Enhancing natural killer cell function with gp41-targeting bispecific antibodies to combat HIV infection

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Objective(s): The aim of this study was to develop and evaluate the activity of bispecific antibodies (bsAbs) to enhance natural killer (NK) cell antibody-dependent cellular cytotoxicity (ADCC) against HIV-infected cells.

Design: These bsAbs are based on patient-derived antibodies targeting the conserved gp41 stump of HIV Env, and also incorporate a high-affinity single chain variable fragment (scFv) targeting the activating receptor CD16 on NK cells. Overall, we expect the bsAbs to provide increased affinity and avidity over their corresponding mAbs, allowing for improved ADCC activity against Env-expressing target cells.

Methods: bsAbs and their corresponding mAbs were expressed in 293T cells and purified. The binding of bsAbs and mAbs to their intended targets was determined using Bio-Layer Interferometry, as well as flow cytometry based binding assays on in-vitro infected cells. The ability of these bsAbs to improve NK cell activity against HIV-infected cells was tested using in-vitro co-culture assays, using flow cytometry and calcein release to analyse NK cell degranulation and target cell killing, respectively.

Results: The bsAbs-bound gp41 with similar affinity to their corresponding mAbs had increased affinity for CD16. The bsAbs also bound to primary CD4\textsuperscript{+} T cells infected in vitro with two different strains of HIV. In addition, the bsAbs induce increased NK cell degranulation and killing of autologous HIV-infected CD4\textsuperscript{+} T cells.

Conclusion: On the basis of their in-vitro killing efficacy, bsAbs may provide a promising strategy to improve NK-mediated immune targeting of infected cells during HIV infection.

Introduction

Natural killer (NK) cells are effector cells of the innate immune system that are equipped to recognize and rapidly eradicate tumour or virus-infected cells. Healthy cells escape NK cell killing because major histocompatibility complex class I (MHC-I) proteins on healthy cells engage NK cell inhibitory receptors [1]. In the context of HIV infection, downregulation of MHC-I proteins can activate NK cells; in addition, upregulation of stress-induced ligands on infected cells can trigger engagement of activating receptors on NK cells.
cells such as NKG2D [2]. However, HIV possesses mechanisms to counteract NK cell surveillance, such as downregulation of activating ligands [3,4]. Thus, although NK cells have the potential to control HIV, enhancing their responses could optimize their ability to control HIV.

NK cell effector function can also be antibody-dependent, a process termed antibody-dependent cellular cytotoxicity (ADCC). NK cells expressing the activating cell surface receptor Fc?RIIA (CD16) can bind antibodies via their constant (Fc) domain; these antibodies can in turn bind cell surface viral antigens. CD16-antibody binding activates NK cells, triggering the secretion of antiviral cytokines such as interferon-gamma (IFN-γ) and the release of perforin and granzymes into target cells, resulting in cell death. ADCC may provide a protective or therapeutic benefit in HIV infection; ADCC antibody titres inversely correlate with viral load and rate of disease progression in HIV-infected individuals [5,6], and are higher in elite controllers than in patients that progress to disease [7]. Furthermore, in mother–infant transmission of HIV, ADCC antibodies in breast milk correlate with reduced infection risk in infants via breastfeeding, while increasing survival rates in infected infants [8,9]. In nonhuman primates, vaccine-induced ADCC antibody titres correlate with decreased viral loads and/or delayed disease progression after challenge with simian immunodeficiency virus (SIV) [10,11]. In humans, the RV144 clinical trial, which was the first HIV vaccine trial to demonstrate some degree of efficacy [12], induced ADCC responses in vaccine recipients [13,14]. Binding of IgGs to V1V2 of Env correlated inversely with the rate of infection, suggesting that ADCC-mediating antibodies could have contributed to the observed protection [15]. However, despite the evidence for the protective role of ADCC responses against HIV, it remains unclear whether the key driver of ADCC efficacy in infection is the binding to viral targets, the triggering of effector cell activation or both.

