Chapter 28

Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells

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Abstract

Cancer immunotherapies have gained significant momentum over the past decade, particularly with the advent of checkpoint inhibitors and CAR T-cells. While the latter personalized targeted immunotherapy has revolutionized the field, a need for off-the-shelf therapies remains. The ability of NK cells to quickly lyse antibody-coated tumors and potently secrete cytokines without prior priming has made NK cells ideal candidates for antigen-specific immunotherapy. NK cells have been targeted to tumors through two main strategies: mono-specific antibodies and bi/tri-specific antibodies. Mono-specific antibodies drive NK cell antibody-dependent cell-mediated cytotoxicity (ADCC) of tumor cells. Bi/tri-specific antibodies drive re-directed lysis of tumor cells through binding of a tumor antigen and direct binding and crosslinking of the CD16 receptor on NK cells, thus bypassing the need for binding of the Fc portion of mono-specific antibodies. This chapter focuses on the generation of bi- and tri-specific killer engagers (BiKEs and TriKEs) meant to target NK cells to tumors. BiKEs and TriKEs are smaller molecules composed of 2–3 variable portions of antibodies with different specificities, and represent a novel and more versatile strategy compared to traditional bi- and tri-specific antibody platforms.

Key words BiKE, TriKE, Bi-specific, Tri-specific, Targeted immunotherapy, NK, Natural killer, ADCC, Redirected lysis

1 Overview

Targeted cancer immunotherapies are currently a subject of great clinical interest and potential [1]. While a great deal of interest has recently been placed upon generation of chimeric antigen receptor (CAR) expressing T cells from monoclonal antibodies shown to target human malignancies [2], and even more recently upon generation of CAR-expressing natural killer (NK) cells [3, 4], these approaches require a personalized approach that is expensive, time consuming, and difficult to apply on a large scale. There is a clear

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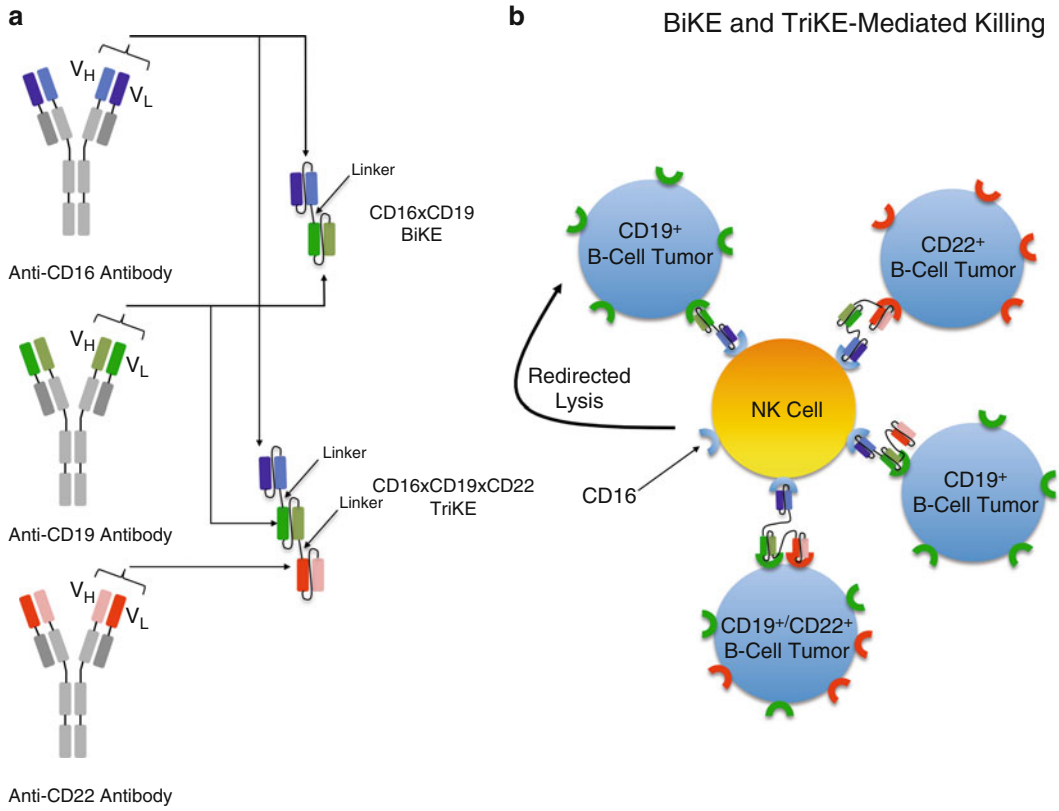

