 BiKE enhances NK cell activation against CD33⁺ AML cells using the HL60 cell line and primary AML cells. In addition, we examined whether ADAM17 inhibition, preventing CD16 shedding, enhances the effector function of NK cells against leukemia targets when combined with CD16×33 BiKE.

Materials and Methods

Cell isolation and purification

Adult peripheral blood was obtained from the Memorial Blood Center (Minneapolis, MN). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation using a Histopaque gradient (Sigma-Aldrich), and monocytes and NK cells were selected using the magnetic-activated cell sorting (MACS) CD14 Isolation Kit and NK Cell Isolation Kit protocol (Miltenyi Biotec), respectively. Deidentified human samples were obtained from the Leukemia-Myelodysplastic Syndrome Tissue Bank (LMTB) at the University of Minnesota (Minneapolis, MN; a shared Masonic Cancer Center resource). Samples included peripheral blood blasts from a patient with *de novo* AML, bone marrow samples from patients with relapse/refractory AML, and PBMCs from patients who underwent double umbilical cord blood transplantation (UCBT). All samples were obtained after informed consent and in accordance with the Declaration of Helsinki and the guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Before their use, samples were thawed and incubated overnight at 37°C, 5% CO₂ and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin.

Cell lines

The human acute promyelocytic leukemia cell line HL60 and the human Burkitt's lymphoma cell line Raji were used as positive and negative controls for CD33 expression, respectively, and were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin.

Flow cytometry

Single-cell suspensions were stained with the following monoclonal antibodies (mAb): PE/Cy7-conjugated CD56 (HCD56; BioLegend), ECD-conjugated CD3 (UCHT1; Beckman Coulter), fluorescein isothiocyanate (FITC)-conjugated CD14 (M5E2; BioLegend), APC/Cy7-conjugated CD16 (3G8; BioLegend), FITC-conjugated CD33 (P67.6; BD Biosciences), PerCP/Cy5.5-conjugated anti-human CD107a (LAMP-1; H4A3; BioLegend), Pacific Blue-conjugated anti-human IFN-γ (4S.B3; BioLegend), Alexa Fluor 647-conjugated anti-human TNF-α (Mab11; BioLegend), phycoerythrin (PE) anti-human CD158a/h (KIR2DL1/S1; HPMA4; BioLegend), PE anti-human CD158b (KIR2DL2/L3, NKAT2; DX27; BioLegend), and PE anti-human CD158e1 (KIR3DL1, NKB1; DX9; BioLegend). HLA expression was analyzed using FITC-conjugated antihuman HLA

ABC (MCA81F; AbD Serotec). Flow cytometry assays were conducted on a LSRII 11-color flow cytometer (BD Biosciences), and all data were analyzed with FlowJo 9.3.2 software (TreeStar).

Construction, expression, and purification of the bscFv CD16×33 BiKE

Synthesis and assembly of hybrid genes encoding the CD16×33 BiKE reagent were accomplished by using DNA shuffling and DNA ligation techniques as previously described (14). The fully assembled gene (from 5'-end to 3'-end) consisted of an *NcoI* restriction site, an ATG initiation codon, the VH and VL regions of anti-human CD16 (NM3E2) derived from a phage display library produced by McCall and colleagues (a gift of L. Weiner, Georgetown University, Washington, DC; ref. 15), a 20-amino acid segment of human muscle aldolase (PSCQAGAAASESLFVSNHAY), the VH and VL regions of human anti-CD33, and an *XhoI* restriction site. The resultant 1,526 base pair *NcoI/XhoI* fragment gene was spliced into the pET21d expression vector under control of an isopropyl- β -D-thio-B-[scap]d[r]-galactopyranoside (IPTG)-inducible T7 promoter. DNA-sequencing analysis (Biomedical Genomics Center, University of Minnesota) was used to verify that the gene sequence was correct and had been cloned in frame. Bacterial protein expression and purification by ion exchange and size exclusion chromatography were conducted as previously described (14).

ADAM17 inhibition and MHC class I antibody-blocking experiments

A highly selective ADAM17 inhibitor (INCB003619) from the Incyte Corporation (Robert C. Newton, Wilmington, DE) was added at a concentration of 2 μ mol/L to purified NK cells 30 minutes before testing. Mouse mAb immunoglobulin G1 (IgG1) clone HP-1F7 (kindly provided by M. Lopez Botet, Universitat Pompeu Fabra, Barcelona, Spain) was used as a positive control for pan-HLA blocking. The mAb HP-1F7 effectively binds to the α -chain of HLA-A, -B, -C, -E, and -G, and blocks all NK cell inhibitory receptor engagement (16–18). Target cells were preincubated for 30 minutes with anti-HLA mAb to a final concentration of 20 μ g/mL.

Cytokine/chemokine production and CD107a degranulation assay

Purified NK cells and PBMCs were incubated overnight at 37°C, 5% CO₂ in basal medium (RPMI supplemented with 10% fetal calf serum and 1% penicillin/streptomycin). Cells were washed and treated with 5 μ g/mL of bscFv CD16×33 BiKE (or as described in figures) and incubated for an extra 30 minutes. Anti-human CD107a mAb was added alone or with target cells [HL60, Raji, *de novo* primary AML, or relapse/refractory AML; effector:target (E:T) ratios 2:1 or as described in figures] and incubated for 1 hour. GolgiStop (1:1,500) and GolgiPlug (1:1,000; both from BD Biosciences) were added to cell cultures, which were incubated for an additional 4 hours. Cells were then harvested and stained with mAb CD56, CD3, CD16, and KIR (CD158a, CD158b, and NKB1) before fixation and per-

meabilization. Permeabilized cells were stained for intracellular TNF- α and IFN- γ with an anti-human TNF- α antibody and an anti-human IFN- γ antibody, respectively. CD3, CD56, CD16, KIR, CD107a, TNF- α , and IFN- γ expression was evaluated by fluorescence-activated cell sorting (FACS) analysis.

⁵¹Chromium release cytotoxicity assay

Direct cytotoxicity assays were conducted by standard 4-hour ⁵¹Cr-release assays using NK cells pretreated with bscFv CD16×33 BiKE and HL60 or Raji targets as indicated. ⁵¹Cr released by specific target cell lysis was measured by a gamma scintillation counter, and the percentage target cell lysis was calculated as follows: [(experimental lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis)] × 100.

Statistical analysis

Data were summarized with mean and SE (mean \pm SEM). For comparisons between independent samples, Student *t* test was used. For comparisons of matched samples, paired *t* test was used. Statistical analyses were conducted using SAS version 9.3 (SAS Institute).

