Slaying the Trojan Horse: Natural Killer Cells Exhibit Robust Anti-HIV-1 Antibody-Dependent Activation and Cytolysis against Allogeneic T Cells

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ABSTRACT

Many attempts to design prophylactic human immunodeficiency virus type 1 (HIV-1) vaccines have focused on the induction of neutralizing antibodies (Abs) that block infection by free virions. Despite the focus on viral particles, virus-infected cells, which can be found within mucosal secretions, are more infectious than free virus both \textit{in vitro} and \textit{in vivo}. Furthermore, assessment of human transmission couples suggests infected seminal lymphocytes might be responsible for a proportion of HIV-1 transmissions. Although vaccines that induce neutralizing Abs are sought, only some broadly neutralizing Abs efficiently block cell-to-cell transmission of HIV-1. As HIV-1 vaccines need to elicit immune responses capable of controlling both free and cell-associated virus, we evaluated the potential of natural killer (NK) cells to respond in an Ab-dependent manner to allogeneic T cells bearing HIV-1 antigens. This study presents data measuring Ab-dependent anti-HIV-1 NK cell responses to primary and transformed allogeneic T-cell targets. We found that NK cells are robustly activated in an anti-HIV-1 Ab-dependent manner against allogeneic targets and that tested target cells are subject to Ab-dependent cytolysis. Furthermore, the educated KIR3DL1+ NK cell subset from HLA-Bw4+ individuals exhibits an activation advantage over the KIR3DL1– subset that contains both NK cells educated through other receptor/ligand combinations and uneducated NK cells. These results are intriguing and important for understanding the regulation of Ab-dependent NK cell responses and are potentially valuable for designing Ab-dependent therapies and/or vaccines.

IMPORTANCE

NK cell-mediated anti-HIV-1 antibody-dependent functions have been associated with protection from infection and disease progression; however, their role in protecting from infection with allogeneic cells infected with HIV-1 is unknown. We found that HIV-1-specific ADCC antibodies bound to allogeneic cells infected with HIV-1 or coated with HIV-1 gp120 were capable of activating NK cells and/or trigging cytolysis of the allogeneic target cells. This suggests ADCC may be able to assist in preventing infection with cell-associated HIV-1. In order to fully utilize NK cell-mediated Ab-dependent effector functions, it might also be important that educated NK cells, which hold the highest activation potential, can become activated against targets bearing HIV-1 antigens and expressing the ligands for self-inhibitory receptors. Here, we show that with Ab-dependent stimulation, NK cells expressing inhibitory receptors can mediate robust activation against targets expressing the ligands for those receptors.

A preventative vaccine is urgently needed to curb the growing human immunodeficiency virus type 1 (HIV-1) epidemic and reduce the global economic burden of supplying lifelong antiretroviral therapies. The induction of antibodies (Abs) capable of neutralizing a broad range of viral strains is a major goal of HIV-1 vaccine research. Such broadly neutralizing Abs (BnAbs) have been isolated from HIV-1-infected individuals, and passive transfer of BnAbs to nonhuman primates has proven sufficient to prevent infection upon challenge with cell-free chimeric simian-human immunodeficiency viruses (SHIV) (1–3). Despite the potential of BnAbs to prevent HIV-1 infection, a vaccine capable of eliciting BnAbs in HIV-1-uninfected individuals has not yet been designed. The lack of appropriate immunogens and the extensive somatic hypermutation within the variable regions of BnAbs are major impediments to their induction via vaccination (4). This inability to elicit BnAbs through immunization has spurred research interest into the potential of nonneutralizing effector functions of Abs to provide protection against HIV-1.