Fig. 1 Structure and function of BiKEs and TriKEs. (**a**) BiKEs and TriKEs are constructed from a single heavy (V_H) and light (V_L) chain of the variable region of each antibody of interest. V_H and V_L domains are joined by a short flexible polypeptide linker to prevent dissociation. Shown here is a BiKE constructed from the variable regions of anti-CD16 and anti-CD19 and a TriKE constructed from the variable regions of anti-CD16, anti-CD19, and anti-CD22. (**b**) BiKE and TriKE binding to NK cells and their targets result in the formation of an immunological synapse and triggers NK killing of the target cell through activation of the low affinity Fc receptor, CD16, on NK cells. The CD16 × CD19 × CD22 TriKE can recognize targets expressing CD19 (*green receptors*), CD22 (*red receptors*) or both receptors simultaneously allowing for more versatile target recognition than the CD16 × CD19 BiKE

need for targeted off-the-shelf therapies that augment the current monoclonal antibody approach. This chapter focuses on generation of bi- and tri-specific killer engagers (BiKEs and TriKEs) meant to target NK cells to the tumor synapse and induce their activation at that site (Fig. 1). Unlike full-length bi- and tri-specific antibodies, BiKEs and TriKEs are small molecules containing two (BiKE) or three (TriKE) single chain variable fragments (scFv) from antibodies of different specificity.

1.1 Mediation of NK Cell Function Through CD16 and ADCC

NK cells are ideal candidates for immune cell-targeted therapies because they do not require prior sensitization to lyse tumor targets and to release pro-inflammatory cytokines, are not HLA-restricted, and can mediate graft-versus-leukemia (or tumor) without inducing graft-versus-host disease [5, 6]. Although NK cells possess a variety of activating receptors and can mediate

function in several different ways, their role in antibody-dependent cell-mediated cytotoxicity (ADCC) is of particular relevance in this chapter. ADCC is mediated by CD16 (FcγRIII), the low affinity receptor for IgG Fc [7]. Two isoforms of CD16 exist in humans, CD16A and CD16B [8]. CD16A is expressed in NK cells, macrophages, and placental trophoblasts as a polypeptide-anchored transmembrane protein while CD16B is expressed in neutrophils in a GPI-anchored form [9–12]. Although the extracellular portion of CD16A and CD16B share a high level of homology (95–97%), CD16A can trigger killing of tumor targets and cytokine production while CD16B cannot [9, 13–15].

In human NK cells, CD16 is mostly expressed in the CD56dim subset, although populations of CD56bright CD16+ NK cells have been observed after transplant [16, 17]. Engagement of CD16 through encounter with the Fc portion of antibodies or direct crosslinking by anti-CD16 antibody results in signals through the immunoreceptor tyrosine-based activation motif (ITAM) of the associated FcεRIγ and CD3ζ chain subunits, leading to cytokine and cytotoxic responses [18–20]. Unlike other activating receptors present in human NK cells, CD16 can robustly mediate activation without the need for co-engagement of other receptors [21]. These signaling properties allow for NK CD16-mediated targeting of antibody-coated cells in natural settings of viral infection, autoimmunity and the onset of some forms of tumors [22–24]. The latter has been exploited in the clinic by generating monoclonal antibodies (mAbs) targeting specific tumor antigens to drive ADCC against those tumors [25–29].

