



Published in final edited form as:

Clin Cancer Res. 2013 July 15; 19(14): 3844–3855. doi:10.1158/1078-0432.CCR-13-0505.

Targeting Natural Killer cells to Acute Myeloid Leukemia *in vitro* with a CD16x33 bispecific killer cell engager (BiKE) and ADAM17 inhibition

Andres Wiernik¹, Bree Foley¹, Bin Zhang¹, Michael R. Verneris^{2,3}, Erica Warlick^{1,3}, Michelle K. Gleason^{3,4}, Julie A. Ross^{3,4}, Xianghua Luo^{3,5}, Daniel J. Weisdorf^{1,3}, Bruce Walcheck⁶, Daniel A Vallera^{3,7}, and Jeffrey S. Miller^{1,3}

¹Department of Medicine, Division of Hematology, Oncology, and Transplantation, University of Minnesota, Minneapolis, MN

²Department of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN

³Masonic Cancer Center, University of Minnesota, Minneapolis, MN

⁴Department of Pediatrics, Division of Epidemiology and Clinical Research, University of Minnesota, Minneapolis, MN

⁵Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis, MN

⁶Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN

⁷Department of Therapeutic Radiology-Radiation Oncology, University of Minnesota Cancer Center, Minneapolis, MN

Abstract

Purpose—The graft versus leukemia (GVL) effect by Natural Killer (NK) cells prevents relapse following hematopoietic stem cell transplantation. We determined whether a novel bi-specific killer cell engager (BiKE) signaling through CD16 and targeting CD33 could activate NK cells at high potency against AML targets.

Experimental Design—We investigated the ability of our fully humanized CD16x33 BiKE to trigger *in vitro* NK cell activation against HL60 (CD33⁺), RAJI (CD33⁻), and primary AML targets (de novo, refractory and post transplant) to determine whether treatment with CD16x33 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 CD16x33 BiKE when cells were cultured with HL60 targets, CD33⁺ de novo and refractory AML targets. Combination treatment with CD16x33 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 CD16x33 BiKE resulted in activation, especially in those recipients with CMV reactivation.

Corresponding Author: Jeffrey S. Miller, MD, Professor of Medicine, University of Minnesota Masonic Cancer Center, MMC 806, Division of Hematology, Oncology, and Transplantation, Harvard Street at East River Road, Minneapolis, MN 55455, mille011@umn.edu, Phone number: 612-625-7409; Fax number: 612-626-3941.

COI Statement: There are no conflicts of interest to on behalf of the co-authors.

Conclusion—CD16x33 BiKE can overcome self inhibitory signals and effectively elicit NK cell effector activity against AML. These *in vitro* studies highlight the potential of CD16x33 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 anti-leukemic therapy post-transplantation.

Keywords

NK cells; AML; CD16; BiKE; ADAM17

Introduction

Patients with intermediate and poor-risk acute myeloid leukemia (AML) who achieve complete remission after induction chemotherapy benefit from allogeneic hematopoietic cell transplantation (HCT)(2). 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 non-transplantation setting (3, 4). 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 (5, 6). 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.

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 (ITAMs), triggering the release of lytic granules and cytokines such as interferon (IFN-γ) and tumor necrosis factor (TNF-α)(7–9). In addition, CD16 downregulation in NK cells occurs when these cells interact with target cells (10), an effect that we demonstrated to be a result of ectodomain shedding of CD16 upon NK cell activation. CD16 shedding by NK cells is mediated by the trans-membrane glycoprotein, a disintegrin and metalloprotease-17 or ADAM17 (11). 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 demonstrated that CD16-directed bi-specific (CD16x19) and tri-specific (CD16x19x22) 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 (12, 13). In the present study, we designed a novel bi-specific killer cell engager antibody (BiKE), developed specifically to signal through CD16 and target the myeloid differentiation antigen CD33 (named CD16x33 BiKE). CD33 is a myeloid surface antigen expressed on the AML blasts of approximately 85% of patients (14). We tested whether CD16x33 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 CD16x33 BiKE.

Materials and Methods

Cell isolation and purification

Adult peripheral blood (PB) 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. De-identified human samples were obtained from the Leukemia-Myelodysplastic Syndrome Tissue Bank (LMTB) at the University of Minnesota (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. Prior to 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), 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 Fluor647-conjugated anti-human TNF- α (Mab11; Biolegend), 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 performed 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 bscFv CD16 x CD33 (CD16x33) BiKE

Synthesis and assembly of hybrid genes encoding the CD16x33 BiKE reagent were accomplished by using DNA shuffling and DNA ligation techniques as previously described (15). 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 et al (a gift of L. Weiner, Georgetown University, Washington DC) (16), a 20-amino acid segment of human muscle aldolase (PSGQAGAAASESLFVSNHAY), the VH and VL regions of human anti-CD33, and an

XhoI restriction site. The resultant 1526 base pair NcoI/XhoI fragment gene was spliced into the pET21d expression vector under control of an isopropyl- β -D-thiogalactopyranoside (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 performed as previously described (15).

