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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 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. Co-culture 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 (E:T) ratios and maintained functional stability after 24 and 48 hours of culture in human serum. NK cell production of IFN- γ , TNF- α , GM-CSF, IL-8, MIP-1 α and 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 ALL and CLL 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, demonstrating their therapeutic potential for enhancing NK cell immunotherapies for leukemias and lymphomas.

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Author Contributions

MKG designed the research plan, performed experiments, data analysis and interpretation, and wrote the paper; MRV performed data analysis, interpretation and paper preparation; DAT generated recombinant reagents and performed experiments; BZ, VM and SZ performed experiments; AP-M performed chemokine/cytokine assays, data analysis, interpretation and paper preparation; LMW provided concept development, experimental platform design and the scFv anti-CD16 reagent; JSM and DAV designed the research plan, performed data analysis, interpretation and paper preparation, and was responsible for all aspects of the work.

Keywords

NK cells; immunotherapy; CD16 signaling; B-cell leukemia; B-cell lymphoma

Introduction

With over 70,000 cases anticipated in the US for 2012, non-Hodgkin's lymphoma (NHL) is the most common adult hematologic malignancy, 85% of which are of B-cell origin (1, 2). Monoclonal antibodies (mAbs), 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 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 (NCRs), or by antibodies, such as rituximab, to trigger antibody dependent cell-mediated cytotoxicity (ADCC) through CD16, the activating low affinity Fc γ receptor for IgG highly expressed by the CD56^{dim} 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 (ITAMs) of the associated Fc ϵ RI γ chain and CD3 ζ 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 co-engagement of other activating receptors (12).

In this study, we demonstrate 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 erythroid cells via magnetic beads per 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. 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 monoclonal antibodies (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 (2H7; BioLegend), FITC-conjugated CD22 (H1B22; BioLegend), PerCP/Cy5.5-conjugated anti-human CD107a (LAMP-1) (H4A3; BioLegend), Pacific Blue-conjugated anti-human IFN- γ (4S.B3; BioLegend) and AF488-conjugated cleaved caspase-3 (9669; Cell Signaling Technology). Phenotypic acquisition of cells was performed 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 NcoI restriction site, an ATG initiation codon, the V_H and V_L regions of anti-human CD16 (NM3E2) (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 XhoI 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's lymphoma Raji cell line (ATCC) was cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). 2×10⁴ Raji cells were treated with varying concentrations of CD16/CD19/CD22 or non-specific control CD16/EpCAM and 1 nM of a targeted diphtheria toxin-CD19/CD22 (DT-CD19/CD22) was added and a ³H-thymidine proliferation was performed 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 RFB4 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 performed as previously described (15). For evaluation of caspase-3 activation, Raji target cell cleaved caspase 3 expression was evaluated by FACS analysis using a live forward/side scatter gate and a CD56⁻/CD20⁺ gate to determine cleaved caspase 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 co-cultured with Raji cells were treated with the BiKE, TriKE, rituximab, negative controls or no reagent for 6 hours at 37°C, 5% CO₂. Supernatant levels of IFN- γ , TNF- α , GM-CSF, IL-8, MIP-1 α , 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 final pg/mL values adjusted for background for each condition were

determined using the following equation: $[\text{NK cells} + \text{Raji cells pg/mL}] - [(\text{NK cells alone pg/mL}) + (\text{Raji cells alone pg/mL})]$.

51-Chromium Release Cytotoxicity Assay

Direct cytotoxicity assays were performed by standard 4-hour ^{51}Cr -release assays using effector cells pre-treated with reagents and Raji cells as targets. For examination of reagent stability, reagents were pre-incubated for 24 and 48 hours at 37°C , 5% CO_2 in 100% human serum. ^{51}Cr released by specific target cell lysis was measured by a gamma scintillation counter and the percent specific cell lysis was calculated.