1.2 Potential Advantages of BiKEs and TriKEs Over Conventional Antibodies

Driving ADCC through mAbs has resulted in significant clinical success. Specific targeting of tumors with BiKEs and TriKEs has the potential to build upon this success and improve efficacy. Binding affinity appears to be an important component of ADCC. This impression is supported by increased rituximab-driven cytotoxicity of B cell tumors mediated by NK cells containing the CD16A-158VV or VF allotypes which, when compared to the CD16A-158FF allotype, display decreased affinity for the Fc portions of antibodies [30]. Therefore, BiKEs and TriKEs might improve NK cell function by generating a stronger interaction through binding with anti-CD16 than that produced by binding of CD16 to the natural Fc portion of antibodies. This increase in affinity and cytotoxicity was demonstrated in a study comparing natural binding of CD16 to the Fc portion of an anti-HER2 antibody versus binding of CD16 through an anti-HER2 x anti-CD16 bi-specific antibody. Data showed a 3.4 fold increase in affinity in the bi-specific antibody versus binding of the native anti-HER2 Fc [31]. The efficacy of therapeutic mAbs in vivo, in contrast to their high ADCC efficacy in vitro, is further attenuated by the presence of physiologic serum IgG levels in plasma. In the in vivo setting, ADCC potency is diminished by saturation of CD16 receptors,

thus competing for binding with the therapeutic mAb [32]. Such competition for binding of the Fc portion of therapeutic antibodies requires high serum levels of the mAb to be sustained over several months of treatment in order to achieve in vivo efficacy [33, 34]. BiKEs and TriKEs bypass this obstacle by binding the CD16 receptor directly. An additional benefit that BiKEs and TriKEs may have over mAbs is superior biodistribution as a consequence of their smaller size, particularly in the treatment of solid tumors [35–37]. In addition to these advantages, BiKEs and TriKEs are non-immunogenic, have quick clearance properties and can be engineered quickly to target known tumor antigens. These attributes make them an ideal pharmaceutical platform for potentiated NK cell-based immunotherapies.

1.3 Bi- and Tri-specific Reagents Targeting NK Cells and Tumors

Over the past two decades multivalent antibodies, and more recently BiKEs and TriKEs, have been used to target tumor antigens and CD16 on NK cells [38]. While approaches for assembly of multivalent antibodies and the current methodology for BiKE and TriKE engineering has evolved, the function of these reagents remains unchanged. Bi-specific and Tri-specific reagents have been generated to engage CD16 on the NK cell and the following tumor antigens: CD20 and CD19 on B cell non-Hodgkin's lymphomas [39–47], CD19 and CD33 on mixed lineage leukemia [48], CD33 or CD33 and CD123 on acute myelogenous leukemia (AML) [49–51], HLA Class II on lymphoma [52], CD30 on Hodgkin's disease [53–62], EGF-R on EGF-R+ tumors [63, 64], HER2/neu on metastatic breast cancer and other HER2 expressing tumors [31, 65–71], and MOV19 on ovarian cancer [72]. Our group has contributed to the field through generation and testing of BiKEs and TriKEs that target CD16 and CD19/CD22 on B cell non-Hodgkin's lymphomas [73], CD33 on AML [74] and MDS/MDSCs [75], and EpCAM on prostate, breast, colon, head, and neck carcinomas [76]. Activation through the BiKEs and TriKEs elicited potent cytotoxicity and cytokine secretion. In the case of the CD16×CD19×CD22 TriKE, the CD107a response to primary CLL and ALL exceeded that of rituximab. The CD16×CD33 BiKE was capable of overcoming HLA-mediated inhibition with primary refractory AML blasts and restored function of NK cells from MDS patients. Encouraged by their translational potential, we are currently producing some versions of these reagents for clinical use. Basic reagent production methods are described in the next section.

2 Methodology

2.1 Selection of Variable Fragment Source and Linkers

BiKE design is a complex process. This section provides an overview of the entire methodology (summarized in Fig. 2). Once a target of interest has been defined, the first step in the design of BiKEs requires selection of a source for the variable fragments.