Investigation into the epitopes of ADCC-mediating mAbs in HIV have revealed multiple viral targets, including Env, Gag, Pol, Vpu and Nef [16–20]. Within Env, many ADCC-mediating mAbs target gp120 [21]. Recently, however, a larger number of gp41-targeting mAbs that mediate ADCC have also been characterized [22,23]. During viral membrane fusion, gp120 is shed, and gp41 undergoes a series of conformational changes that exposes various targetable epitopes such as the fusion peptide, the loop region that connects the two heptad regions and the membrane proximal region [24]. The postfusion state of gp41, a six-helix bundle [25–28], remains exposed at the cell surface after viral and host membranes fuse and is often referred to as a stump [29]. How long stumps remain on the surface of infected cells postfusion has been poorly studied; however, there are some data to suggest that they can remain on the cell surface for at least an hour (longer time points were not tested in this study) [30]. Stumps can also occur on cell surfaces, as gp120 can be shed when virus buds from infected cells [29]. The cell-surface exposed nature of the stumps make them targetable by antigp41, ADCC-mediating mAbs. However, unlike influenza or dengue virions that are densely studded with their envelope glycoproteins, HIV is sparsely coated with Env [31,32]. This presumably results in very few targetable stumps per cell on the surfaces of infected cells, decreasing the overall efficacy of ADCC.

By engineering known antistump, ADCC-mediating mAbs into bispecific antibodies (bsAbs) that adopt a tetravalent IgG-scFv format [33], we rationalized that we would achieve the following. First, we would bridge NK cells to HIV-infected targets via CD16, a potent trigger of NK cell activation [34], thus forcing NK cells to contact and be activated specifically against these cells. Second, the bsAb would show increased avidity over a mAb due to its ability to simultaneously engage two CD16 receptors on the NK cell surface. The resulting increase in local concentration of bsAb at the NK cell surface can enable more efficient crosslinking to the stumps on the infected cell surface. As such, we tested these bsAbs for their ability to activate NK cells and clear HIV-infected primary CD4+ T cells in vitro.

Materials and methods

Expression of bispecific antibodies and mAbs

mAbs heavy and light chain plasmids were obtained from Dr Julie Overbaugh (Fred Hutchinson Cancer Research Center). For the bsAbs, the variable heavy chain sequence connected to the NM3E2 scFv sequence via the linker LES(GGGGS)3 (IDT gBlocks) were cloned the VRC01 plasmid backbone (retaining the plasmid's signal sequence and constant heavy chain regions) by In-Fusion. Light chain plasmids from the Overbaugh lab were used as is for bsAb expression. NM3E2 scFv was expressed and purified from Escherichia coli as described previously [35].

bsAbs and mAbs were expressed in Exp293 cells, a well established cell line for the production of recombinant antibodies [36,37], at a 2:1 Heavy:Light chain ratio, tracking viability (until < 70%) or until day 6, whichever was sooner. Media was harvested and filtered, before purifying bsAbs and mAbs on Protein A columns, followed by size exclusion chromatography (S200 pg or S200 increase for 5 ml or 500 µl scale purification, respectively; GE Healthcare, Chicago, Illinois, USA). All proteins were analysed by reducing and nonreducing SDS-PAGE.

Antigens for biolayer interferometry

Six-helix containing a cysteine in the first HR2 helix (Ser68→Cys68) was expressed and purified as described
co-culture assays

T cells were isolated from healthy donors using the CD4+ T cell Isolation Kit (Miltenyi Biotec). NK cells were isolated from donor PBMCs using the NK Cell Isolation Kit and the CD4+ T cell Isolation Kit respectively (Miltenyi Biotec). All flow cytometry analysis was performed using FlowJo version 9.9.6 (Tree Star, Ashland, Oregon, USA).

Binding assays by biolayer interferometry

BLI is a label-free optical analytical technique used to measure biomolecular interactions, based on interference patterns of white light reflected from a biosensor tip. Samples in assay buffer (1x TBS with 0.5% BSA) were dispensed into 96–well black flat-bottom plates (Greiner Bio-One, Kremsmünster, Austria) at a volume of 180–200 μl per well; all measurements were performed on the Octet Red96 (Pall ForteBio, Menlo Park, California, USA) at room temperature at 1000 rpm agitation. Streptavidin-coated biosensor tips (Pall ForteBio) were used to capture biotinylated antigens, and typical immobilization levels captured on the AMC sensors varied up to 0.5 nm. Antigen loading was carried out for 30 s, followed by regeneration to remove nonspecifically bound antigen. After two baseline steps of 20 s each, association was performed for 60 s in wells containing antibody dilutions, followed by dissociation in assay buffer for 90 s. Between each antibody sample, a regeneration step was included to eliminate carryover. Raw data were exported and fitted using Prism's (GraphPad Software, San Diego, California, USA) association then dissociation function.

