Bispecific and Trispecific Killer Cell Engagers Directly Activate Human NK Cells through CD16 Signaling and Induce Cytotoxicity and Cytokine Production

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Abstract

This study evaluates the mechanism by which bispecific and trispecific killer cell engagers (BiKEs and TriKEs) act to trigger human natural killer (NK) cell effector function and investigates their ability to induce NK cell cytokine and chemokine production against human B-cell leukemia. We examined the ability of BiKEs and TriKEs to trigger NK cell activation through direct CD16 signaling, measuring intracellular Ca2+ mobilization, secretion of lytic granules, induction of target cell apoptosis, and production of cytokine and chemokines in response to the Raji cell line and primary leukemia targets. Resting NK cells triggered by the recombinant reagents led to intracellular Ca2+ mobilization through direct CD16 signaling. Coculture of reagent-treated resting NK cells with Raji targets resulted in significant increases in NK cell degranulation and target cell death. BiKEs and TriKEs effectively mediated NK cytotoxicity of Raji targets at high and low effector-to-target ratios and maintained functional stability after 24 and 48 hours of culture in human serum. NK cell production of IFN-γ, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, macrophage inflammatory protein (MIP)-1α, and regulated and normal T cell expressed and secreted (RANTES) was differentially induced in the presence of recombinant reagents and Raji targets. Moreover, significant increases in NK cell degranulation and enhancement of IFN-γ production against primary acute lymphoblastic leukemia and chronic lymphocytic leukemia targets were induced with reagent treatment of resting NK cells. In conclusion, BiKEs and TriKEs directly trigger NK cell activation through CD16, significantly increasing NK cell cytolytic activity and cytokine production against tumor targets, showing their therapeutic potential for enhancing NK cell immunotherapies for leukemias and lymphomas. Mol Cancer Ther; 11(12); 2674–84. ©2012 AACR.

Introduction

With more than 70,000 cases anticipated in the United States for 2012, non-Hodgkins lymphoma (NHL) is the most common adult hematologic malignancy, of which 85% of cases are of B-cell origin (1, 2). Monoclonal antibodies (mAb), such as rituximab, have shown to be therapeutic for the treatment of NHL (3). Despite this success, there are limitations that decrease the overall efficiency of mAb therapies (4). With the development of CD16-directed bispecific and trispecific single-chain fragment variable (bscFv and tscFv) recombinant reagents, most of these undesired limitations are avoided while eliciting high effector function as they lack the Fc portion of whole antibodies and have a targeted specificity for CD16 (5–7). As a result, recombinant reagents are attractive for clinical use in enhancing natural killer (NK) cell immunotherapies.

The ability of NK cells to recognize and kill targets is regulated by a sophisticated repertoire of inhibitory and activating cell surface receptors. NK cell cytotoxicity can occur by natural cytotoxicity, mediated via the natural cytotoxicity receptors (NCR), or by antibodies, such as rituximab, to trigger antibody-dependent cell-mediated cytotoxicity (ADCC) through CD16, the activating low-affinity Fc-γ receptor for immunoglobulin G (IgG) highly expressed by the CD56dim subset of NK cells (8–11). Natural cytotoxicity is triggered via NCRs by de novo expression of NK cell activating receptor ligands on target cells. In the absence of cytokine stimulation, these receptors inefficiently elicit a cytotoxic or cytokine response independently, but together they are able to function synergistically to activate a resting NK cell and promote effector function (9, 12). In contrast, ADCC is mediated when CD16 binds to opsonized targets through Fc...
engagement and signals through immunoreceptor tyrosine-based activation motifs (ITAM) of the associated FcγRIγ chain and CD3ε chain subunits (13). The signal delivered via CD16 is potent and induces both a cytotoxic and cytokine response in the absence of cytokine stimulation that can further be enhanced by coengagement of other activating receptors (12).