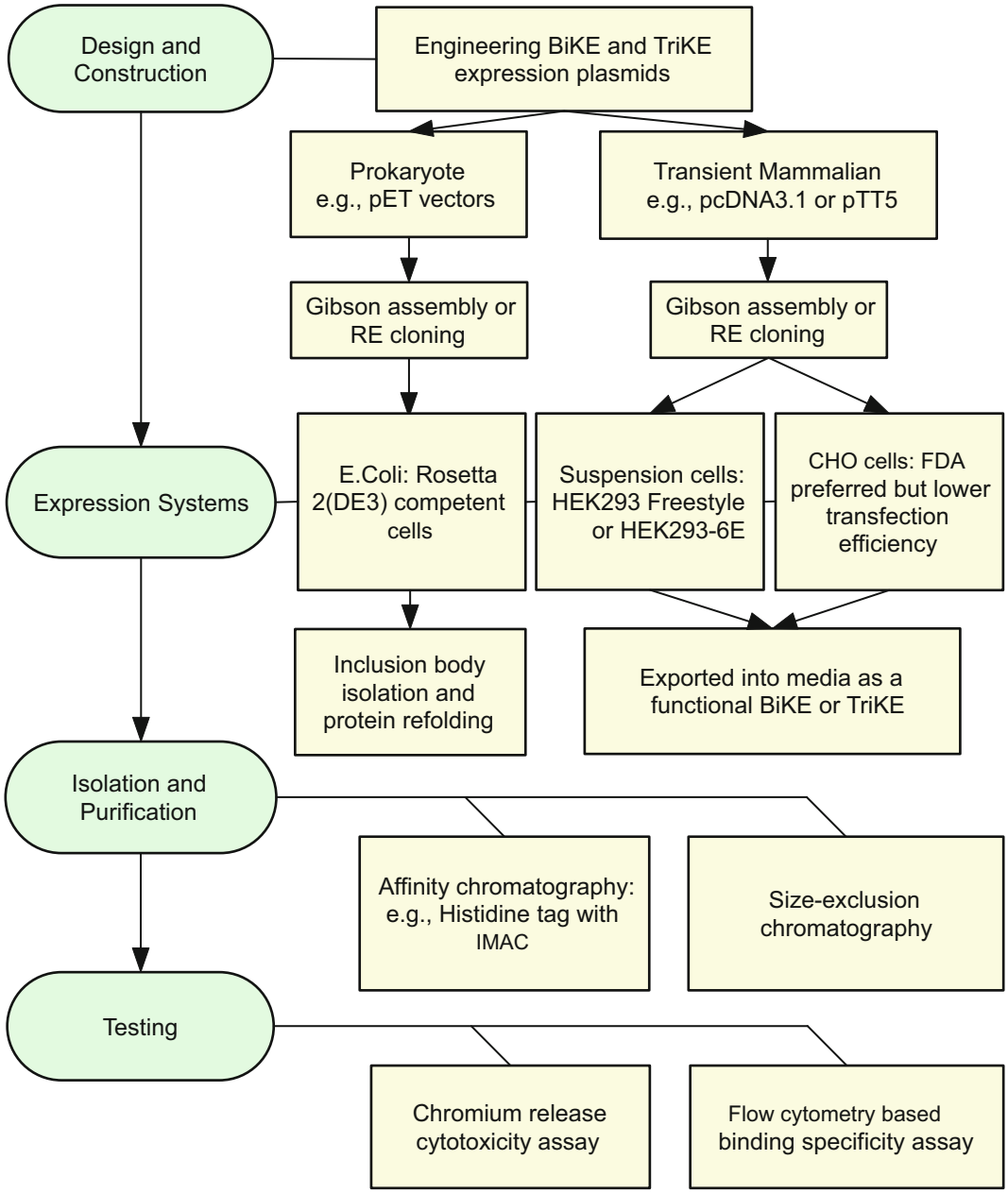

Fig. 2 Workflow for generation of BiKEs and TriKEs. In the left (*green*) are the four steps necessary for generation and validation of the BiKE/TriKE constructs. In the right (*yellow*) are possible options for each of the steps. CHO: Chinese hamster ovary cells. IMAC: immobilized metal affinity chromatography. RE: restriction enzyme