ADAM17 inhibition and MHC-class I antibody-blocking experiments

A highly selective ADAM17 inhibitor (INCB003619) from Incyte (Wilmington, DE) was added at a concentration of 2 μ M to purified NK cells 30 minutes before testing. Mouse mAb IgG1 clone HP-1F7 (kindly provided by M. Lopez Botet) was used as a positive control for pan-HLA blocking. The mAb HP-1F7 effectively binds to the alpha chain of HLA-A, -B, -C, -E and -G, and blocks all NK cell inhibitory receptor engagement (17–19). 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 CD16x33 BiKE (or as described in figures) and incubated for an extra 30 mins. Anti-human CD107a mAb was added alone or with target cells (HL60, Raji, de novo primary AML, or relapse/refractory AML; E:T ratios 2:1 or as described in figures) and incubated for 1 hour. GolgiStop (1:1500) and GolgiPlug (1:1000; 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 permeabilization. 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 FACS analysis.

⁵¹Chromium Release Cytotoxicity Assay

Direct cytotoxicity assays were performed by standard 4-hour ⁵¹Cr-release assays using NK cells pre-treated with bscFv CD16x33 BiKE and HL60 or Raji targets as indicated. ⁵¹Cr released by specific target cell lysis was measured by a gamma scintillation counter, and the percent target cell lysis was calculated as follows: [(experimental lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis)] \times 100.

Statistical Analysis

Data were summarized with mean and standard error (mean \pm SEM). For comparisons between independent samples, Student's T-test was used. For comparisons of matched samples, paired T-test was used. Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

Results

CD16x33 BiKE specificity enhances NK cell effector functions against CD33⁺ HL60 AML cells

We first determined whether the CD16x33 BiKE induces NK cell effector function against the HL60 cell line (CD33⁺). CD16x33 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, CD16x33 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 CD16x33 BiKE, with a plateau at 5 ug/mL and above. On the basis of these initial results, we selected a dose of 5 ug/mL of the CD16x33 BiKE for future experiments.

For NK cells to be effective therapeutically, they must function at low effector:target (E:T) ratios. CD16x33 BiKE at 5 ug/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 CD16x33 BiKE at all E:T ratios, with diminished absolute killing at lower E:T ratios as expected (Fig. 1D). Further, CD16x33 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(20). To determine whether CD16A⁺ cells other than NK cells could contribute to the observed CD16x33 BiKE-mediated cytotoxicity, we performed a 4-hour chromium release assay with or without CD16x33 BiKE added to PBMC-containing NK cells or monocytes (Fig. 2A). CD16x33 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.

In addition to CD33 expression on AML cells, CD33 expression on other myeloid differentiated cells, such as monocytes (14, 21), has the potential to interfere with target cell killing. We tested whether autologous CD33⁺ monocytes interfere with the ability of CD16x33 BiKE to induce NK cell cytotoxicity and cytokine release. Flow cytometry based function indicated that CD16x33 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 CD16x33 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 CD16x33 BiKE treatment (Fig 2A). Lastly, 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 CD16x33 BiKE does not activate NK cells sufficient to mediate a bystander effect against CD33⁻ targets.

CD16 downregulation on NK cells occurs after exposure to CD16x33 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 while others are inhibitory, where recognition of MHC suppresses NK cell cytotoxic activity (5). CD16 is an important activating receptor that mediates ADCC. It has been previously recognized that CD56^{dim} NK cells downregulate CD16 after target cell-induced activation (10). To determine if BiKE activation induces changes in NK cell receptor expression, we analyzed the expression of CD16 and KIR (KIR2DL1, KIR2DL2, KIR2DL3, 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 demonstrate 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 3B).

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 3C). More importantly, ADAM17 inhibition enhanced CD16x33 BiKE-mediated NK cell effector functions against de novo CD33⁺ AML blasts (Fig. 3A and 3B). 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 CD16 shedding by an ADAM17 inhibitor translates into markedly stronger BiKE activation of NK cells.

CD16x33 BiKE overcomes major histocompatibility complex (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 (5). It is well recognized that AML cells express MHC class I ligands (22). 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 CD16x33 BiKE activation of NK cells. The monoclonal antibody (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 (17) and we have demonstrated 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 (23), validating the HP-F1 mAb to block MHC class I interactions. Our results demonstrate 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 CD16x33 BiKE alone. In addition, blocking of class I further enhances NK cell activation when cells are treated with the CD16x33 BiKE (Fig 4). Together, these results suggest that the CD16x33 BiKE has the ability to overcome inhibitory signals, which under physiologic conditions inhibit AML recognition by NK cells.

CD16x33 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). CD16x33 BiKE substantially activated NK cells against these CD33⁺ refractory AML targets (Fig 5B). Combination treatment with CD16x33 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 CD16x33 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 CD16x33 BiKE, but that CD33⁺ AML blasts from relapse/refractory disease also remain sensitive to the BiKE.

CD16x33 BiKE-stimulated NK cell effector functions are enhanced by cytomegalovirus (CMV) reactivation in UCBT recipients

CMV reactivation after allogeneic HCT is reportedly associated with a decreased risk of relapse in patients with AML (24). We have previously described a more mature NK cell phenotype in transplant recipients who reactivate CMV (25). To determine whether CMV reactivation affects the ability of the CD16x33 BiKE to activate NK cells from UCBT recipients, we analyzed PBMC samples from patients that underwent UCBT at 3 months and 1 year post-transplant. Samples were selected based on 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 performed on these samples and compared to NK cell CD16 expression from healthy donors (Fig. 6A). Our results demonstrate 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 CD16x33 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 CD16x33 BiKE-induced NK cell activation as compared to 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 CD16x33 BiKE in CMV-reactive UCBT recipients, and that other mechanisms are likely involved that result in increase NK cell effector functions. Overall, these data demonstrate that CMV reactivation after allogeneic HCT enhances CD16x33 BiKE-mediated NK cell cytotoxicity and cytokine release.