Binding assays by flow cytometry

CHO-WT was obtained through the NIH AIDS Reagent Program, from Dr Carol Weiss and Dr Judith White. These cells express HIV-1 HXB2 gp120 envelope glycoprotein on their surface, are highly fusogenic and readily form syncytia with human CD4+ cells [39]. Cells were plated at 105 cells/well in a 96–well flat-bottomed plate (Corning, New York, USA). After 24 h, supernatant media was removed and replaced with FACS buffer. Cells were stained with 1:100 dilution of each antibody for 1 h at 4°C, washed, and stained with Alexa 647-conjugated antihuman IgG (H+L) antibody (Thermo Fisher) for 30 min at 4°C. Cells were further washed, fixed with FACS Lyse (BD Biosciences) and permeabilized with FACS Permeabilization Buffer 2 (BD Biosciences), stained for HIV p24 (KC57–FITC; Beckman Coulter) and analysed by flow cytometry on a MACSQuant Analyzer (Miltenyi Biotec). All flow cytometry analysis was performed using FlowJo version 9.9.6 (Tree Star, Ashland, Oregon, USA).

Natural killer-CD4+ co-culture assays

NK cells and CD4+ T cells were isolated from donor PBMCs by negative selection with the CD4+ T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), and activated with plate-bound anti-CD3 (clone OKT3; eBioscience, San Diego, California, USA), anti–CD28/CD49d (BD Biosciences) and PHA-L (eBioscience). After 48 h, CD4+ T cells were mock-infected, or infected with either Q23 [40] or NL4–3 (infectious molecular clone obtained through the NIH AIDS Reagent Program, from Dr Malcom Martin [41]) virus, using Viromag magnetofection (OZ Biosciences, San Diego, California, USA). Both Q23 and NL4–3 viruses were grown in 293T cells and titrated as previously described [42]. Twenty-four hours post infection, cells were stained at a 10 nmol/l concentration of each mAb/bsAb for 30 min at 4°C, washed, and then stained with Alexa 647-conjugated antihuman IgG (H+L) antibody (Thermo Fisher) for 30 min at 4°C. Cells were further washed, fixed with FACS Lyse (BD Biosciences) and permeabilized with FACS Permeabilization Buffer 2 (BD Biosciences), stained for HIV p24 (KC57–FITC; Beckman Coulter) and analysed by flow cytometry on a MACSQuant Analyzer (Miltenyi Biotec). All flow cytometry analysis was performed using FlowJo version 9.9.6 (Tree Star, Ashland, Oregon, USA).

Statistical analysis

For co-culture assays, linear mixed effects models were used. The models incorporated fixed effects for
concentration, bsAb vs. mAb, and mock vs. HIV infection, and a random effect for donor. This model takes into account that there are multiple observations per donor and accounts for variability between donors. Statistical analyses were performed using SPSS and the nlme package in R (R Foundation, Vienna, Austria).

Results

Expression and purification of stump-targeting bispecific antibodies

To improve ADCC activity of stump-targeting mAbs, we designed bsAbs in the IgG-scFv or Morrison format [33,44], in which the variable regions of the antibody target gp41 stumps on HIV-infected cells, while the high-affinity scFvs tethered to the heavy chains bind CD16 on NK cells. These bsAbs can engage two CD16 receptors on the NK cell while binding to the target antigen, resulting in increased avidity. We chose the IgG-scFv format to retain full Fc effector function on the bsAb, so that we could directly compare bsAb activity to the corresponding mAb. We postulated that the combination of high affinity and avidity would drive high efficacy of the bsAbs compared with the mAbs, which have lower efficacy due to low-affinity Fcs engaging one CD16 on the NK cell surface at a time (Fig. 1a).