Sequences for relevant fragments can be obtained from published work, hybridomas, B cells from immunized animals, phage display and other such display technology. For bacterial expression systems, phage display is ideal because the constructs are selected in bacteria, essentially pre-screening their function in the system of expression. The next step involves selection of a proper linker. BiKEs combine two different antigen-binding sites with a short flexible linker. The antigen-binding domains are single-chain variable fragments (scFv), which consist of heavy and light variable domains, also fused with a flexible linker (V_H-linker-V_L) [35]. The main linker design is important to the function of the BiKE by allowing separation of the functional domains as well as providing flexibility to bind the two (or three in the case of the TriKE) epitopes on the different targeted cells [77]. The (SGGG)4 linker is one of the first flexible linkers used in the construction of single-chain variable fragments (scFv) [78]. Another commonly used linker, the 218s linker (GSTSGSGKPGSGEGSTKG), is reported to improve proteolytic stability and reduce aggregation [79]. To reduce immunogenicity, our group utilized an HMA linker (PSGQAGAAASESLFVSNHAY) between the antiCD16 and the tumor antigen scFv [76].

2.2 Selection of Vector and Expression System

Once the components of the BiKE have been determined, selection of an appropriate vector for expression follows. We and other investigators focus on plasmid expression systems in bacteria and mammalian cells to create BiKEs, but there are other less utilized expression systems, such as lentivirus or sleeping beauty, which will not be discussed in this section. For bacterial expression systems, the pET vector is the system most commonly used in conjunction with the Rosetta 2(DE3) host cells (Novagen). The Rosetta 2(DE3) cells contain an IPTG- inducible T7 RNA polymerase, which is compatible with the pET vectors. Another feature of this strain is that it has been engineered to express a "universal" set of transfer RNAs as a way to mitigate the need for codon optimization. For transient mammalian expression systems, the pTT5 vector can be utilized in conjunction with the HEK293-E6 suspension cells or the pcDNA3.1 system can be used with the HEK293 Freestyle cells (Invitrogen). Reported yields have been higher in the HEK293-E6 system [80]. These cells express a truncated variant of the Epstein Barr virus (EBV) for which pTT5 vector contains the short EBV oriP for episomal replication. These two systems display advantages in yields and ease of use but a number of other systems utilizing different vectors can also be applied [80, 81].

2.3 Cloning the BiKE/TriKE Components into Expression Vector

Upon selection of a vector, one can begin cloning the BiKE components into the vector backbone. Significant advances have been made in the recombination technique. While there are several ways to clone DNA fragments into the vector backbone, we and others

favor Gibson assembly because it is cost and time efficient [82]. Gibson assembly utilizes in vitro homologous recombination through insertion of a DNA fragment into a vector, where insertion is directed by homologous regions that are present at the end of the insert DNA and the linearized vector DNA [83]. An advantage of Gibson assembly over standard restriction cloning is that it requires little to no restriction enzyme utilization and multiple pieces can be cloned in one reaction. With the advent of this method, together with recent access to inexpensive high-fidelity synthetic DNA, it is now possible to construct BiKE expression plasmids in a few days of labor.

2.4 Expression and Isolation of the BiKEs/TriKEs

Following preparation, the BiKE expression vector can then be chemically transduced into E. coli or transfected into mammalian cells through lipid or chemical means and, to a lesser extent, through electroporation. The advantage of E. coli versus the mammalian system is that it allows for quick, easy, robust, and inexpensive expression of the BiKEs [84]. An important difference between the bacterial and mammalian systems is that in the mammalian system, fully functional proteins are secreted and can be harvested from the supernatant. A disadvantage of bacteria is that most recombinant proteins are found in an insoluble form, termed an inclusion body [85]. To resolve this problem, lysis of the bacteria and isolation of the inclusion bodies through centrifugation followed by solubilization with strong denaturing reagents is required. The protein then must be refolded. Refolding is carried out at low protein concentrations. Conditions for refolding of the recombinant protein must be optimized (i.e., pH, ionic strength, temperature, and redox environment). The protein can then be isolated through size exclusion chromatography or through the use of an affinity tag, such as histidine-tags [85, 86]. As discussed, both systems have advantages and disadvantages. While the bacterial system is quick, easy, and robust, the mammalian system does not require re-folding and can be utilized to generate smaller amounts of functional protein quickly for initial screening. Another consideration possibly favoring the mammalian approach is that most therapeutic recombinant proteins gaining FDA approval are made in Chinese hamster ovary (CHO) cells [87].