Discussion

We addressed whether a fully humanized bispecific killer cell engager antibody containing binding sites for CD16 and CD33 (CD16x33 BiKE) could enhance the potency and specificity of CD16⁺ NK cell effector functions against CD33⁺ leukemic cells. Collectively, our experiments demonstrate 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 CD16x33 BiKE, enhances NK cell cytokine production and cytotoxicity. Lastly, our data indicate that activation of NK cells by CD16x33 BiKE is diminished early after UCBT, but that CMV reactivation increases BiKE-induced NK cell targeting against AML.

Our experiments establish two 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 demonstrate that the use of the CD16x33 BiKE is specific for NK cell activation and exclusively targets CD33⁺ cells. Since CD33 is expressed in healthy myeloid cells, we showed that CD16x33 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 versus 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 to HL60 targets. Despite this, if CD16x33 BiKE is used to treat AML patients, myelosuppression from targeting healthy CD33⁺ cells would be expected. Since 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 CD16x33 BiKE treatment could target both CD33⁺ AML blasts and CD33⁺ monocytes. This is not a surprise since monocytes can express NK activating ligands such as CD48, the ligand for the activating receptor 2B4 (26). To minimize this potential “monocyte sink”, treating AML patients 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 acute myeloid leukemia blasts at diagnosis and at relapse (22, 27). 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 killing. While treatment of AML targets with MHC class I blockade alone is able to trigger NK cell function to some degree, treatment with CD16x33 BiKE alone was far more potent. Our results demonstrate that treatment with CD16x33 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 CD16x33 BiKE since this reagent alone is able to induce potent CD16 signaling as demonstrated by intracellular Ca² mobilization (12).

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 (13). 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 (11), but has also been recognized as a potential target for cancer therapy because it is widely overexpressed by many tumors (1, 28, 29). ADAM17 has been reported to have a direct functional role in controlling proliferation and migration of tumor 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 anti-tumor activity by ADAM17 inhibition. We found that ADAM17 inhibition and NK cell activation with CD16x33 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 CD16x33 BiKE and ADAM17 inhibition in tumor bearing animals. Since ADAM17 was originally recognized for being the major protein responsible for the cleavage of the trans-membrane proteins TNF- α (33), inhibitors of ADAM17 have been used in animal models and have showed 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 (11)

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 post-transplantation is urgently needed. After allogeneic HCT, NK cells mediate GVL by the production of inflammatory cytokines and by direct target lysis. We have previously demonstrated 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 CD16x33 BiKE to induce GVL after UCBT. Elmaagacli et al. 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 (24). The mechanism by which CMV reactivation is protective in the setting of allogeneic transplantation is poorly understood. We recently demonstrated that NK cells from patients who reactivate CMV post-transplant have a more mature phenotype, with an increased percentage of CD56^{dim} NK cells and increased expression of the activating receptor NKG2C, as compared to NK cells from patients who did not reactivate CMV post-allogeneic HCT (25). 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 et al. 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 to healthy donors, but this percentage recovers over time. Moreover, CMV reactivation post-transplant confers an increase in NK cell responsiveness to CD16x33 BiKE. Together, these findings raise the possibility that treatment with the CD16x33 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 monoclonal antibody, failed to demonstrate improvement in response rates or overall survival in patients with refractory or relapsed AML in a phase III study (41). Gemtuzumab ozogamicin (GO), 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 Food and Drug Administration (FDA) in 2000. Unfortunately, GO was eventually withdrawn from the market in 2010 after a post-approval 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 GO 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⁺. Based on this, escape by CD33-negative 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 CD16x33 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 CD16x33 BiKE in AML patients with CD33 expressing leukemic stem cells and in combination with chemotherapy after haploidentical NK cell infusion or early after HCT to enhance CD33⁺ stem cell 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.

While it is possible that treatment of AML patients with the CD16x33 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 CD16x33 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, monoclonal antibodies 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 two 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 two 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 CD16x33 BiKE ± ADAM17 inhibition against myeloid disorders and their potential role in cancer therapeutics.

Acknowledgments

We would like to acknowledge Michael Franklin for editorial assistance, Julie Curtsinger and Liz Narten for assistance with sample collections and the personnel at the Leukemia-Myelodysplastic Syndrome Tissue Bank (LMTB) at the University of Minnesota Masonic Cancer Center.

Grants and financial support: These studies were supported in part by P01 CA65493 (JSM, MRV), P01 CA111412 (JSM, DJW), K05 CA157439 (JAR), R01 CA142714 (JAR), R01 CA107143 (JAR), R01 CA36725 (DAV), T32 CA099936 (MKG), the Minnesota Masonic Charities (JSM, DAV), the US Public Health Service Grants, the William Lawrence and Blanche Hughes Fund (DAV), and the University of Minnesota/May Clinic Partnership Grant (JSM, JAR, DJW, EW).