In this study, we show the ability of a CD16/CD19 BiKE and a CD16/CD19/CD22 TriKE to trigger NK cell activation through direct signaling of CD16 and induce directed secretion of lytic granules and target cell death. Furthermore, we show for the first time the ability of these reagents to induce NK cell activation that leads to cytokine and chemokine production.

Materials and Methods

Cell isolation and purification

Peripheral blood mononuclear cells (PBMC) were isolated from adult blood (Memorial Blood Center, Minneapolis, MN) by centrifugation using a Histopaque gradient (Sigma-Aldrich), and NK cells were purified by removing T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythrocyds via magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). Primary acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic lymphoblastic leukemia (CLL) samples were obtained from the Leukemia MDS Tissue Bank (LMTB) at the University of Minnesota (Minneapolis, MN). All samples were obtained after informed consent and in accordance with the Declaration of Helsinki, using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

Flow cytometry

Cell suspensions were stained with the following mAbs: PE/Cy7-conjugated CD56 (HCD56; BioLegend), ECD-conjugated CD3 (UCHT1; Beckman Coulter), FITC-conjugated CD16 (3G8; BD Biosciences), PE-conjugated CD19 (SJ25C1; BD Biosciences), APC-conjugated CD20 (L243; BD Biosciences), PE-conjugated CD16 (3G8; BD Biosciences), ECD-conjugated CD3 (UCHT1; Beckman Coulter), FITC-conjugated CD22 (H1B22; BioLegend), PE/Cy7-conjugated CD56 (HCD56; BioLegend), and AF488-conjugated cleaved caspase-3 (9669; Cell Signaling Technology). Phenotypic acquisition of cells was carried out on the LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

Construction, expression, and purification of bscFv CD16/CD19 and tscFv CD16/CD19/CD22 reagents

Synthesis of hybrid genes encoding the bscFv and tscFv reagents were accomplished using DNA shuffling and DNA ligation techniques as previously described (14). The fully assembled gene (from 5’ end to 3’ end) consisted of an Ncol restriction site, an ATG initiation codon, the 

\[ V_h \]  and 

\[ V_l \]  regions of anti-human CD16 (NM3E2; ref. 6), a 20-amino acid segment of human muscle aldolase, the 

\[ V_h \]  and 

\[ V_l \]  regions of humanized anti-CD22 (14), humanized anti-CD19 (14), and a Khol restriction site. The resultant gene fragment was spliced into the pET21d expression vector, and DNA sequencing analysis (Biomedical Genomics Center, University of Minnesota) was used to verify that the gene was correct in sequence and cloned in frame. Genes encoding the BiKEs and monospecific scFv controls were created in the same manner. For bacterial protein expression and purification by ion exchange and size-exclusion chromatography, methods were used as previously described (14).

Proliferation assay

The Burkitt lymphoma Raji cell line (American Type Culture Collection) was cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco). Then, 2 × 10⁴ Raji cells were treated with varying concentrations of CD16/CD19/CD22 or nonspecific control CD16/EpCAM and 1 nmol/L of a targeted diphtheria toxin-CD19/CD22 (DT-CD19/CD22) was added and a ³H-thymidine proliferation was carried out as previously described (14).