Results

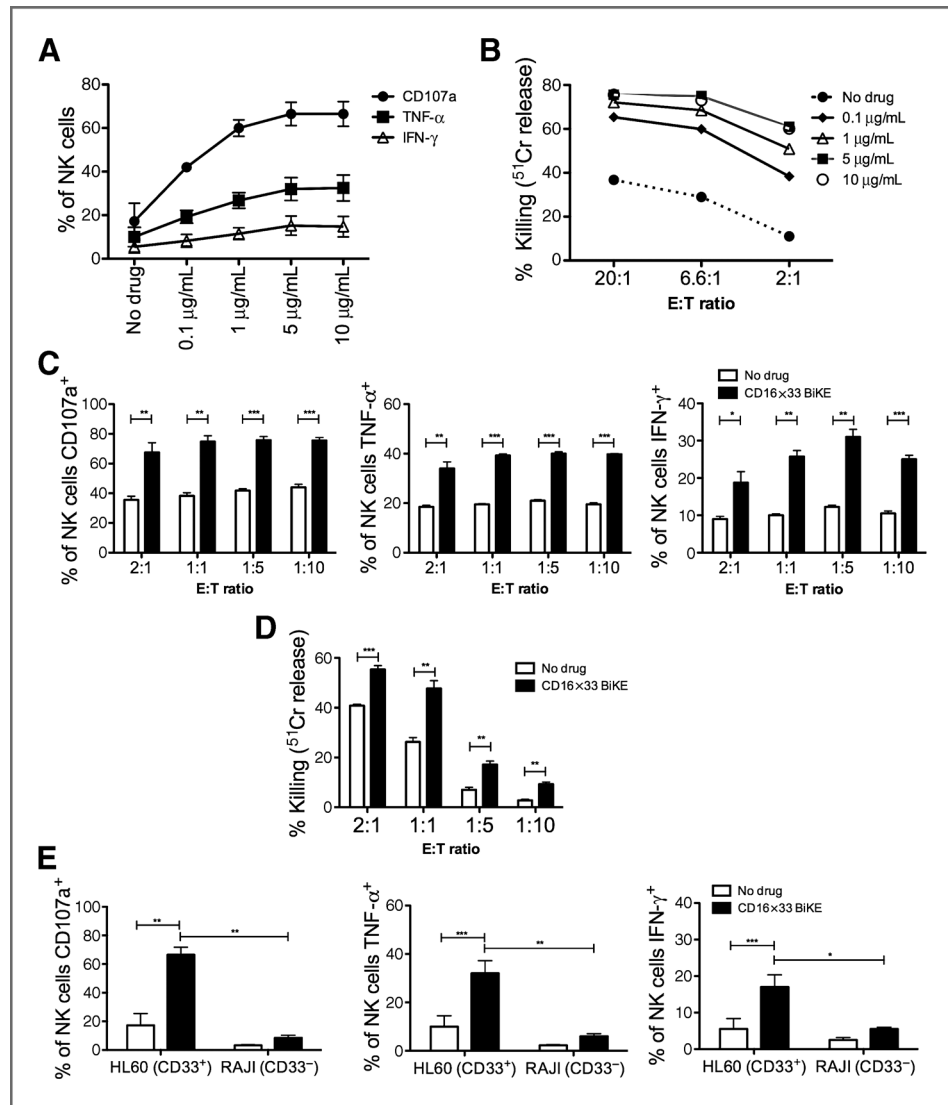
CD16×33 BiKE specificity enhances NK cell effector functions against CD33⁺ HL60 AML cells

We first determined whether the CD16×33 BiKE induces NK cell effector function against the HL60 cell line (CD33⁺). CD16×33 BiKE at the indicated concentrations induced NK cells to release lytic granules (CD107a) and produce cytokines (TNF- α and IFN- γ ; Fig. 1A). In addition, CD16×33 BiKE induced target cell death as measured by chromium release assay (Fig. 1B). Moreover, a dose-dependent increase in NK cell function was observed in response to CD16×33 BiKE, with a plateau at 5 μ g/mL and above. On the basis of these initial results, we selected a dose of 5 μ g/mL of the CD16×33 BiKE for future experiments.

For NK cells to be effective therapeutically, they must function at low E:T ratios. CD16×33 BiKE at 5 μ g/mL enhanced NK cell degranulation (CD107a expression) and TNF- α and IFN- γ production, independently of the E:T target ratio (Fig. 1C). Using a standard chromium release assay and HL60 targets, we observed enhanced killing with the CD16×33 BiKE at all E:T ratios, with diminished absolute killing at lower E:T ratios as expected (Fig. 1D). Furthermore, CD16×33 BiKE-mediated killing was specific as this antibody only potentiated NK cell functional activity against CD33⁺ HL60 cells but not CD33⁻ RAJI cells (Fig. 1E).

In addition to NK cells, the Fc receptor CD16A is expressed on the surface of monocytes and macrophages (19). To determine whether CD16A⁺ cells other than NK cells could contribute to the observed CD16×33 BiKE-mediated cytotoxicity, we conducted a 4-hour chromium release assay with or without CD16×33 BiKE added to PBMC-containing NK cells or monocytes (Fig. 2A). CD16×33 BiKE mediated cytotoxicity was specific for NK cells, suggesting that this reagent activates CD16⁺ NK cells to directly lyse CD33⁺ targets but not CD16A⁺ monocytes.

Figure 1. CD16×33 BiKE enhances NK cell function against CD33⁺ targets in a dose-dependent manner. Purified NK cells from healthy donors (*n* = 4) were cultured with HL60 (CD33⁺) target cells (E:T ratio of 2:1) and treated with the indicated concentrations of CD16×33 BiKE. **A**, after 4 hours in culture, intracellular CD107a degranulation, TNF- α , and IFN- γ were measured by flow cytometry, and **(B)** cytotoxicity was measured in a 4-hour chromium release assay. **C**, NK cell function (intracellular CD107a degranulation, TNF- α , and IFN- γ measured by flow cytometry) and **(D)** cytotoxicity were determined at the indicated E:T ratios (*n* = 2) with or without 5 μ g/mL of CD16×33 BiKE. **E**, specificity of the CD16×33 BiKE was tested by culturing NK cells with HL60 (CD33⁺) and Raji targets (CD33⁻) for 4 hours (E:T ratio of 2:1) with or without 5 μ g/mL of CD16×33 BiKE (*n* = 4; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).