References

1. Blanchot-Jossic F, Jarry A, Masson D, Bach-Ngohou K, Paineau J, Denis MG, et al. Up-regulated expression of ADAM17 in human colon carcinoma: co-expression with EGFR in neoplastic and endothelial cells. *The Journal of pathology*. 2005; 207:156–63. [PubMed: 16041691]
2. Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA: the journal of the American Medical Association*. 2009; 301:2349–61. [PubMed: 19509382]
3. Fuchs EJ, Huang XJ, Miller JS. HLA-haploidentical stem cell transplantation for hematologic malignancies. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2010; 16:S57–63. [PubMed: 19892024]

4. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005; 105:3051–7. [PubMed: 15632206]
5. Lanier LL. NK cell recognition. *Annu Rev Immunol*. 2005; 23:225–74. [PubMed: 15771571]
6. Ljunggren HG, Karre K. In search of the ‘missing self’: MHC molecules and NK cell recognition. *Immunology today*. 1990; 11:237–44. [PubMed: 2201309]
7. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *J Immunol*. 1988; 141:3478–85. [PubMed: 2903193]
8. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nature reviews Immunology*. 2003; 3:304–16.
9. Lanier LL. Natural killer cell receptor signaling. *Current opinion in immunology*. 2003; 15:308–14. [PubMed: 12787756]
10. Grzywacz B, Kataria N, Verneris MR. CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2007; 21:356–9. author reply 9.
11. Romee R, Foley B, Lenvik T, Wang Y, Zhang B, Ankarlo D, et al. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood*. 2013
12. Gleason MK, Verneris MR, Todhunter DA, Zhang B, McCullar V, Zhou SX, et al. Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Molecular cancer therapeutics*. 2012; 11:2674–84. [PubMed: 23075808]
13. Bruenke J, Barbin K, Kunert S, Lang P, Pfeiffer M, Stieglmaier K, et al. Effective lysis of lymphoma cells with a stabilised bispecific single-chain Fv antibody against CD19 and FcγRIII (CD16). *British journal of haematology*. 2005; 130:218–28. [PubMed: 16029450]
14. Duffy MJ, Mullooly M, O’Donovan N, Sukor S, Crown J, Pierce A, et al. The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? *Clinical proteomics*. 2011; 8:9. [PubMed: 21906355]
15. Vallera DA, Todhunter DA, Kuroki DW, Shu Y, Sicheneder A, Chen H. A bispecific recombinant immunotoxin, DT2219, targeting human CD19 and CD22 receptors in a mouse xenograft model of B-cell leukemia/lymphoma. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2005; 11:3879–88. [PubMed: 15897589]
16. McCall AM, Adams GP, Amoroso AR, Nielsen UB, Zhang L, Horak E, et al. Isolation and characterization of an anti-CD16 single-chain Fv fragment and construction of an anti-HER2/neu/anti-CD16 bispecific scFv that triggers CD16-dependent tumor cytotoxicity. *Molecular immunology*. 1999; 36:433–45. [PubMed: 10449096]
17. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *The Journal of experimental medicine*. 1997; 186:1809–18. [PubMed: 9382880]
18. Perez-Villar JJ, Melero I, Navarro F, Carretero M, Bellon T, Llano M, et al. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. *J Immunol*. 1997; 158:5736–43. [PubMed: 9190923]
19. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *European journal of immunology*. 1999; 29:277–83. [PubMed: 9933109]
20. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. *Nature reviews Immunology*. 2008; 8:34–47.
21. Freeman SD, Kelm S, Barber EK, Crocker PR. Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. *Blood*. 1995; 85:2005–12. [PubMed: 7718872]
22. Wetzler M, Baer MR, Stewart SJ, Donohue K, Ford L, Stewart CC, et al. HLA class I antigen cell surface expression is preserved on acute myeloid leukemia blasts at diagnosis and at relapse.

- Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2001; 15:128–33.
23. Godal R, Bachanova V, Gleason M, McCullar V, Yun GH, Cooley S, et al. Natural killer cell killing of acute myelogenous leukemia and acute lymphoblastic leukemia blasts by killer cell immunoglobulin-like receptor-negative natural killer cells after NKG2A and LIR-1 blockade. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2010; 16:612–21. [PubMed: 20139023]
 24. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenscher R, Ditschkowski M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood*. 2011; 118:1402–12. [PubMed: 21540462]
 25. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. Human Cytomegalovirus (CMV)-Induced Memory-like NKG2C+ NK Cells Are Transplantable and Expand In Vivo in Response to Recipient CMV Antigen. *J Immunol*. 2012; 189:5082–8. [PubMed: 23077239]
 26. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nature reviews Immunology*. 2005; 5:112–24.
 27. Lion E, Willems Y, Berneman ZN, Van Tendeloo VF, Smits EL. Natural killer cell immune escape in acute myeloid leukemia. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2012; 26:2019–26.
 28. Sinnathamby G, Zerfass J, Hafner J, Block P, Nickens Z, Hobeika A, et al. ADAM metalloproteinase domain 17 (ADAM17) is naturally processed through major histocompatibility complex (MHC) class I molecules and is a potential immunotherapeutic target in breast, ovarian and prostate cancers. *Clinical and experimental immunology*. 2011; 163:324–32. [PubMed: 21175594]
 29. Ringel J, Jesnowski R, Moniaux N, Luttes J, Ringel J, Choudhury A, et al. Aberrant expression of a disintegrin and metalloproteinase 17/tumor necrosis factor-alpha converting enzyme increases the malignant potential in human pancreatic ductal adenocarcinoma. *Cancer research*. 2006; 66:9045–53. [PubMed: 16982746]
 30. Richards FM, Tape CJ, Jodrell DI, Murphy G. Anti-tumour effects of a specific anti-ADAM17 antibody in an ovarian cancer model in vivo. *PloS one*. 2012; 7:e40597. [PubMed: 22792380]
 31. Maretzky T, Zhou W, Huang XY, Blobel CP. A transforming Src mutant increases the bioavailability of EGFR ligands via stimulation of the cell-surface metalloproteinase ADAM17. *Oncogene*. 2011; 30:611–8. [PubMed: 20871631]
 32. Weskamp G, Mendelson K, Swendeman S, Le Gall S, Ma Y, Lyman S, et al. Pathological neovascularization is reduced by inactivation of ADAM17 in endothelial cells but not in pericytes. *Circulation research*. 2010; 106:932–40. [PubMed: 20110534]
 33. Rose-John S. ADAM17, shedding, TACE as therapeutic targets. *Pharmacological research: the official journal of the Italian Pharmacological Society*. 2013; 71C:19–22. [PubMed: 23415892]
 34. Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, et al. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature*. 1994; 370:218–20. [PubMed: 8028669]
 35. Moss ML, Sklair-Tavron L, Nudelman R. Drug insight: tumor necrosis factor-converting enzyme as a pharmaceutical target for rheumatoid arthritis. *Nature clinical practice Rheumatology*. 2008; 4:300–9.
 36. Gupta V, Tallman MS, He W, Logan BR, Copelan E, Gale RP, et al. Comparable survival after HLA-well-matched unrelated or matched sibling donor transplantation for acute myeloid leukemia in first remission with unfavorable cytogenetics at diagnosis. *Blood*. 2010; 116:1839–48. [PubMed: 20538804]
 37. Farag SS, Maharry K, Zhang MJ, Perez WS, George SL, Mrozek K, et al. Comparison of reduced-intensity hematopoietic cell transplantation with chemotherapy in patients age 60–70 years with acute myelogenous leukemia in first remission. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2011; 17:1796–803. [PubMed: 21699879]

38. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. NK cell education after allogeneic transplantation: dissociation between recovery of cytokine-producing and cytotoxic functions. *Blood*. 2011; 118:2784–92. [PubMed: 21757615]
39. Beck JC, Wagner JE, DeFor TE, Brunstein CG, Schleiss MR, Young JA, et al. Impact of cytomegalovirus (CMV) reactivation after umbilical cord blood transplantation. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2010; 16:215–22. [PubMed: 19786112]
40. Jacobson CA, Turki AT, McDonough SM, Stevenson KE, Kim HT, Kao G, et al. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2012; 18:565–74. [PubMed: 21875503]
41. Feldman EJ, Brandwein J, Stone R, Kalaycio M, Moore J, O'Connor J, et al. Phase III randomized multicenter study of a humanized anti-CD33 monoclonal antibody, lintuzumab, in combination with chemotherapy, versus chemotherapy alone in patients with refractory or first-relapsed acute myeloid leukemia. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2005; 23:4110–6. [PubMed: 15961759]
42. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H, et al. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2001; 19:3244–54. [PubMed: 11432892]
43. Castaigne S, Pautas C, Terre C, Raffoux E, Bordessoule D, Bastie JN, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. *Lancet*. 2012; 379:1508–16. [PubMed: 22482940]
44. Burnett AK, Hills RK, Milligan D, Kjeldsen L, Kell J, Russell NH, et al. Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2011; 29:369–77. [PubMed: 21172891]
45. Burnett AK, Russell NH, Hills RK, Kell J, Freeman S, Kjeldsen L, et al. Addition of gemtuzumab ozogamicin to induction chemotherapy improves survival in older patients with acute myeloid leukemia. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2012; 30:3924–31. [PubMed: 22851554]
46. Walter RB, Appelbaum FR, Estey EH, Bernstein ID. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. *Blood*. 2012; 119:6198–208. [PubMed: 22286199]
47. Lee SC, Srivastava RM, Lopez-Albaitero A, Ferrone S, Ferris RL. Natural killer (NK): dendritic cell (DC) cross talk induced by therapeutic monoclonal antibody triggers tumor antigen-specific T cell immunity. *Immunologic research*. 2011; 50:248–54. [PubMed: 21717064]
48. Stasi R, Evangelista ML, Buccisano F, Venditti A, Amadori S. Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. *Cancer treatment reviews*. 2008; 34:49–60. [PubMed: 17942233]
49. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *British journal of pharmacology*. 2009; 157:220–33. [PubMed: 19459844]

STATEMENT OF 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 down-regulated by ADAM17, a trans-membrane CD16 sheddase. This manuscript reports on a novel bi-specific killer cell engager (BiKE) signaling through CD16 and targeting the myeloid antigen CD33 (CD16x33 BiKE), which activates NK cells specificity against de novo and refractory Acute Myeloid 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 CD16x33 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 CMV reactivation, is warranted.