Cytokine/chemokine production, CD107a, and caspase-3 assay

PBMC or purified NK cells were incubated overnight at 37°C, 5% CO₂ in basal medium (RPMI supplemented with 10% fetal calf serum). Cells were washed, treated with bscFv CD16/CD19, tscFv CD16/CD19/CD22, rituximab (Genentech), CD22 parental antibody (derived from the RF84 hybridoma), CD19 parental antibody (derived from the HD37 hybridoma), scFv anti-CD16 or scFv CD19/CD22 (negative controls), and CD107a and intracellular IFN-γ assays were conducted as previously described (15). For evaluation of caspase-3 activation, Raji target cell cleaved caspase-3 expression was evaluated by fluorescent-activated cell sorting (FACS) analysis using a live forward/side scatter gate and a CD56+/CD20+ gate to determine cleaved casapse-3–positive Raji populations relative to an isotype control (rabbit IgG; Cell Signaling Technology). For Luminex analysis of cytokines/chemokines, NK cells, Raji cells, and NK cells cocultured with Raji cells were treated with the BiKE, TriKE, rituximab, negative controls, or no reagent for 6 hours at 37°C in 5% CO₂. Supernatant levels of IFN-γ, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL-8), macrophage inflammatory protein (MIP-1α), and RANTES were determined by multiplex assay using the Luminex system (Luminex Co.) and human-specific bead sets (R&D systems; sensitivity 0.5–3.0 pg/mL) and the final pg/mL values adjusted for background for each condition were determined using the following equation: (NK cells + Raji cells pg/mL) – [NK cells alone pg/mL] + [Raji cells alone pg/mL].
**51Chromium release cytotoxicity assay**

Direct cytotoxicity assays were conducted by standard 4-hour 51Chromium (51Cr) release assays using effector
cells pretreated with reagents and Raji cells as targets. For
examination of reagent stability, reagents were preincu-
bated for 24 and 48 hours at 37°C, 5% CO₂ in 100% human
serum. The 51Cr released by specific target cell lysis was
measured by a gamma scintillation counter and the per-
centage of specific cell lysis was calculated.

**Ca²⁺ flux assay**

Ca²⁺ flux was measured using the Fluo-4 NW Calcium
Assay Kit (Invitrogen) within effector NK cells according
to the manufacturer’s protocol. Ca²⁺ mobilization was
evaluated by FACS analysis. After 30 seconds of acquisi-
tion, 10 μg/mL of purified anti-CD16 mAb (3G8; Becton
Dickinson Biosciences), biotinylated scFv anti-CD16, puri-
ified mlgG (negative control; R&D Systems), or 1 μg/mL of
ionomycin (positive control; Sigma) was added and events
were acquired for 1 minute. After 1 minute, 10 μg/mL of
purified goat anti-mouse (GAM; BioLegend) or purified
streptavidin (SA; Pierce Thermo Scientific) was added to
cross-link receptors and events were acquired for an
additional 4 to 5 minutes. The median Fluo-4 relative
fluorescence was analyzed as a function of time in seconds.

**Results**

**Generation of bscFv CD16/CD19 BiKE and tscFv CD16/CD19/CD22 TriKE**

Recombinant bscFv and tscFv reagents were generated
targeting the NK cell receptor CD16 and B-cell antigens
CD19 and CD22, antigens that have been shown to be
therapeutic for the treatment of diffuse large B-cell lymphoma
and ALL (refs. 16, 17; Fig. 1A). BiKEs and TriKEs

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**Figure 1.** BiKEs and TriKEs specifically target both effector and target cells. A, construct diagrams of bscFv CD16/CD19 and tscFv CD16/CD19/CD22
reagents. B, ion exchange and size-exclusion chromatography purification trace of the CD16/CD19/CD22 TriKE. C, Coomassie Blue staining of TriKE protein
isolates (molecular weight of 96 kDa). Lane 1, nonreduced recombinant protein; Lane 2, reduced recombinant protein; and Lane 3, molecular weight
ladder. D, proliferation assay of 3H-pulsed Raji cells treated with CD16/CD19/CD22 (top graph) or a nonspecific control (bottom). E, intracellular Ca²⁺ flux
assay in resting NK cells after CD16 receptor cross-linking. Plots display a representative donor (of 4 experiments) showing the median Fluo-4 relative
fluorescence plotted as a function of time in seconds.
were purified by ion exchange and size-exclusion chromatography. The fractions collected during the final step of purification of the CD16/CD19/CD22 TriKE are shown (Fig. 1B). High (95%) purity was obtained as shown by Coomassie Blue staining (Fig. 1C). Similar results were obtained for the CD16/CD19 BiKE (data not shown).