In addition to CD33 expression on AML cells, CD33 expression on other myeloid differentiated cells, such as monocytes (13, 20), has the potential to interfere with target cell killing. We tested whether autologous CD33⁺ monocytes interfere with the ability of CD16×33 BiKE to induce NK cell cytotoxicity and cytokine release. Flow cytometry-based function indicated that CD16×33 BiKE induced lower level degranulation and cytokine release against autologous CD33⁺ monocytes but more robust NK cell function was induced by HL60 targets (Fig. 2B). Furthermore, when PBMCs (containing NK cell and CD33⁺ monocytes) were treated with CD16×33 BiKE, we observed enhanced CD107a degranulation and INF- γ secretion against HL60 targets than when NK cells were cultured with CD33⁺ monocytes alone under similar conditions. These results suggest that the presence of CD33⁺ monocytes does not inhibit HL60 target activation of NK cells and that NK cells could be targeting both the HL60 and the monocyte population leading to enhance NK cell activation. Despite

this potential target competition, cytotoxicity from PBMCs (containing NK cells and monocytes) against HL60 targets is significantly enhanced by CD16×33 BiKE treatment (Fig. 2A). Finally, CD33⁺ unlabeled HL60 targets were admixed with chromium-labeled CD33⁻ Raji targets and no killing occurred (data not shown). This leads to the conclusion that CD16×33 BiKE does not activate NK cells sufficient to mediate a bystander effect against CD33⁻ targets.

CD16 downregulation on NK cells occurs after exposure to CD16×33 BiKE

In humans, the most diverse NK cell inhibitory MHC class I-specific receptors belong to the killer cell immunoglobulin-like receptor (KIR) family. Depending on their immunoglobulin-like domains, some KIRs are activating whereas others are inhibitory, where recognition of MHC suppresses NK cell cytotoxic activity (4). CD16 is an important activating receptor that mediates ADCC. It has been previously

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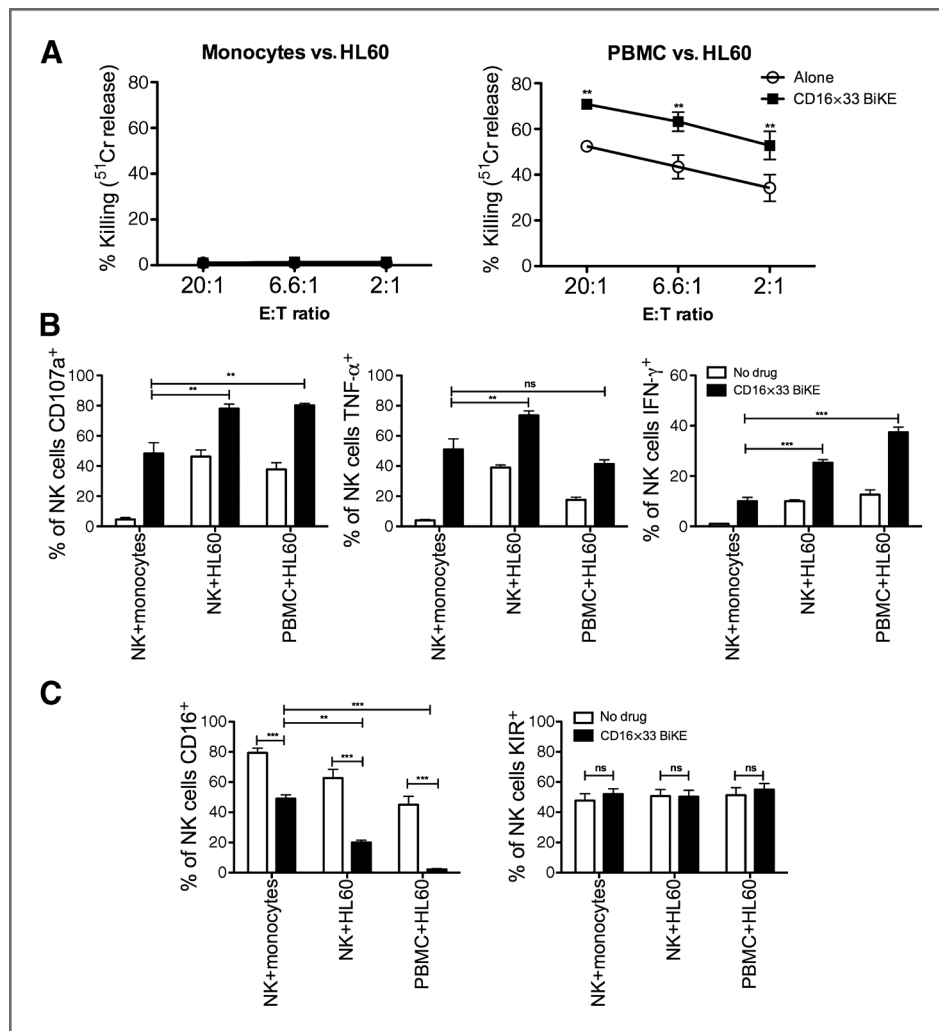


Figure 2. CD16x33 BiKE activates NK cells against CD33⁺ HL60 targets in the presence of CD33⁺ monocytes. A, monocytes (CD33⁺) and PBMC-containing NK cells [NK cells⁺ PBMC (NK⁻) [NK: PBMC; ratio of 2:1] were tested against HL60 cells at the indicated effector-to-HL60 ratios after treatment with or without 5 μg/mL of the CD16x33 BiKE. Cytotoxicity against HL60 targets was determined by 4-hour chromium release assay. B, purified NK cells from healthy donors were cultured with autologous monocytes (CD33⁺) and with HL60 (CD33⁺) target cells (E:T ratio of 2:1), and PBMC (containing NK cells and monocytes) were cultured with HL60 targets (E:T ratio of 2:1) for 4 hours with or without 5 μg/mL of CD16x33 BiKE. Flow cytometry was used to determine intracellular CD107a degranulation, TNF-α, and IFN-γ. C, the same conditions were used to determine CD16 and KIR expression on NK cells after 4 hours of activation (n = 3 for all experiments, **, P < 0.01; ***, P < 0.001; ns, not significant).

recognized that CD56^{dim} NK cells downregulate CD16 after target cell-induced activation (9). To determine if BiKE activation induces changes in NK cell receptor expression, we analyzed the expression of CD16 and KIR (KIR2DL1, KIR2DL2, KIR2DL3, and 3DL1) on the surface of the NK cells after 4 hours of activation with CD16x33 BiKE. KIR expression was not affected by the activation of NK cells with the CD16x33 BiKE (Fig. 2C). In contrast, CD16 expression was markedly downregulated after treatment with the CD16x33 BiKE, an effect which was greater when NK cells were also cultured with HL60 targets than when NK cells were cultured with autologous CD33⁺ monocytes. Our data show a direct correlation between NK cell activation and loss of CD16 expression after exposure to the CD16x33 BiKE.