2.5 Testing the BiKEs and TriKEs

Flow cytometry is used to evaluate binding of the constructs to their respective targets. Prior to incorporation into the full bi- or tri-specific constructs containing the anti-CD16 variable portion and the linker/s, individual variable portions containing a His-tag or similar small tag are incubated with cells expressing the antigen of interest or cells expressing an irrelevant antigen, to evaluate non-specific binding. A biotinylated anti-His antibody is then used to recognize the His-tag on the variable portion, followed by addition of fluorescently labeled streptavidin to attain fluorescent

conjugation. To ensure that the variable fragment is binding to the desired antigen, binding is then compared to fluorescently labeled commercial antibodies to the antigen of choice. Alternatively, the variable portion can be biotinylated or fluorescently labeled directly. However, this approach may increase risk of altering binding to the antigen. If the variable construct is designed from a known antibody for which fluorescently labeled forms already exist, the construct can be tested in a competition assay. In such assays, increasing concentrations of construct are bound to the cells expressing the specific antigen prior to addition of the known fluorescently labeled antibody. Specific binding is then measured by a decrease in binding of the fluorescently labeled full antibody form, indicating binding of the variable fragment to the antigen.

Once specific binding has been confirmed, the variable portion is incorporated into a full BiKE or TriKE construct and the functional activity of the construct is evaluated by two different methods. First, the ability of NK cells to degranulate in response to targets coated in the construct is assessed by a redirected lysis assay. Peripheral blood mononuclear cells (PBMCs) or purified NK cells are co-cultured with targets at a range of effector to target (E:T) ratios (1:1 to 20:1) in the presence or absence of a saturating concentration of the BiKE/TriKE of interest. Higher ratios are required for PMBCs when compared to purified NK cells. Effectors, targets, and constructs are incubated together for several hours (usually 4) and then surface LAMP-1 (CD107a), used to evaluate degranulation, and intracellular IFN-γ and TNF-α, used to evaluate cytokine secretion, are assessed on the NK cells by flow cytometry. Irrelevant targets are used as a negative control in this assay, while full length antibodies that direct ADCC towards the antigen of choice are used as a positive/comparative control.

While this assay determines the level to which NK cells are activated, it does not reflect the level of target cell killing in response to the NK cell activation. To evaluate target cell killing a cytotoxicity assay, such as a chromium release assay, is performed. In this assay, target cells are labeled with radioactive Chromium-51 (^{51}Cr) prior to co-culture with PBMCs or purified NK cells and the BiKE/TriKE. E:T ratios in this assay range from 20:1 to 0.625:1. Wells containing targets without NK cells are plated for use as maximum (10% SDS mediated lysis) and minimum (no treatment) release groups. These groups are used for the calculation of percent targets killed. During the incubation, as target cells are killed they release ^{51}Cr into the supernatant while the targets that remain alive keep the ^{51}Cr sequestered inside the cell. ^{51}Cr release is then assessed on a gamma counter and the percent of targets killed is calculated. Controls similar to those mentioned in the flow-based assay are also included. Once the specificity and efficacy of the BiKEs/TriKEs has been determined, the constructs can now be tested with clinical samples and/or in more complex in vivo killing assays utilizing NSG mice, engrafted xenogeneic tumors, and transferred human NK cells.