**BiKE and TriKE antigen binding is cell specific and directly signals through CD16 to induce NK cell activation**

Raji cells were coated with varying concentrations of the TriKE, treated with a CD19/CD22-targeted diphtheria toxin (DT-CD19/CD22), and a ³H-thymidine proliferation assay was conducted (Fig. 1D). All concentrations of the TriKE blocked the effects of the inhibitory toxin, whereas the nonspecific control reagent (CD16/EpCAM) had no effect, showing effective targeting of the B-cell antigens by the CD19 and CD22 end of the reagents.

We next evaluated the ability of the BiKE and TriKE to directly target and trigger NK cell activation. Ca²⁺ mobilization, a primary indicator of cell activation, was measured after specifically cross-linking CD16 on the surface of NK cells. Resting NK cells were loaded with a Ca²⁺ binding dye solution and a time-course analysis of changes in intracellular Ca²⁺ concentration was carried out by flow cytometry (Fig. 1E). Without cross-linking, both the CD16 mAb and the biotinylated scFv CD16 elevated baseline Ca²⁺ levels, which were further enhanced upon cross-linking with the secondary antibody. The Ca²⁺ flux kinetics induced by the biotinylated scFv CD16 were faster than the response elicited by the CD16 mAb, which may be explained by the high-affinity interaction of streptavidin and biotin molecules. These results show that the CD16 end of the BiKE and TriKE is capable of engaging the NK cell and inducing a potent signal through CD16, which results in Ca²⁺ mobilization.

**BiKEs and TriKEs induce directed secretion of NK cell lytic granules and lysis of B-cell targets**

As Ca²⁺ flux is a prerequisite for function (18), we evaluated the ability of these recombinant reagents to mediate killing of Raji targets, a lymphoma cell line with high expression of CD19, CD20, and CD22 (Fig. 2A). Resting NK cells were cocultured with Raji targets with or without reagent treatment and CD107a expression, a marker of NK cell cytotoxicity (19, 20), was measured via FACS analysis (Fig. 2B). NK cell CD107a expression significantly increased in the presence of the BiKE and TriKE compared with untreated NK cells or parental antibodies. This response was target cell restricted as no functional response was induced with treated NK cells in the absence of targets (data not shown). The level of induced degranulation was equal to the function induced by the mAb rituximab. To analyze the correlation of NK cell CD107a expression induced by the recombinant reagents with direct target cell lysis (19, 20), ⁵¹Cr-labeled Raji targets were cocultured with resting NK cells treated with or without reagents and target cell killing was determined (Fig. 2C). Both the BiKE (for effector-to-target (E:T) ratio = 2.2:1, 70.6% ± 4.5%; P < 0.01) and TriKE (59.8% ± 3.9%; P < 0.01) mediated significant lysis of the target cells at all E:T ratios compared with the untreated (18.7% ± 3.0%) and control-treated NK cells. Furthermore, analysis of caspase activation in Raji target cells after 2 or 4 hours of coculture with NK cells led to increases in cleaved caspase-3 expression in BiKE and TriKE coated-Raji targets remaining in the live cell gate compared with the uncoated-Raji targets (Fig. 2D). These results show that BiKE and TriKE reagents specifically engage both targets and effectors to mediate target cell killing, activating NK cells to secrete lytic granules and induce target cell death via a caspase-3 apoptosis pathway.

**BiKEs and TriKEs are stable and mediate target cell killing at high and low E:T ratios in a dose-dependent manner**

To determine the potency of the BiKE and TriKE reagents, resting PBMC were pretreated with or without reagents at varying concentrations, exposed to Raji target cells, and NK cell function was measured by FACS analysis (Fig. 3A). Both NK cell degranulation and IFN-γ production increased in a dose-dependent manner. While rituximab induced NK cell function equally well at low and high concentrations, it peaked without reaching the functional levels seen for the higher concentrations of the BiKE and TriKE reagents. The human FcγR family consists of 6 known members, which are expressed by various effector immune cells (4). As PBMCs contain multiple FcγR receptor-expressing populations in addition to NK cells (21), these cells may compete with NK cells for engagement of the Fc portion of rituximab diluting the NK cell effect. As the effector-targeting end of BiKEs and TriKEs is specific for the low affinity FcγRIII (CD16; ref. 6), this further supports the notion of increased NK cell specificity of the bscFv and tscFv recombinant reagents.