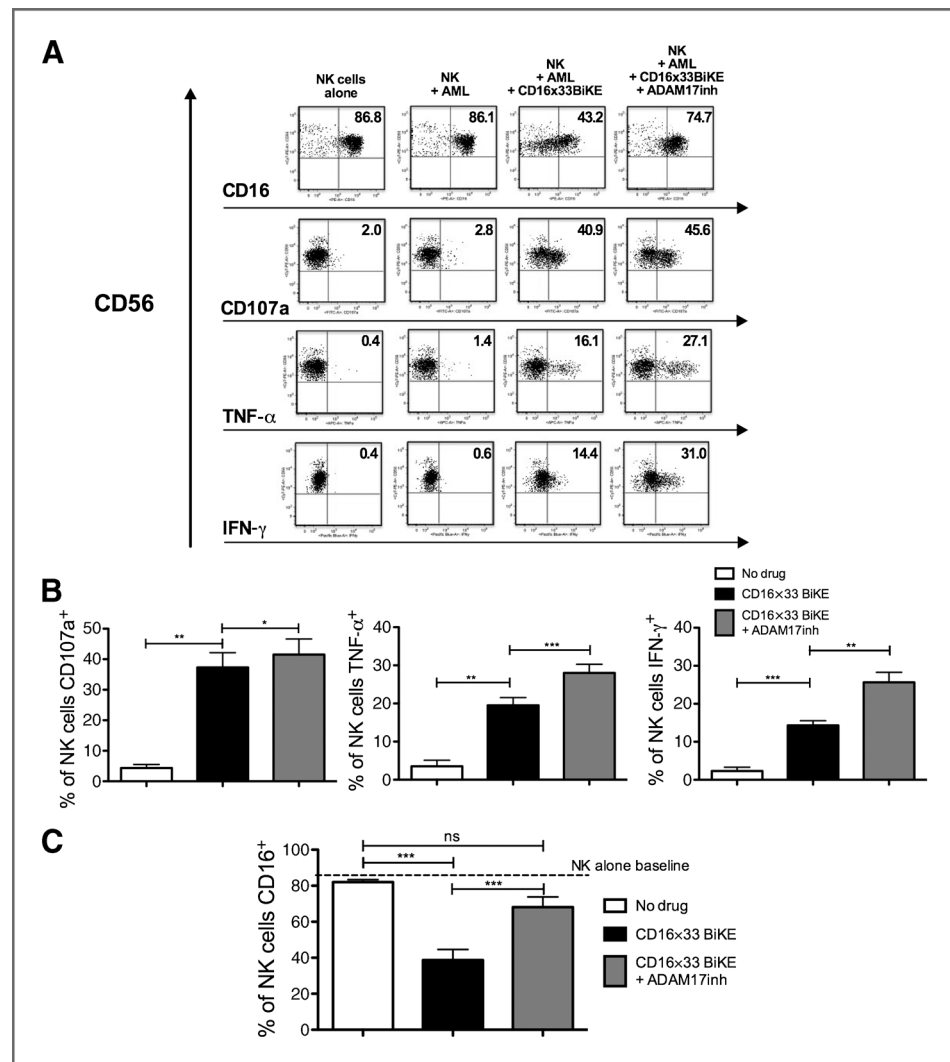
CD16x33 BiKE induces NK cell activation against *de novo* CD33⁺ AML cells, an effect enhanced by ADAM17 inhibition

To explore whether NK cell function against CD33⁺ AML blasts is enhanced by treatment with the CD16x33 BiKE, we

purified NK cells from healthy donors and cultured them for 4 hours with or without CD33⁺ AML blasts obtained from a patient with *de novo* AML. NK cells alone had no cytotoxic or cytokine release activity against *de novo* AML targets (Fig. 3A). In contrast, the addition of CD16x33 BiKE to the cell culture resulted in significant NK cell cytolytic activity and cytokine release against CD33⁺ AML blasts (Fig. 3A and B).

We next explored whether inhibition of ADAM17 (with the purpose of preventing CD16 shedding) affects the ability of CD16x33 BiKE to activate NK cells by treating NK cells and CD33⁺ AML blasts with CD16x33 BiKE and an ADAM17 inhibitor (INCB003619). Treatment of NK cells with the BiKE reagent plus the ADAM17 inhibitor prevented CD16 shedding from the NK cell surface (Fig. 3A and C). More importantly, ADAM17 inhibition enhanced CD16x33 BiKE-mediated NK cell effector functions against *de novo* CD33⁺ AML blasts (Fig. 3A and B). Of note, when the NK cells were treated with the ADAM17 inhibitor alone, NK cell activation was not observed (data not shown). Our results indicate that inhibition of

Figure 3. CD16×33 BiKE triggers NK cell degranulation and cytokine production against *de novo* CD33⁺ AML targets, an effect that is enhanced by ADAM17 inhibition. Purified NK cells isolated from healthy donors (*n* = 6) were cultured with *de novo* CD33⁺ AML cells (E:T ratio of 2:1). Cells were treated with or without 5 μg/mL of CD16×33 BiKE and with or without 2 μmol/L of an ADAM17 inhibitor (INCB003619). A, representative flow cytometry analysis of CD56⁺ NK cells after 4 hours in culture. Aggregate data of (B) intracellular CD107a degranulation, TNF-α, and IFN-γ for the indicated conditions and (C) CD16 expression (baseline CD16 expression of NK cells shown in the dotted line; *n* = 6; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant).



CD16 shedding by an ADAM17 inhibitor translates into markedly stronger BiKE activation of NK cells.