For the stump binding antibodies, we previously identified patient-derived antistump antibodies, QA255.006 and QA255.072, referred to hereafter as 006 and 072, respectively [23]. The 006 antibody binds gp41 stumps and as a stump mimetic, six-helix [23]. The 006 epitope has not been precisely mapped, but is thought to be a discontinuous epitope that includes parts of the C-heptad repeat and N-heptad repeat regions of gp41, and potentially the fusion proximal peptide region (FPPR) of gp41 [23]. The 072 antibody binds a highly conserved linear epitope in the loop region of gp41 [23]. This linear epitope overlaps with the binding epitope of another patient-derived mAb 7B2 [45], as well as other known loop binding mAbs [46,47]. The stump-binding antibodies demonstrate cross-clade binding to HIV-1 strains. For the CD16-targeting scFv portion of the bsAbs, we used NM3E2, a phage-display derived antibody [35]. After expression in mammalian cells, bsAbs and mAbs were isolated using Protein A resin, followed by size exclusion chromatography. As expected, the bsAbs eluted earlier than the mAbs on the size exclusion column due to their larger molecular weights (Fig. 1c, Supplemental Fig 1, http://links.lww.com/QAD/B711).

Bispecific antibodies show increased affinity to CD16 over mAbs, while engaging stumps with similar affinity

To determine whether bsAbs showed improved binding to CD16 over mAbs, we used biolayer interferometry (BLI), and compared binding of the bsAbs, mAbs and the NM3E2 scFv with CD16. The bsAbs demonstrated binding in the nanomolar range to CD16, which were two to three orders of magnitude improved over that of the mAbs (low to sub-micromolar range) (Fig. 2a, Table 1). NM3E2 scFv affinity was determined to be 75.1 ± 15.1 nmol/l, which is within an order of magnitude of the published value of 20 nmol/l for this scFv [35].

To determine whether bsAbs retained their binding specificities and affinities to their targets, we used BLI and compared binding of the bsAbs, mAbs and NM3E2 with six-helix, the stump mimetic that has been shown to be targeted by 006 but not 072 (as six-helix does not contain the immunodominant loop that 072 binds to), as well as two 19-mer loop peptides that both contain the previously determined linear epitope of 072 [23], but that do not bind 006. Recombinantly expressed gp41 ectodomain was insoluble in the BLI assay buffer, and therefore not used in this assay. bsAbs and IgGs demonstrated similar levels of binding (Table 1), and retained their binding specificities to their corresponding targets, that is, 006 bsAb and IgG bound six-helix but not loop peptides, while 072 bsAb and IgG-bound loop peptides but not 006 (Fig. 2b, c).

Bispecific antibodies bind to cells expressing Env, as well as to HIV-infected primary CD4⁺ T cells

The stump-binding mAbs have previously been shown to bind to Env in a cell-based ELISA assay [23]. To determine whether the constructs could engage their targets on the surface of cells, we first tested binding of the mAbs and bsAbs in adherent CHO cells expressing HXB2 gp120. mAbs and bsAbs bind 9–12% of Env-expressing CHO cells, six to seven-fold greater than the background secondary only control (Fig. 3a). These results correlate with the binding of bsAbs and mAbs in vitro, showing that in the absence of NK cells or CD16, binding profiles of the bsAbs and mAbs to the target antigen are similar. We then tested dose-dependence of cell-surface binding with the 006 and 072 mAbs. Binding to Env-expressing CHO cells was dose-dependent for both 006 and 072 in the 0–500 nmol/l range (Supplemental Figure 2A, http://links.lww.com/QAD/B711).

To evaluate binding of these bsAbs to Env in a more physiological system, we tested the binding of the mAbs and bsAbs to primary CD4⁺ T cells infected in vitro with either a clone from early, subtype A infection (Q23) or a commonly used laboratory-adapted, subtype B strain (NL4–3). The Q23 strain was used as the 006 and 072 mAbs were derived from an individual with subtype A infection [23]; the NL4–3 strain was used to test cross-reactivity in a different subtype. To exclude bystander uninfected cells, we only included HIV p24⁺ cells in the analysis. Both the 072 and 006 bsAbs and mAbs demonstrated binding to HIV–1-infected CD4⁺ T cells, with 60–80% of infected cells staining positively in Q23-infected cells and 13–40% in NL4–3 infected cells;
binding was similar between bsAbs and mAbs (Fig. 3c). No binding higher than 1% was observed for either bsAb or mAb to mock-infected cells (Supplemental Figure 2B, http://links.lww.com/QAD/B711).