NK cells were next tested at varying E:T ratios (Fig. 3B). NK cells efficiently degranulated at both high and low E:T ratios. The BiKE induced greater CD107a expression compared with the TriKE at the 1:1, 1:5, and 1:10 E:T ratios, suggesting that the BiKE may be more efficient at recruiting effector NK cells for target cell lysis. Furthermore, production of IFN-γ by NK cells against Raji targets in the presence of the BiKE and TriKE was also maintained at both high and low E:T ratios (Fig. 3B). As the activation threshold for NK cell cytokine production differs from that of NK cell cytotoxicity (12, 22, 23), these results indicate the recombinant reagents are capable of inducing both activation requirements and function consistently at both low and high E:T ratios.

The stability of antibody-derived proteins is a vital property that greatly affects the therapeutic efficacy. To evaluate the functional stability, BiKEs and TriKEs were incubated in 100% human serum for 24 or 48 hours, after which resting NK cells were treated with or without reagents, cocultured with ⁵¹Cr-labeled Raji target cells...
and target cell lysis was measured (Fig. 4A). As the results show, the reagents remain stable and are capable of mediating target cell lysis. In addition, after human serum incubation, the TriKE induced greater degranulation (Fig. 4B), target cell lysis, and IFN-γ production (Fig. 4C) compared with the BiKE, implying structural variations in the recombinant reagents may impact their biologic properties, which has been suggested (24). In addition, these data support the premise that degranulation and cytotoxicity data correlate well as readouts for CD16 triggering.

BiKEs and TriKEs induce NK cell chemokine and cytokine production against B-cell targets

Analysis of intracellular IFN-γ production of resting NK cells with or without reagent treatment against Raji target cells showed significant increases in cytokine production for both BiKE- and TriKE-treated NK cells compared with untreated NK cells (Fig. 5A). Further analysis of cytokine and chemokine production was carried out via Luminex on supernatants harvested from resting NK cells cocultured with Raji targets. The BiKE induced significant increases in NK cell production of IFN-γ, GM-CSF, TNF-α, RANTES, MIP-1α, and IL-8 and treatment with the TriKE resulted in a different profile with significant increases in GM-CSF, TNF-α, RANTES, and MIP-1α compared with untreated NK cells (Fig. 5B). Interestingly, the potency with which rituximab induced NK cell IFN-γ (rituximab vs. bsFcV, \( P = 0.003 \); vs. tscFv, \( P = 0.001 \), GM-CSF (\( P = 0.026; P = 0.003 \)), TNF-α (\( P = 0.108; P = 0.0002 \)), and MIP-1α (\( P = 0.207; P = 0.03 \)) production was greater than that of the recombinant reagents. Furthermore, there were significant differences observed between the BiKE and TriKE reagents for NK cell production of GM-CSF (\( P < 0.05 \)) and RANTES (\( P < 0.01 \), showing that CD16 triggering by each reagent generates a unique profile of significantly increased chemokine and cytokine production. As differences in signaling thresholds for the induction of NK cell chemokine and cytokine responses have been shown (25), the differential effects induced by BiKEs, TriKEs, and the mAb
rituximab suggest other receptor–ligand interactions may be differentially engaged to ultimately determine effector function.