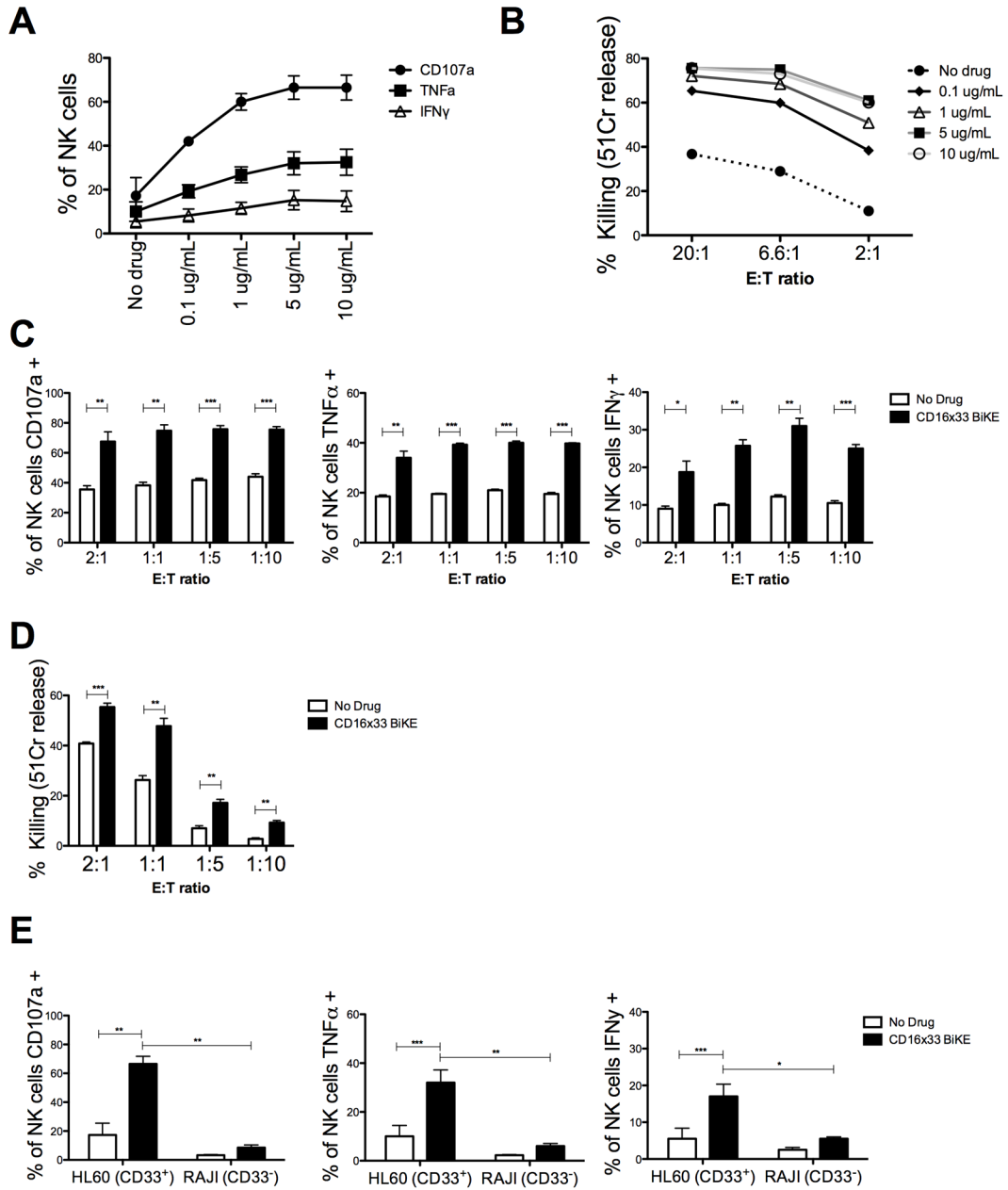


Fig 1. CD16x33 BiKE enhances NK cell function against CD33⁺ targets in a dose-dependent manner

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 CD16x33 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 ug/mL of CD16 x CD33 BiKE. (E) Specificity of the CD16 x CD33 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 ug/mL of CD16 x CD33 BiKE (n=4). (*P<0.05; **P<0.01, ***P<0.001).