**Bispecific antibodies improve natural killer cell mediated killing of autologous HIV-infected cells**

To determine if the bsAbs could improve NK cell targeting of autologous HIV-1-infected cells, we used an in-vitro NK-CD4⁺ co-culture assay to test the effect of the bsAbs on NK cell degranulation and target cell killing. The range of concentrations tested, from 0.1 to 10 nmol/1, was chosen to be within the testing concentrations previously documented [35]. NK cell degranulation significantly increased, in co-culture with CD4⁺ T cells infected with either the Q23 or NL4–3 strains, in the presence of increasing concentrations of the bsAb (Fig. 4a). A similar increase was not observed with
mock-infected cells. We also tested the 072 and 006 mAbs in the same assay, to determine if the bsAbs’ enhanced affinity and avidity improved NK cell targeting compared with the corresponding mAbs. As expected, the 072 and 006 bsAbs induced much stronger NK cell degranulation responses than the mAbs - at 10 nmol/l, NK degranulation was between 2.5 and 3.5-fold higher in the presence of the bsAbs than their matched mAbs (Fig. 4a). For each of the 072 and 006 constructs, we used a linear mixed model [48], with a random effect for donor to

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**Table 1.** Apparent binding constants (Kapp)\(^a\) of Abs to six-helix, loop peptides and CD16.

<table>
<thead>
<tr>
<th></th>
<th>006 IgG</th>
<th>006 IgG-Fv</th>
<th>072 IgG</th>
<th>072 IgG-Fv</th>
<th>NM3E2 scFv</th>
</tr>
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<tbody>
<tr>
<td>CD16</td>
<td>0.37 ± 0.26 μmol/l</td>
<td>4.6 ± 2.4 μmol/l</td>
<td>4.9 ± 4.4 μmol/l</td>
<td>9.7 ± 6.1 μmol/l</td>
<td>72 ± 14 nmol/l</td>
</tr>
<tr>
<td>Six-helix</td>
<td>2.5 ± 0.55 nmol/l</td>
<td>1.6 ± 0.20 nmol/l</td>
<td>No binding</td>
<td>No binding</td>
<td>No binding</td>
</tr>
<tr>
<td>Loop peptide 1</td>
<td>No binding</td>
<td>No binding</td>
<td>3.2 ± 1.1 nmol/l</td>
<td>2.5 ± 0.67 nmol/l</td>
<td>No binding</td>
</tr>
<tr>
<td>Loop peptide 2</td>
<td>No binding</td>
<td>No binding</td>
<td>3.1 ± 1.4 nmol/l</td>
<td>6.4 ± 3.9 nmol/l</td>
<td>No binding</td>
</tr>
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*Values reported as mean ± SD for three biological repeats.
Fig. 3. Binding of antibodies (bsAbs and mAbs) to (a) CHO-WT cells expressing Env and (b) primary CD4+ T cells infected \textit{in vitro} with either a clone from early subtype A HIV infection (Q23) or laboratory-adapted (NL4–3) strain of HIV. For each, histograms of antibody binding are shown for one representative repeat; the dotted line indicates the gate set based on the secondary only stained control. Mean percentage positive binding and corresponding SEM are shown numerically for each antibody. In the bar graph, percentage positive binding of each antibody is shown for each of (a) three separate biological repeats, each with two replicates and (b) six biological replicates, and mean values for each antibody are shown in the overlaid bar graph. Shapes denote individual biological replicates.
account for inter-individual variability, to test the contributions of concentration, HIV infection and bsAb/mAb on NK cell degranulation. In the model, concentration, infection (with either strain of HIV-1) and bsAb (vs. mAb) were all found to have a statistically significant effect on NK cell degranulation, for both bsAbs.

To determine whether the increases in NK cell degranulation also led to increased killing of HIV-1-infected target cells, we measured target cell death using calcein release [43]. Both bsAbs significantly enhanced NK killing of HIV-infected cells with increasing concentration, for both strains of HIV-1 tested, and had little nonspecific targeting, as killing of mock-infected cells was not increased (Fig. 4b). Once again, the 072 and 006 bsAbs induced higher levels of target cell death in the presence of NK cells compared with their corresponding mAbs at matched concentrations (Fig. 4b). In linear mixed models, infection (with either strain of HIV) and bsAb (vs mAb) were both found to have a statistically significant effect on NK cell degranulation;
concentration had a statistically significant effect on the 006 bsAb but not the 072. The bsAb format enabled improved NK cell targeting and killing of infected cells over the mAb; this improved targeting was HIV-specific, and was observed across two HIV-1 strains of different subtypes.