Ca^{2+} Flux Assay

Ca^{2+} flux was measured using the Fluo-4 NW Calcium Assay Kit (Invitrogen) within effector NK cells as per manufacturer's protocol. Ca^{2+} mobilization was evaluated by FACS analysis. After 30 seconds of acquisition, $10\mu\text{g/mL}$ of purified anti-CD16 mAb (3G8; BD Biosciences), biotinylated scFv anti-CD16, purified mIgG (negative control; R&D Systems), or $1\mu\text{g/mL}$ of ionomycin (positive control; Sigma) was added and events were acquired for 1 minute. After 1 minute, $10\mu\text{g/mL}$ 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 4–5 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 (16, 17) (Figure 1A). BiKEs and TriKEs 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 (Figure 1B). High (95%) purity was obtained as demonstrated by Coomassie Blue staining (Figure 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 performed (Figure 1D). All concentrations of the TriKE blocked the effects of the inhibitory toxin while the non-specific control reagent (CD16/EpCAM) had no effect, demonstrating 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. Ca^{2+} 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 Ca^{2+} binding dye solution and a time course analysis of changes in intracellular Ca^{2+} concentration was performed by flow cytometry (Figure 1E). Without cross-linking, both the CD16 mAb and the biotinylated scFv CD16 elevated baseline Ca^{2+} levels, which were further enhanced upon cross-linking with the secondary antibody. The Ca^{2+} 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 demonstrate 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 Ca^{2+} mobilization.

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

As Ca^{2+} 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 (Figure 2A). Resting NK cells were co-cultured with Raji targets with or without reagent treatment and CD107a expression, a marker of NK cell cytotoxicity (19, 20), was measured via FACS analysis (Figure 2B). NK cell CD107a expression significantly increased in the presence of the BiKE and TriKE compared to 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), ^{51}Cr -labeled Raji targets were co-cultured with resting NK cells treated with or without reagents and target cell killing was determined (Figure 2C). Both the BiKE (for E:T=2.2:1, $70.6\% \pm 4.5\%$; $P < 0.01$) and TriKE ($59.8\% \pm 3.9\%$; $P < 0.01$) mediated significant lysis of the target cells at all E:T ratios compared to the untreated ($18.7\% \pm 3.0\%$) and control treated NK cells. Furthermore, analysis of caspase activation in Raji target cells after two or four hours of co-culture with NK cells led to increases in cleaved caspase-3 expression in BiKE and TriKE coated-Raji target remaining in the live cell gate compared to the uncoated-Raji targets (Figure 2D). These results demonstrate 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 pre-treated with or without reagents at varying concentrations, exposed to Raji target cells and NK cell function was measured by FACS analysis (Figure 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 Fc γ R family consists of six known members which are expressed by various effector immune cells (4). As PBMC contain multiple Fc γ R 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 Fc γ RIII (CD16) (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 (Figure 3B). NK cells efficiently degranulated at both high and low E:T ratios. The BiKE induced greater CD107a expression compared to the TriKE at the 1:1, 1:5 and 1:10 E:T ratios suggesting 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 (Figure 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, co-cultured with ^{51}Cr -labeled Raji target cells and target cell lysis was measured (Figure 4A). As the results demonstrate, the reagents remain stable and are capable of mediating target cell lysis. In addition, after human serum incubation the TriKE induced greater degranulation (Figure 4B), target cell lysis and IFN- γ production (Figure 4C) compared to the BiKE, implying structural variations in the recombinant reagents may impact their biological properties, which has been suggested (24). These data also 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 to untreated NK cells (Figure 5A). Further analysis of cytokine and chemokine production was performed via Luminex on supernatants harvested from resting NK cells co-cultured with Raji targets. The BiKE induced significant increases in NK cell production of IFN- γ , GM-CSF, TNF- α , RANTES, MIP-1 α and IL-8 and treatment with the TriKE resulted in a different profile with significant increases in GM-CSF, TNF- α , RANTES and MIP-1 α compared to untreated NK cells (Figure 5B). Interestingly, the potency with which rituximab induced NK cell IFN- γ (rituximab vs. bscFv $P=0.003$; vs. tscFv $P=0.001$), GM-CSF ($P=0.026$; $P=0.003$), TNF- α ($P=0.108$; $P=0.0002$) and MIP-1 α ($P=0.207$; $P=0.03$) 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 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 demonstrated (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 to untreated NK cells with no increases observed for the primary AML targets further demonstrating B-cell target specificity (Figure 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 (Figures 6B and 6C). 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 there may be an advantage to targeting two tumor-antigens rather than one. Lastly, evaluation of NK cell IFN- γ production against primary leukemia targets was also performed. 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 to untreated NK cells (Figure 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 demonstrate engagement of CD16 by the CD16 end of the recombinant reagents actively induces Ca^{2+} mobilization in the effector NK cell. As triggering of CD16 leads to phospholipase C- γ activation and Ca^{2+} mobilization via signaling through the associated ITAM-bearing Fc ϵ RI γ chain and CD3 ζ chain (13), our data definitively demonstrate BiKE and TriKE reagents directly signal through CD16 to activate NK cells. Furthermore, it has been demonstrated 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 demonstrates 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 pro-inflammatory 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 to the recombinant reagents. It has been demonstrated that CD16 engagement is sufficient to induce significant TNF- α secretion, but requires the addition of co-activating 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 co-activating 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 co-receptor involved in the positive regulation of B-cell function and up-regulation of B-cell co-stimulatory 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 a CD19 triggering event in the target cell occurs upon cross-linking that up-regulates B-cell co-stimulatory molecules that serve to further drive NK cell activation through interactions with activating co-receptors, 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 demonstrate the BiKE induced greater NK cell degranulation and cytokine production compared to 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 Figure 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 co-stimulatory ligand expression and thus lower reagent-induced effector function as observed for the TriKE.