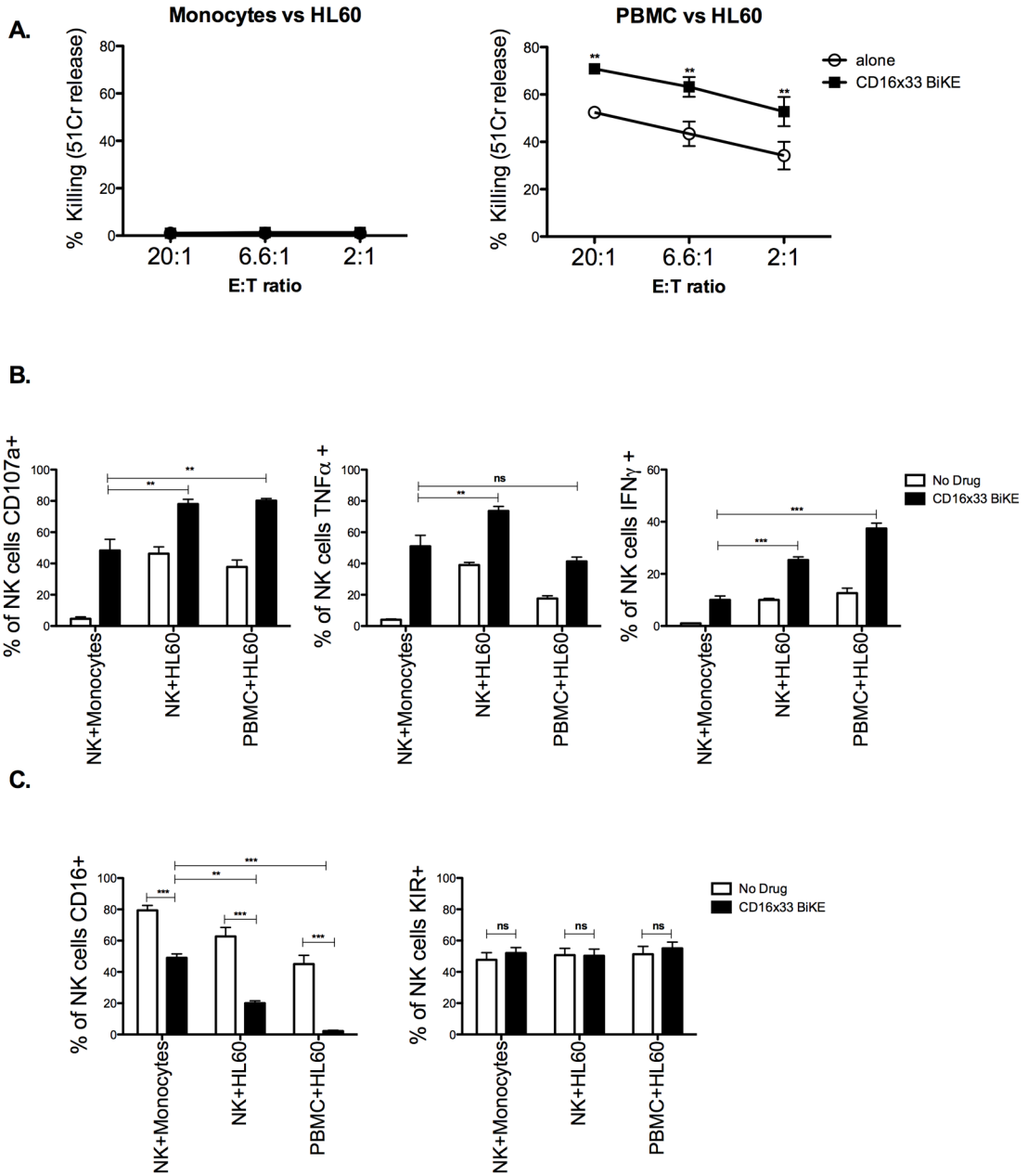


Fig 2. CD16x33 BiKE activates NK cells against CD33⁺ HL60 targets in the presence of CD33⁺ monocytes

(A) **Monocytes (CD33⁺)** and PBMC containing NK cells (PBMC [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 μ g/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 μ g/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<0.05; **P<0.01, ***P<0.001).

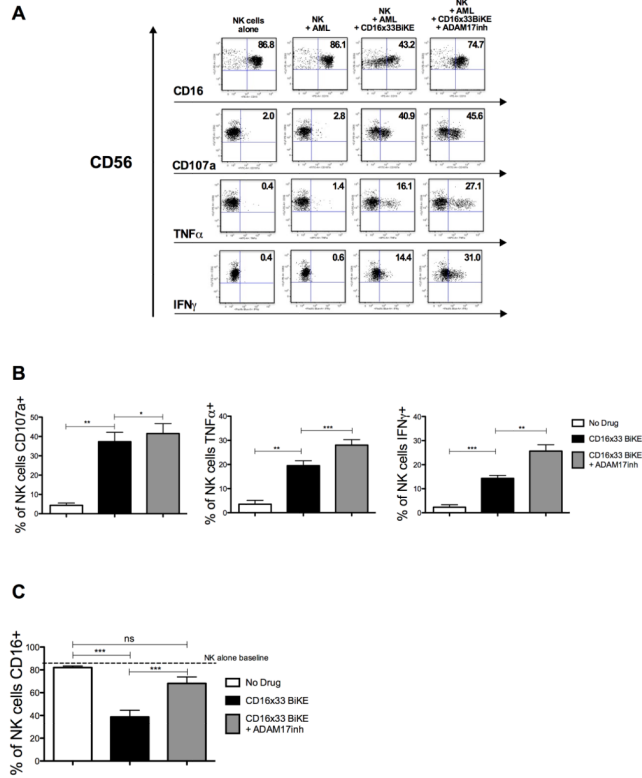


Fig 3. CD16x33 BiKE triggers NK cell degranulation and cytokine production against de-novo CD33⁺ AML targets, an effect that is enhanced by ADAM17 inhibition

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 ug/mL of CD16x33 BiKE and with or without 2uM 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).

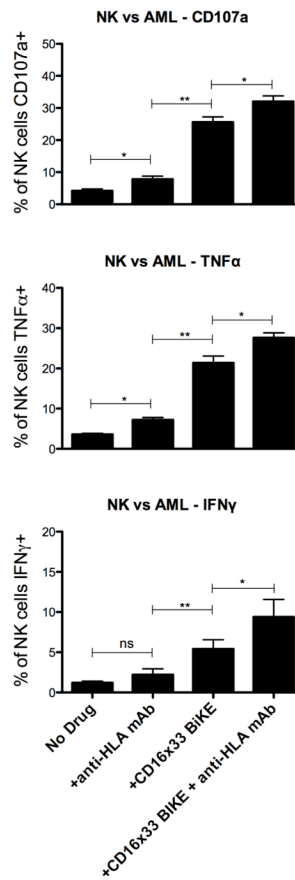


Fig 4. MHC class I blockade in AML targets triggers NK cell activation, effect enhanced with CD16x33 BiKE activation

AML target cells were pre-incubated with 20 ug/mL of a pan-HLA blocking antibody (HP-1F7) for 30 mins prior to 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 ug/mL of CD16x33 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, ***P<0.001).