Discussion

NK cells can rapidly recognize and lyse virus-infected or malignant cells; however, cancers and viruses have developed strategies to avoid NK cell surveillance. Thus, there is a need to specifically activate NK cells to overcome these evasion strategies. bsAbs have previously been used successfully in the cancer setting to enhance NK cell activity and clear tumours such as non-Hodgkin's lymphoma [49] and acute myeloid leukaemia [50]. In contrast, little work has been done in the field of bsAbs to recruit NK cells to treat viral infections. This work shows that NK cell activity against HIV-1 can be specifically enhanced using bsAbs that simultaneously target gp41 on HIV-infected cells and the activating receptor CD16 on NK cells. As CD16 is expressed on other effector cell types such as macrophages and neutrophils [51,52], activation of non-NK cells might be a concern; however, even if this were to occur, these effectors would likely augment the clearance of infected cells. In addition, effector cells can express phenotypic variants of the CD16 receptor. To this end, we tested binding of our bsAbs to two CD16a variants using ELISAs and found that the binding remained unchanged between the two variants (Supplemental Fig 3, http://links.lww.com/QAD/B711).

Recently, one study has linked single domain soluble CD4 to an anti-CD16 antibody to exploit the high-affinity interaction between gp120 and its native receptor CD4 [53]. Soluble CD4 confers broad-spectrum recognition of envelope glycoprotein across different HIV strains. However, a potential limitation of this strategy is that CD4 can induce gp120 shedding, which can decrease binding over time. Our alternative approach of targeting gp41 avoids this limitation. Another abstract described NK cell activation in the context of HIV by linking a known gp120-binding broadly neutralizing antibody, VRC01, to an anti-CD16 single chain nanobody [54].

Our study differs from this aforementioned work as a proof of principle to determine the feasibility of using tetravalent bsAbs to enhance NK cell function against HIV, particularly by using gp41 antibodies to target infected cells. Presumably, this strategy can be extended to enhancing NK cell function in other disease contexts, such as influenza, wherein ADCC responses are also known to be protective [55].

The gp41 antibodies used in the bsAb constructs in this study were recently shown in a separate study to mediate ADCC in the commonly used RF-ADCC assay that employs cells coated with HIV Env [23]. In the context of in-vitro infected cells, the binding of these mAbs was much lower (Fig. 3b), which is unsurprising given the low density of Env on infected cell surfaces compared with protein-coated cell surfaces. In addition, we observed differences in binding between cells infected with Q23 or NL4–3; this may reflect differences in binding of the gp41 antibodies between Env proteins between the two strains, or differences in the level of expression of Env between the two strains. Binding of the gp41 mAbs was increased when infecting cells with Vpu/Nef-deficient virus (which results in higher Env surface expression), or in a cell-based ELISA wherein soluble CD4 was used to induce gp120 shedding to expose the gp41 epitopes [23]. However, to overcome the reduced ADCC activity due to low binding of the mAbs in in-vitro infected cells, we employed a more therapeutically translatable approach and enhanced ADCC activity by the tetravalent bsAb format to increase affinity and avidity to CD16, compared with the corresponding mAbs. The bsAb platform is an exciting tool to modulate NK cell function, with the potential for improved activity and specificity in future studies. For instance, studies targeting other activating receptors on NK cells, such as NKG2D, are warranted. In addition, two distinct activating receptors on the NK cell can be targeted to engineer a trispecific antibody (two specificities to the NK cell, and one to the infected target) to fine tune NK activation and the magnitude of effector responses. Much of the focus for enhancing ADCC has previously only been on the level of the antibody, either on its Fab affinity to its target, or on its Fc type. Our work emphasizes the importance of improving effector cell activation to boost therapeutic outcomes.

Overall, we have demonstrated that NK cell cytotoxicity against HIV-1 can be specifically enhanced using bsAbs targeting both the gp41 region of HIV Env and the activating receptor CD16 on NK cells, providing a new avenue for immune targeting of HIV infection.

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Conflicts of interest

There are no conflicts of interest.

References