CD16×33 BiKE overcomes MHC class I inhibition by AML targets resulting in NK cell activation

Expression of MHC class I molecules by tumors triggers inhibitory signals that prevent NK cell activation against targets (4). It is well recognized that AML cells express MHC class I ligands (21). We confirmed MHC class I expression on blasts from *de novo* AML by flow cytometry (data not shown). We then determined whether inhibition of class I ligands in AML targets would influence NK cell activation and whether MHC class I blocking would enhance the CD16×33 BiKE activation of NK cells. The mAb HP-F1 used for these experiments was developed against common MHC class I epitopes that recognize inhibitory receptors on NK cells. Both native mAb and Fab fragments of HP-F1 can interrupt MHC class I molecules to enhance NK cell killing (16), and we have shown that blockade of NK cell receptors that recognize class I (KIR, ILT-2, and NKG2A)

results in similar target cell killing than when HP-F1 blockade is used alone (22), validating the HP-F1 mAb to block MHC class I interactions. Our results show that blocking of MHC class I ligands in AML cells triggers NK cell activation, but this effect is inferior to the activation observed with treatment with the CD16×33 BiKE alone. In addition, blocking of class I further enhances NK cell activation when cells are treated with the CD16×33 BiKE (Fig. 4). Together, these results suggest that the CD16×33 BiKE has the ability to overcome inhibitory signals, which under physiologic conditions inhibit AML recognition by NK cells.

CD16×33 BiKE and ADAM17 inhibition leads to potent NK cell activity against refractory CD33⁺ AML cells

Immune sensitivity against *de novo* AML and refractory disease may differ. We obtained 10 bone marrow samples from patients with refractory AML; 8 of 10 samples expressed CD33 (Fig. 5A). CD16×33 BiKE substantially

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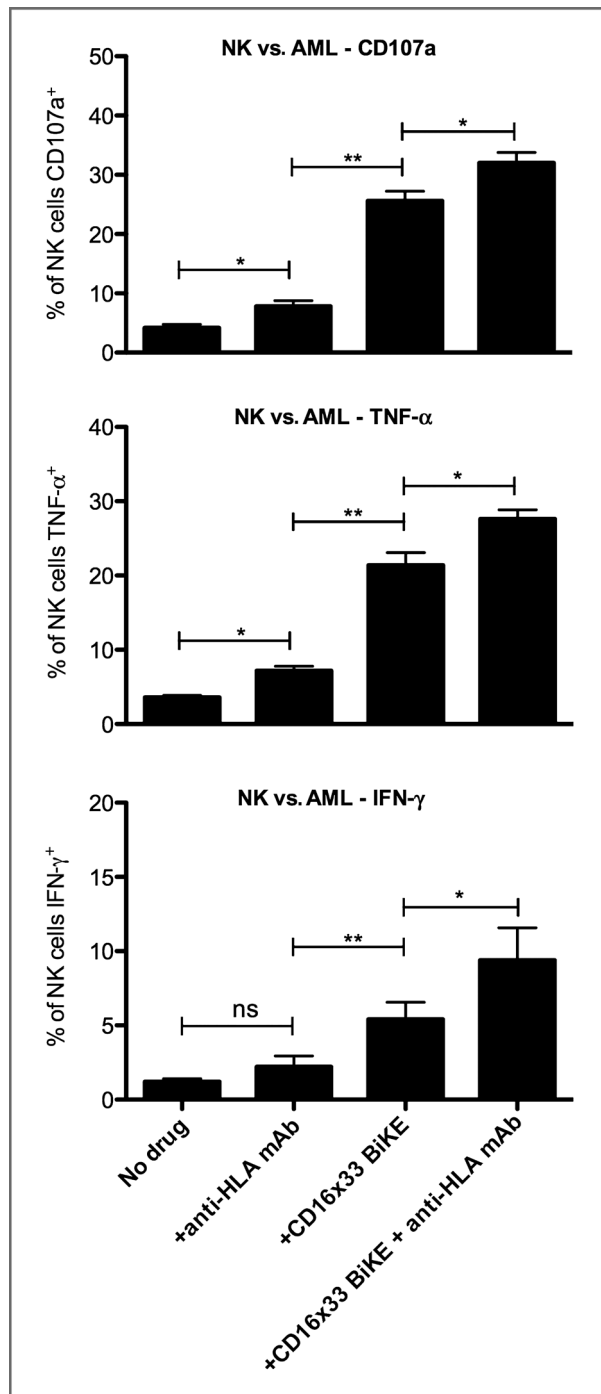


Figure 4. CD16×33 BiKE enhances NK cell responses to AML overcoming MHC class I inhibition. AML target cells were preincubated with 20 μg/mL of a pan-HLA-blocking antibody (HP-1F7) for 30 minutes before culturing with NK cells. Purified NK cells isolated from healthy donors (*n* = 5) were cultured with *de novo* CD33⁺ AML cells (E:T ratio of 2:1). Cells were treated with or without 5 μg/mL of CD16×33 BiKE as indicated and cultured for 4 hours. Aggregate data of NK cell intracellular CD107a degranulation, TNF-α, and IFN-γ for the indicated conditions were determined by flow cytometry (*n* = 5; *, *P* < 0.05; **, *P* < 0.01; ns, not significant).

activated NK cells against these CD33⁺ refractory AML targets (Fig. 5B). Combination treatment with CD16×33 BiKE plus the ADAM17 inhibitor resulted in enhanced NK cell activation, especially cytokine production. In contrast, no significant NK cell activation was observed with the CD16×33 BiKE against the AML samples that lacked CD33 expression (CD33⁻ AML) targets (Fig. 5C). Our results show that not only does CD33 expression correlate well with sensitivity to NK cells stimulated by CD16×33 BiKE, but that CD33⁺ AML blasts from relapse/refractory disease also remain sensitive to the BiKE.

CD16×33 BiKE-stimulated NK cell effector functions are enhanced by cytomegalovirus reactivation in UCBT recipients

Cytomegalovirus (CMV) reactivation after allogeneic HCT is reportedly associated with a decreased risk of relapse in patients with AML (23). We have previously described a more mature NK cell phenotype in transplant recipients who reactivate CMV (24). To determine whether CMV reactivation affects the ability of the CD16×33 BiKE to activate NK cells from UCBT recipients, we analyzed PBMC samples from patients who underwent UCBT at 3 months and 1 year posttransplant. Samples were selected on the basis of CMV reactivation status (6 patients with no CMV reactivation and 6 patients with CMV reactivation in the first 100 days after transplant).