3 Future Directions

Although current BiKE and TriKE constructs display great translational potential, efforts are currently underway to further improve their efficacy. One obstacle that could limit the efficacy of BiKEs and TriKEs, as well as all other antibody therapy mediated through NK cells, is CD16 expression. NK cell-mediated ADCC by therapeutic antibodies depends on ligation of CD16, on the NK cell, with the Fc portion of the antibody [88]. BiKEs and TriKEs, as well as other formats of bi- and tri-specific antibodies, mediate redirected lysis of the target and NK cell function through direct binding and crosslinking of the CD16 receptor. This bears relevance because CD16 is rapidly clipped from the surface of NK cells activated through CD16 by matrix metalloproteinases (MMPs), in particular ADAM-17 [89–92]. Activation through cytokines can also result in the clipping of CD16 [93]. Loss of surface CD16 expression on activated NK likely results in a diminished capacity to mediate subsequent rounds of ADCC. To address this concern, we and others are currently evaluating MMP-specific inhibitors as a means to prevent CD16 clipping during NK cell activation [94, 95]. We have demonstrated that inhibition of ADAM-17 results in superior function post CD16 crosslinking and can potentiate rituximab-mediated responses in vitro. We have also shown that ADAM-17 inhibition can enhance BiKE mediated killing against myeloid targets in vitro [74]. These results indicate that co-treatment with ADAM-17 inhibitor may be a good strategy to enhance BiKE/TriKE function in the clinic.

A different approach to circumvent the CD16 problem is to target other receptors on the NK cells with the BiKEs and TriKEs. CD16 was originally selected owing to its ability to potently activate NK cells and overcome inhibitory signaling [21]. This was highlighted in the BiKE system showing that the CD16×CD33 BiKE could overcome HLA-mediated inhibition in primary AML blasts and could restore NK cell function from MDS patients, whose natural cytotoxicity is thought to be impaired [74, 75]. However, co-engagement of other receptors, particularly NKG2D and 2B4, has been shown to induce activation similar to that provided by CD16 alone [21]. There is also potential for TriKEs engaging CD16, a tumor antigen, and another NK cell activating or co-stimulatory receptor. For instance, co-engagement of CD16 with DNAM-1, CD2, or 2B4 was shown to potentiate function in NK cells from MDS patients [75]. Co-administration of cytokines may also enhance BiKE mediated NK cell function. Several cytokines, including IL-15, IL-2, IL-21, and IL-12 have prominent roles in NK cell development, proliferation, survival, and/or activation. Encouraged by these attributes, trials are underway to implement them in the clinic [96]. Besides the aforementioned

attributes, some of these cytokines have been shown to also potentiate ADCC, making them an interesting co-therapeutic approach.

While personalized CAR-T cell therapies have recently enjoyed a great deal of clinical success [2], there remains a clear need for off-the shelf reagents that enhance targeting of the immune system to tumor antigens. Directing targeting of NK cells is a compelling therapeutic approach on the basis of their ability to quickly kill tumors and secrete cytokines without prior priming [5]. BiKEs and TriKEs are an important conduit for achieving this since they are relatively easy to produce, drive potent NK cell activation through CD16 crosslinking, and can be utilized to target almost any tumor antigen for which an antibody has been designed. This is true regardless of whether the antibody displays activating properties because the activation is driven through the CD16 scFv. To date our group has primarily focused on non-Hodgkin's lymphoma (through CD19 and CD22), AML and MDS (through CD33), and breast, colon, and lung carcinomas (through EpCAM) [73–76]. Notably, there are an abundance of promising tumor antigens for which therapeutic antibodies have been designed that could be incorporated into BiKE and TriKE platforms [37]. These include CD30 (Hodgkin's lymphoma), CD52 (CLL), CEA (breast, colon, and lung), gpA33 (colorectal), CAIX (renal cell), Mucins (breast, colon, lung, and ovarian), PSMA (prostate), VEGFR (epithelium-derived solid tumors), VEGF and Integrins αVβ3 and α5β1 (tumor vasculature), EGFR (breast, lung, colon, glioma, and head and neck), and ERBB2 and ERBB3 (breast, lung, colon, ovarian, and prostate). This list, by no means comprehensive, enumerates several important hematological and solid tumors that potentially could be targeted through the powerful BiKE platform.

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