NK cell function against primary ALL and CLL tumors is enhanced in the presence of BiKEs and TriKEs

Primary leukemia cells, pre-B ALL and B-CLL cells, were next tested with allogeneic normal NK cells. NK cell degranulation was significantly enhanced against both ALL and CLL primary targets in the presence of the BiKE and TriKE compared with untreated NK cells with no increases observed for the primary AML targets, further showing B-cell target specificity (Fig. 6A). Moreover, CD107a expression induced by the TriKE was significantly greater than the levels induced by rituximab. To evaluate whether this increased function was due to the surface expression levels of CD19, CD20, and CD22 on the primary ALL, CLL, and AML leukemia targets, expression of these B-cell antigens was measured via FACS analysis (Fig. 6B and C). Variations in surface expression of these receptors between patient samples were observed within each type of primary leukemia. The overall CD19, CD20, and CD22 expression profile of the primary leukemia targets suggests that there may be an advantage to targeting 2 tumor antigens rather than 1. Finally, evaluation of NK cell IFN-γ production against primary leukemia targets was also carried out. NK cells treated with BiKE and TriKE reagents displayed significant increases in IFN-γ production against primary CLL targets and enhancement (not statistically significant) against primary ALL targets compared with untreated NK cells (Fig. 6D).

Discussion

In this study, we examined the ability of CD16/CD19 BiKE and CD16/CD19/CD22 TriKE recombinant reagents to enhance NK cell function against B-cell tumor targets. While in vitro NK cell targeting of B-cell malignancies with bispecific recombinant reagents has been described (5, 7, 24), the mechanism by which these reagents directly function to enhance NK cell activity has not been addressed. Our results show engagement of CD16 by the CD16 end of the recombinant reagents actively induces Ca²⁺ mobilization in the effector NK cell.
As triggering of CD16 leads to phospholipase C-γ activation and Ca²⁺ mobilization via signaling through the associated ITAM-bearing FcγRIγ chain and CD3ζ chain (13), our data definitively show that BiKE and TriKE reagents directly signal through CD16 to activate NK cells. Furthermore, it has been shown that NK cell cytotoxicity results from a combination of distinct signals, which includes CD16 (26, 27). As NK cell engagement by BiKEs and TriKEs led to significant increases in NK cell degranulation and directed target cell lysis, this further shows the ability of these reagents to actively propagate signals for NK cell activation.

Cytokine and chemokine production is another critical component of NK cell function. We definitely show the ability of BiKE and TriKE reagents to induce a proinflammatory profile of cytokines and chemokines. When comparing the effects of the BiKE to the TriKE, there is a general increase in overall cytokine and chemokine production induced by the BiKE. Furthermore, NK cell cytokine and chemokine production induced by the mAb rituximab was significantly enhanced compared with the recombinant reagents. It has been shown that CD16 engagement is sufficient to induce significant TNF-α secretion, but requires the addition of coactivating receptor engagement to induce an equivalent IFN-γ response (25). Therefore, the functional potency of the recombinant reagents is likely influenced by the presence of coactivating receptor ligands present on the engaged target cell, whose expression may be altered by signaling through the target antigen, which has been shown for CD20 engagement by rituximab (28). CD19 is a B-cell coreceptor involved in the positive regulation of B-cell function and upregulation of B-cell costimulatory ligands (29–33). As our data show enhanced NK cell effector function, both in cytotoxicity and cytokine and chemokine production, after engagement of target cells via the recombinant reagents targeting CD19, it is likely that a CD19 triggering event in the target cell occurs upon cross-linking that upregulates B-cell costimulatory molecules that serve to further drive NK cell activation through interactions with activating coreceptors, such as 2B4, ICOS, OX40, 4-1BB, and CD28, all of which are expressed on activated NK cells (34–38). In contrast to CD19, CD22 is an ITIM-containing B-cell receptor involved in the negative regulation of B-cell function (31, 39). Our data show that the BiKE induced greater NK cell degranulation and cytokine production compared with the TriKE. As CD22 and CD19 deliver opposing signals that regulate B-cell activation (39) and as antigen expression on Raji target cells is virtually uniform for both (shown in Fig. 2A), this could suggest engagement of the inhibitory CD22 receptor by the TriKE modulates the CD19 triggering event, which may lead to a lower surface density of costimulatory ligand expression and, thus, lower reagent-induced effector function as observed for the TriKE.