The ability of rituximab to induce significantly greater amounts of pro-inflammatory 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 ($K_d=10^{-8}$ M) (6) than that of IgG ($K_d=10^{-6}$ M) (40), this points to the later. 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 co-activating signals) (25) and as our results show rituximab induces a stronger cytokine and chemokine NK cell response compared to the BiKE despite the lower affinity interaction with CD16, this suggests an important role for target cell co-stimulatory 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 (GvL) reactions against minimal residual disease (MRD) 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 demonstrated 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 to CD19 on the ALL tumors could explain these differences. However, expression of CD20 on CLL was greater than that of CD19, which suggests targeting CD22 in addition to CD19 provided an advantage. This has been demonstrated with the combinatorial use of the epratuzumab, a humanized anti-CD22 mAb, and rituximab, which produced enhanced anti-tumor activity compared with either mAb alone (16). In contrast, as B-cell ALL has been shown to express the inhibitory receptor CD32 (Fc γ RIIA) (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 demonstrate a greater ability of rituximab to induce NK cell effector function against CLL compared to ALL.

Altogether, our data demonstrate BiKEs and TriKEs directly activate NK cells through CD16, overcoming inhibition by MHC class I molecules and inducing target-specific cytotoxicity, 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.

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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 = non-reduced recombinant protein, Lane 2 = reduced recombinant protein, Lane 3 = molecular weight ladder. D, Proliferation assay of ³H-pulsed Raji cells treated with CD16/CD19/CD22 (top graph) or a non-specific control (bottom graph). 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.

Figure 2. BiKEs and TriKEs enhance NK cell killing of Raji tumor target cells

A, FACS plots of CD19, CD20 and CD22 (open histogram; isotype control shaded histogram) receptor expression on Raji targets. B, CD107a expression of resting NK cells co-cultured with Raji target cells (E:T=2:1) with or without (10 μg/mL) reagent treatment (**P*<0.01 for test condition compared to no reagent, *n*=3; error bars represent SEM). C, ⁵¹Cr labeled Raji target cell lysis by resting treated (10 μg/mL) or untreated NK cells treated. Each experiment was carried out in triplicate and repeated with three donors, with aggregate results shown (**P*<0.01 for test condition compared to no reagent; error bars represent SEM). D, Cleaved caspase 3-expression in Raji targets induced by treated or untreated resting NK cells (E:T=2:1; experiment was performed twice with two NK donors; error bars represent SEM).

Figure 5. NK cell chemokine and cytokine production induced by Raji target cells is enhanced in the presence of BiKEs and TriKEs

A, FACS analysis of treated (10 μ g/mL) or untreated NK cell IFN- γ production against Raji targets. (* P <0.01 for test condition compared to no reagent, n =3; error bars represent SEM). B, Luminex analysis of cytokine/chemokine levels on supernatants harvested from resting treated (10 μ g/mL) or untreated NK cells co-cultured with Raji targets. Final pg/mL values were determined for each condition using the following equation: [NK cell + Raji cell pg/mL] – [(NK cell alone pg/mL) + (Raji cell alone pg/mL)] (* P <0.05, n =6; error bars represent SEM).

Figure 6. NK cell function against primary leukemia targets is enhanced in the presence of BiKEs and TriKEs

A–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 one of six donors (A) with the scatter plot displaying aggregate data (B). C–D, Resting NK cells were co-cultured with primary ALL, CLL or AML tumor cells with or without 10µg/mL of reagent and CD107a (C) or IFN-γ (D) expression was evaluated (* $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, six primary leukemia samples of each tumor type were tested against two allogeneic normal NK donors; error bars represent SEM).