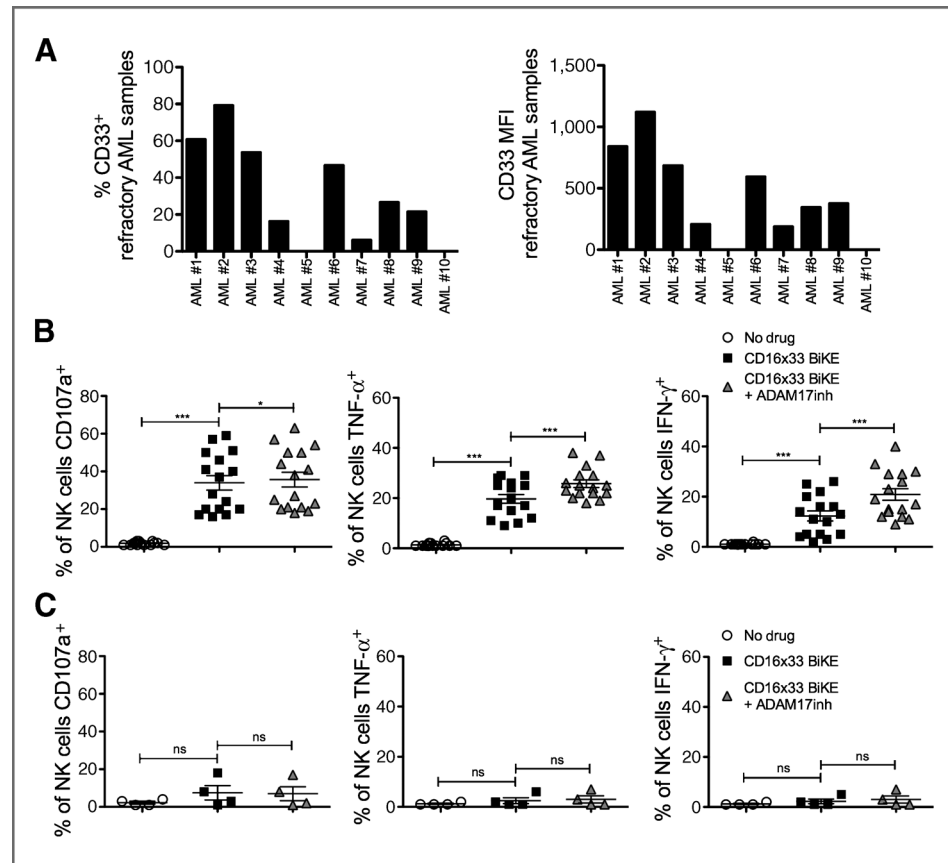
NK cell CD16 expression, measured by flow cytometry, was conducted on these samples and compared with NK cell CD16 expression from healthy donors (Fig. 6A). Our results show that CD16 reconstitution occurs in a time-dependent manner, with higher CD16 expression observed at 1 year than at 3 months post-UCBT. In addition, no differences in CD16 expression were observed between CMV reactivating patients and those without reactivation.

We then analyzed the effector function of the PBMC samples against *de novo* CD33⁺ AML blasts after treatment with the CD16×33 BiKE. Analysis at 3 months and 1 year post-UCBT determined that NK cells from patients who experienced early CMV reactivation post-UCBT had increased CD16×33 BiKE-induced NK cell activation as compared with NK cells from patients without CMV reactivation (Fig. 6B). Nevertheless, overall NK cell activation with the BiKE after UCBT was substantially lower when compared with NK cell activation from healthy donors. However, in transplant recipients with CMV reactivation, BiKE-mediated cytokine release of TNF-α and IFN-γ at 1 year post-UCBT was similar to the cytokine released observed in NK cells from healthy donors, a result that indicates that NK cell cytokine release function has fully recovered in this group of transplant recipients.

Our results suggest that CD16 expression alone is not entirely responsible for activation of NK cells with the CD16×33 BiKE in CMV-reactive UCBT recipients, and that other mechanisms are likely involved that result in increase NK cell effector functions. Overall, these data show that CMV reactivation after allogeneic HCT enhances CD16×33 BiKE-mediated NK cell cytotoxicity and cytokine release.

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Figure 5. CD16×33 BiKE triggers NK cell degranulation and cytokine production against relapse/refractory CD33⁺ AML targets. **A**, CD33 surface expression measured by flow cytometry analysis of bone marrow samples from 10 patients with relapse/refractory AML. Percentage expression and median fluorescence intensity (MFI) are shown for each sample. **B** and **C**, purified NK cells isolated from 2 healthy donors were cultured with each refractory AML sample (E:T ratio of 2:1; *n* = 20 separate assays). Cells were treated with or without 5 μg/mL of CD16×33 BiKE. After 4 hours in culture, intracellular CD107a degranulation, TNF-α, and IFN-γ were determined by flow cytometry for (B) CD33⁺ AML targets (*n* = 16 from 8 CD33⁺ AML targets derived from 2 donors each) and (C) CD33⁻ AML targets (*n* = 4 from 2 CD33⁻ AML targets derived from 2 donors each; *, *P* < 0.05; ***, *P* < 0.001; ns, not significant).



Discussion

We addressed whether a fully humanized bispecific killer cell engager antibody containing binding sites for CD16 and CD33 (CD16×33 BiKE) could enhance the potency and specificity of CD16⁺ NK cell effector functions against CD33⁺ leukemic cells. Collectively, our experiments show the ability of a BiKE antibody to trigger NK cell activation, through direct signaling of CD16, and thereby inducing secretion of cytokines and lytic granules against CD33⁺ AML targets. In addition, an ADAM17 inhibitor prevents activation-induced NK cell CD16 shedding and, when combined with CD16×33 BiKE, enhances NK cell cytokine production and cytotoxicity. Finally, our data indicate that activation of NK cells by CD16×33 BiKE is diminished early after UCBT, but that CMV reactivation increases BiKE-induced NK cell targeting against AML.

Our experiments establish 2 key steps pertinent to the development of a new therapeutic strategy against CD33⁺ AML. First, by developing a molecule that specifically engages CD16 signaling against CD33⁺ targets, we show that the use of the CD16×33 BiKE is specific for NK cell activation and exclusively targets CD33⁺ cells. Because CD33 is expressed in healthy myeloid cells, we showed that CD16×33 BiKE triggers NK cell activation against autologous CD33⁺ monocytes suggesting that our BiKE reagent is specific for CD33⁺ targets independently of the

nature of the target cell (malignant vs. healthy). Because KIR expression is sustained after activation of NK cells, we believe that inhibitory ligands or lack of expression of activating ligands expressed by healthy monocytes (and recognized by KIR) could contribute to the limited effector activity observed against autologous myeloid expressing CD33⁺ cells as compared with HL60 targets. Despite this, if CD16×33 BiKE is used to treat patients with AML, myelosuppression from targeting healthy CD33⁺ cells would be expected. Because CD33 is not expressed by the hematopoietic stem cell (HSC), bone marrow recovery could be delayed but should not induce irreversible bone marrow aplasia. Furthermore, our results suggest that CD16×33 BiKE treatment could target both CD33⁺ AML blasts and CD33⁺ monocytes. This is not a surprise as monocytes can express NK-activating ligands such as CD48, the ligand for the activating receptor 2B4 (25). To minimize this potential "monocyte sink," treating patients with AML with chemotherapy to minimize CD33 target competition may be warranted, but this needs to be balanced with the chemotherapy effect on the NK cells.