The ability of rituximab to induce significantly greater amounts of proinflammatory cytokines suggests it either mediates stronger cross-linking of CD16 or CD20 ligation
leads to target cell modifications capable of enhancing effector function. As surface densities of CD19, CD20, and CD22 were essentially the same on the Raji target cells and the binding affinity of the scFv CD16 is higher \( (K_d = 10^{-8} \text{ mol/L}; \text{ref. 6}) \) than that of IgG \( (K_d = 10^{-6} \text{ mol/L}; \text{ref. 40}) \), this points to the latter. Activated B cells express CD40.
and the CD40-CD40L ligand interaction has been shown to induce B-cell IL-12 production (41, 42). As activated NK cells express CD40L, effector–target cell interaction could promote B-cell production of IL-12, a known stimulus for IFN-γ production and a cytokine that enhances the effectiveness of mAb therapy (43). NK cell IFN-γ production has a greater threshold of activation (i.e., requires the addition of coactivating signals; ref. 25) and, as our results show that rituximab induces a stronger cytokine and chemokine NK cell response compared with the BiKE despite the lower affinity interaction with CD16, this suggests an important role for target cell costimulatory ligand expression for which CD19 engagement may be a weaker stimulus.

Donor-derived NK cells are among the first cells to reconstitute the recipient’s immune cell repertoire after HCT and are key mediators of graft versus leukemia reactions against minimal residual disease providing protection against relapse (44). Consequently, the use of BiKEs and TriKEs to enhance NK cell target recognition and function holds great therapeutic promise for the treatment of cancer. We have shown that BiKEs and TriKEs successfully enhance NK cell function against primary ALL and CLL tumors. Notably, the TriKE was significantly superior to rituximab in targeting of ALL and CLL. Lower expression of CD20 compared with CD19 on the ALL tumors could explain these differences. However, expression of CD20 on CLL was greater than that of CD19, which suggests that targeting CD22 in addition to CD19 provided an advantage. This has been shown with the combinatorial use of the epratuzumab, a humanized anti-CD22 mAb, and rituximab, which produced enhanced antitumor activity compared with either mAb alone (16). In contrast, as B cell ALL has been shown to express the inhibitory receptor CD32 (FcγR II A; ref. 45), it is likely this receptor competes with CD16 for Fc binding of rituximab resulting in decreased effector function. This notion may further be supported by the fact that B cell CLL has been shown to be CD32 negative (46) and our results show a greater ability of rituximab

Figure 6. NK cell function against primary leukemia targets is enhanced in the presence of BiKEs and TriKEs. A and B, FACS analysis of CD19, CD20, and CD22 (open histograms; isotype control, shaded histogram) surface expression on ALL, CLL, and AML primary tumor cells. For each primary tumor, the histogram plots represent 1 of 6 donors (A), with the scatter-plot displaying aggregate data (B). C and D, resting NK cells were cocultured with primary ALL, CLL, or AML tumor cells with or without 10 µg/mL of reagent and CD107a (C) or IFN-γ (D) expression was evaluated (*, P < 0.01; **, P < 0.001; and ***, P < 0.0001; 6 primary leukemia samples of each tumor type were tested against 2 allogeneic normal NK donors; error bars represent SEM).
to induce NK cell effector function against CLL compared with ALL.

Alltogether, our data show that BiKEs and TriKEs directly activate NK cells through CD16, overcoming inhibition by MHC class I molecules and inducing target-specific cytotoxicity and cytokine and chemokine production. Moreover, there are implications that the tumor antigens targeted by the recombinant reagents influence effector function. Therefore, careful evaluation of effector–target cell interactions induced by BiKEs and TriKEs will further serve to optimize their clinical efficacy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.K. Gleason, M.R. Verneris, V. McCullar, A. Panoskaltsis-Mortari, B. Zhang, J.S. Miller

References

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