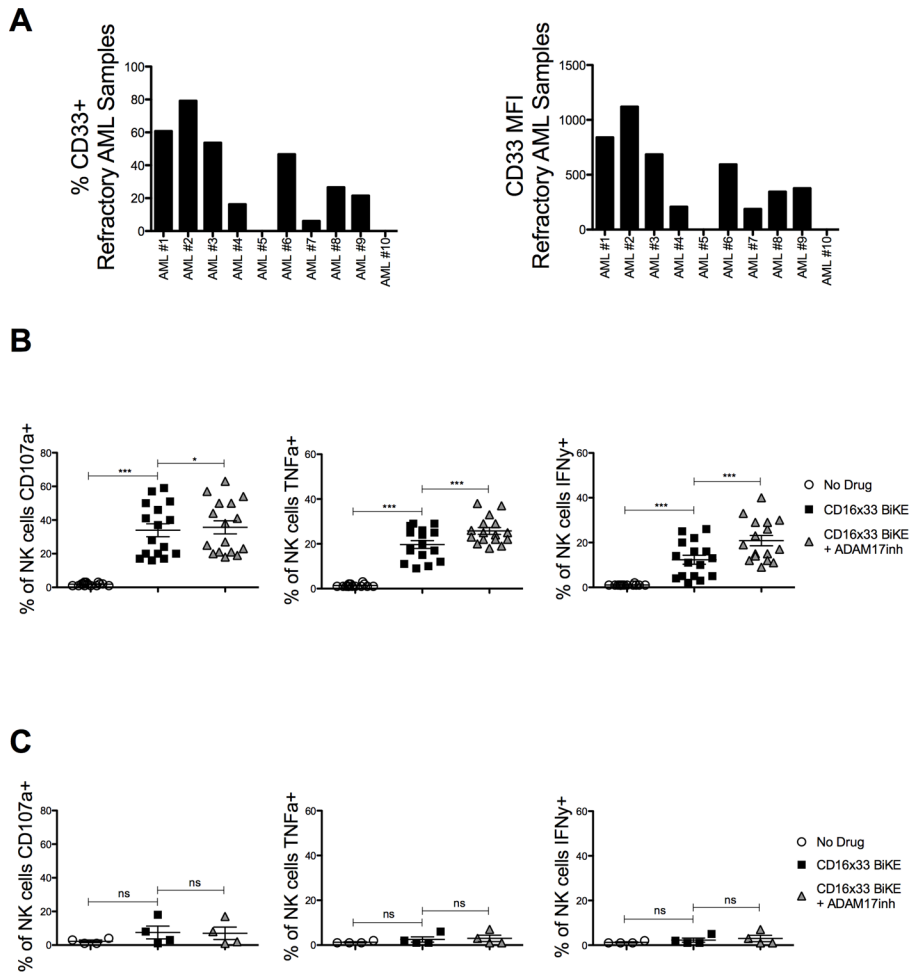


Fig 5. CD16x33 BiKE triggers NK cell degranulation and cytokine production against relapse/refractory CD33⁺ AML targets
(A) CD33 surface expression measured by flow cytometry analysis of bone marrow samples from 10 patients with relapse/refractory AML. Percent expression and median fluorescence intensity (MFI) are shown for each sample. **(B and C)** Purified NK cells isolated from two 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 ug/mL of CD16x33 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<0.01, ***P<0.001).

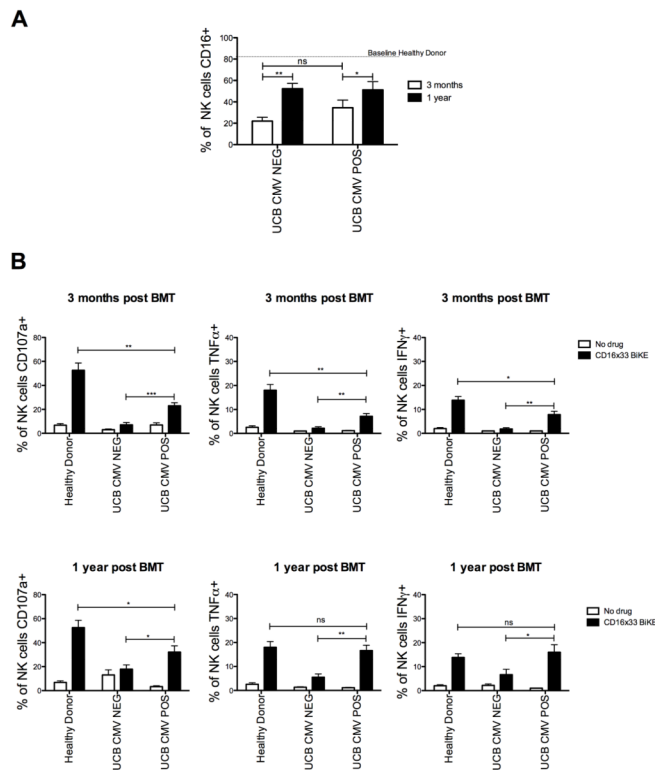


Fig 6. CD16x33 BiKE-stimulated NK cells after SCT exhibit time- and CMV reactivation-dependent activity against primary CD33⁺ AML targets
 PBMCs were collected from double umbilical cord blood (UCB) recipients without CMV reactivation (UCB CMV NEG)(n=6) and from recipients with CMV reactivation (UCB CMV POS)(n=6) who had reactivated CMV in the first one hundred days post-transplant. PBMC samples were collected at 3 months and 1 year post-transplant and compared to 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 post-transplant. **(B)** PBMCs were treated with or without 5 ug/mL of CD16 x CD33 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 to PBMC from healthy donors (*P<0.05; **P<0.01, ***P<0.001).