It has been previously recognized that HLA class I expression, a strong inhibitory ligand, is preserved on AML blasts at diagnosis and at relapse (21, 26). HLA class I expression by the target cell serves as an immune escape mechanism from NK cell recognition. In addition, expression of activating ligands by target cells is required to elicit NK cell

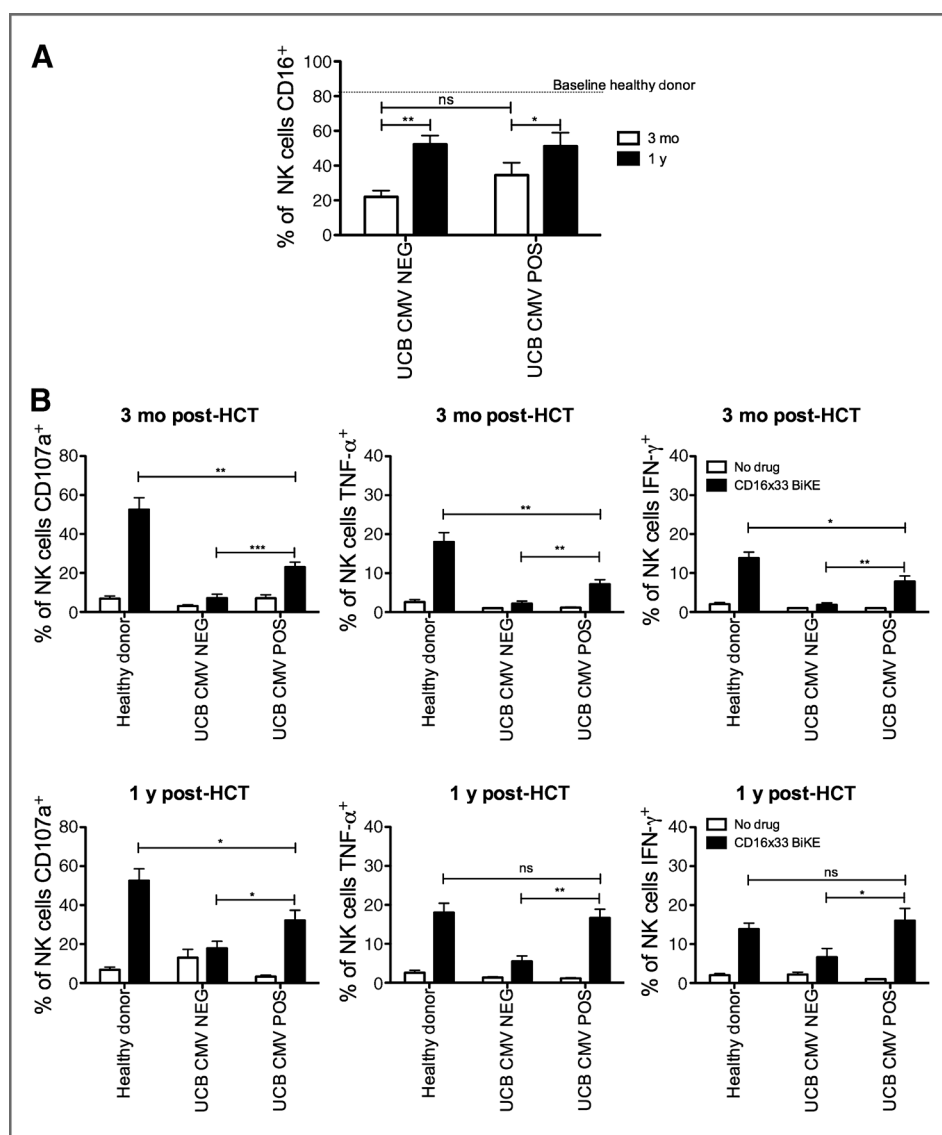


Figure 6. CD16×33 BiKE-stimulated NK cells after HCT exhibit time- and CMV reactivation-dependent activity against primary CD33⁺ AML targets. PBMCs were collected from double UCB recipients without CMV reactivation (UCB CMV NEG; *n* = 6) and from recipients with CMV reactivation (UCB CMV POS; *n* = 6) who had reactivated CMV in the first 100 days posttransplant. PBMC samples were collected at 3 months and 1 year posttransplant and compared with PBMCs obtained from healthy donors (*n* = 7). A, CD16 expression on NK cells was determined by flow cytometry (baseline CD16 expression of NK cells from healthy donors is shown in dotted line) at the indicated time points posttransplant. B, PBMCs were treated with or without 5 μg/mL of CD16×33 BiKE. After 4 hours in culture, levels of NK cell intracellular CD107a degranulation, TNF-α, and IFN-γ were determined by flow cytometry and compared with PBMC from healthy donors (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant).

killing. Although treatment of AML targets with MHC class I blockade alone is able to trigger NK cell function to some degree, treatment with CD16×33 BiKE alone was far more potent. Our results show that treatment with CD16×33 BiKE can overcome the inhibitory signals expressed by leukemic targets. The role of other activating ligands on the target cell may not be required when NK cells are treated with the CD16×33 BiKE as this reagent alone is able to induce potent CD16 signaling as shown by intracellular Ca²⁺ mobilization (11).

We investigated how the effector function of a therapeutic antibody signaling through CD16, can be enhanced by inhibition of ADAM17. The ADAM family of multidomain proteins is involved in both proteolysis and cell adhesion (12). These molecules play a major role in processing or shedding of cell membrane proteins, thereby altering the phenotype (and likely function) of cells. ADAM17 is responsible for shedding of CD16 after NK cell activation

(10), but has also been recognized as a potential target for cancer therapy because it is widely overexpressed by many tumors (27–29). ADAM17 has been reported to have a direct functional role in controlling proliferation and migration of tumors cells and is an important protein involved in controlling endothelial cell migration in angiogenesis (30–32). Our studies show a new immune-mediated mechanism of antitumor activity by ADAM17 inhibition. We found that ADAM17 inhibition and NK cell activation with CD16×33 BiKE resulted in increased cytotoxicity and cytokine secretion of IFN-γ and TNF-α, a combination optimal for therapeutic use. We acknowledge that our results are limited to *in vitro* evaluation and development of *in vivo* models are planned to substantiate the combined treatment with CD16×33 BiKE and ADAM17 inhibition in tumor-bearing animals. Because ADAM17 was originally recognized for being the major protein responsible for the cleavage of the transmembrane proteins

TNF- α (33), inhibitors of ADAM17 have been used in animal models and have shown to be effective in models of septic shock and rheumatoid arthritis (34, 35). There are multiple potential mechanisms, including inhibition of CD16 shedding on NK cells, by which ADAM17 inhibitors can affect immune recognition of malignant targets. We have recently described that CD62L (L-selectin), the cell-adhesion molecule expressed by most leukocytes (including NK cells), is also shed by ADAM17 (10).

Relapse mortality following allogeneic HCT remains a major challenge in the care of patients with AML (36) and is likely to increase now that reduced-intensity regimens are used in older patients who tend to have more aggressive disease (37). Thus, development of new therapeutic strategies to improve GVL posttransplantation is urgently needed. After allogeneic HCT, NK cells mediate GVL by the production of inflammatory cytokines and by direct target lysis. We have previously shown that target cell-induced IFN- γ production is markedly diminished in recipients of allogeneic transplantation (38). The current study explores the potential effect of using a CD16 \times 33 BiKE to induce GVL after UCBT. Elmaagacli and colleagues previously reported that the risk of leukemic relapse after allogeneic HCT was 9% at 10 years as compared with 42% in patients who did not reactivate CMV (23). The mechanism by which CMV reactivation is protective in the setting of allogeneic transplantation is poorly understood. We recently showed that NK cells from patients who reactivate CMV posttransplant have a more mature phenotype, with an increased percentage of CD56^{dim} NK cells and increased expression of the activating receptor NKG2C, as compared with NK cells from patients who did not reactivate CMV after allogeneic HCT (24). In addition, rapid lymphocyte recovery has been associated with CMV reactivation (39), which raises the possibility that CMV infection may induce expression of a ligand that activates T cells or NK cells or both. Jacobson and colleagues recently published that the total number of CD56⁺CD16⁺ NK cells recovered rapidly in double UCB recipients and was similar to their healthy controls (40). Here, we measured the percentage of CD16 expression among bulk NK cells and showed that CD16 expression is diminished in double UCB samples as compared with healthy donors, but this percentage recovers over time. Moreover, CMV reactivation after transplant confers an increase in NK cell responsiveness to CD16 \times 33 BiKE. Together, these findings raise the possibility that treatment with the CD16 \times 33 BiKE after transplantation, could enhance and direct the GVL effect in patients with CD33⁺ AML, especially after CMV reactivation.

Different modalities of anti-CD33-directed therapy have been tested in clinical trials in recent years. Lintuzumab, an anti-CD33 mAb, failed to show improvement in response rates or overall survival in patients with refractory or relapsed AML in a phase III study (41). Gemtuzumab ozogamicin, an anti-CD33 antibody linked to the toxin calicheamicin, was reported to yield 30% response rates in patients with relapsed CD33⁺ AML (42), which led to its approval for use in AML by the U.S. Food and Drug

Administration (FDA) in 2000. Unfortunately, gemtuzumab ozogamicin was eventually withdrawn from the market in 2010 after a postapproval clinical trial (SWOG S0106) showed lack of efficacy and raised safety concerns. Despite these disappointing results, recent randomized European studies have shown a survival advantage in patients who received gemtuzumab ozogamicin in combination with induction chemotherapy (43–45). Effectiveness of CD33-directed therapy depends on expression of CD33 on the leukemic stem cell (46). The models of stem cell heterogeneity in AML proposed by these authors indicate that CD33 is an attractive AML target particularly in leukemias that derive from mutations occurring at the level of the "committed precursor compartment" defined as CD34⁺ and CD33⁺. On the basis of these observations, CD33 remains an attractive target for immune therapy in AML as long as the stem cell clone is CD33⁺. On the basis of this, escape by CD33⁻ leukemic cells or by AML blasts expressing low levels of surface antigens could be a potential mechanism of resistance or lack of efficacy to treatment with CD16 \times 33 BiKE therapy. Despite this, our anti-CD33 strategy differs from previous CD33 immunotherapies in the ability of the BiKE to mediate NK activation, enhancing cytotoxicity and release of TNF- α and IFN- γ , which have the ability to regulate and orchestrate immune responses by enhancing activation of other NK cells, macrophages, and lymphocytes that induce GVL (47). It will be important to test CD16 \times 33 BiKE in patients with AML with CD33-expressing leukemic stem cells and in combination with chemotherapy after haploidentical NK cell infusion or early after HCT to enhance GVL. Furthermore, biomarkers to predict responses to anti-CD33 therapy, other than CD33 expression, will need to be studied and identified as our BiKE is tested in clinical trials.

Although it is possible that treatment of patients with AML with the CD16 \times 33 BiKE could lead to myelosuppression as previously recognized in clinical trials using anti-CD33-targeting therapies (48), there are several advantages to using a bispecific antibody such as CD16 \times 33 BiKE for cancer immune therapy. By increasing the affinity for the CD16 receptor, we predict that the use of a BiKE in an *in vivo* model will more effectively induce ADCC and trigger NK cell effector functions at lower doses. Moreover, mAbs are usually large with an average size of 150 kDa. Our BiKE was engineered using only the single-chain variable fragment (scFv) of an anti-CD16 molecule linked to the scFv of an anti-CD33 molecule. As this molecule has only 2 single chains, its size is approximately 60 kDa. It has been recognized that smaller-sized antibodies have higher "tissue penetration" allowing these molecules to penetrate the extracellular matrix to reach their target tumor cells more effectively (49). The smaller size would likely allow this molecule to have increased distribution and penetration to the bone marrow, although we will need to consider the possibility of renal secretion of the BiKE because it is below the renal clearance threshold (~70 kDa).

In adult patients with AML, relapse and refractory disease shortens the survival of patients and represents a major

challenge to overcome. New treatment options are urgently needed, especially to fight residual clonal disease. Here, we have introduced 2 new therapeutic strategies to enhance the potency and selectivity of NK cells against CD33⁺ AML. Future studies are needed to determine the *in vivo* efficacy of CD16×33 BiKE ± ADAM17 inhibition against myeloid disorders and their potential role in cancer therapeutics.

Disclosure of Potential Conflicts of Interest

J.S. Miller is a consultant/advisory board member of Coronado Biosciences. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: A. Wiernik, J.S. Miller

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