Interest in the potential of the nonneutralizing effector functions of Abs has also been stimulated by the recent phase III human RV144 vaccine trial in Thailand, which provided evidence that nonneutralizing Ab-associated effector functions may be involved in Ab-confferred protection against HIV-1 infection (5, 6). Indeed, correlates of immunity analyses revealed that, in the absence of broadly neutralizing Abs, both high levels of IgG Abs to the V1V2 regions of the HIV-1 Env and low levels of plasma an-
tiviral IgA were linked with lowered risk of infection in vaccinated individuals (5). Furthermore, anti-HIV-1 Abs that elicit Ab-dependent cellular cytotoxicity (ADCC) were associated with lowered risk of infection, but only in vaccinated individuals with low plasma antiviral IgA. The suggestion that ADCC may be important for protection against HIV-1 in humans corroborates several lines of evidence provided by the macaque simian immunodeficiency virus (SIV) and SHIV infection models. For example, the protective potential of live-attenuated SIV vaccines in macaques was associated with the activity of ADCC-mediating Abs (7). Furthermore, the protective potential of the CD4 binding site BnAb, b12, is linked to the ability of the Ab’s constant region (Fc) to bind to Fc receptors (FCRs), as mutation of the Fc to abrogate binding to FCRs reduced the degree of protection observed in passively transferred macaques challenged with SHIV (2). It is possible that the ability of the b12 Ab to mediate functions such as ADCC might confer it with the ability to eliminate early infected autologous lymphocytes. This explanation might also explain how ADCC-competent Abs could provide protection in vaccinated individuals in the RV144 trial. It should be noted, however, that to achieve protection against natural mucosal exposure to HIV-1, it might also be required that these Abs be capable of eliminating HIV-1-infected allogeneic lymphocytes.

The “Trojan horse” hypothesis predicts that mucosal HIV-1 infection is established in the uninfected partner by HIV-1-infected allogeneic lymphocytes received within the genital secretions of the infected partner (8). Much evidence from animal studies, as well as human studies, supports the concept that cell-to-cell HIV-1 transmission can be responsible for initial infection. Lymphocytes infected with SIV and HIV-1 robustly infect macaques and chimpanzees, respectively (9–11). Indeed, virus-infected lymphocytes are more efficient at establishing infection than free virus (8, 10). Further, the number of infected cells required for infection is more reflective of the quantity of these cells that are found in the semen of HIV-1-infected men than is the amount of free virus used to establish infection (8). Evidence of the Trojan horse hypothesis in humans consists of the observation of increased lymphocytes, including HIV-1-infected cells, within the semen of infected men, the demonstration that these infected lymphocytes are more infectious in vitro than free virions within the genital secretions, and the observation that in some newly HIV-1-infected individuals transmitted virus is more genetically similar to virus found in the seminal lymphocytes of the transmitting partner than to free virions within the transmitting partner’s semen (8, 12, 13). Cumulatively, these observations suggest that cell-to-cell transmission accounts for at least a proportion of HIV-1 transmissions. This implies vaccine design should incorporate tactics to eliminate allogeneic HIV-1-infected lymphocytes.

The effector function of ADCC represents an attractive mechanism via which HIV-1-infected allogeneic lymphocytes could be targeted for elimination. Natural killer (NK) cells are a major effector cell that mediates ADCC (14). Theoretically, Abs binding to primary allogeneic lymphocytes bearing HIV-1 antigens could trigger activation of host NK cells, but this has not been sufficiently studied.

How efficiently host NK cells respond to allogeneic lymphocytes via an anti-HIV-1 Ab-dependent fashion is likely to be modulated by several factors. A two-tier process tightly regulates the potential of NK cells to become activated upon stimulation. First, NK cells are subjected to the ontological process of education, where NK cells expressing inhibitory surface receptors, such as killer cell immunoglobulin-like receptors (KIR), specific for self-major histocompatibility complex class I (MHC-I or HLA-I) ligands, are conferred with the potential to mediate effector functions upon encountering appropriate target cells (15, 16). NK cells not expressing inhibitory KIR capable of interacting with self-HLA-I remain hypofunctional or noneducated. Indeed, studies assessing HIV-1 and non-HIV-1 Ab-dependent NK cell activation have demonstrated that NK cells educated by the interaction of inhibitory KIR and HLA-I exhibit higher activation upon stimulation with Ab-coated target cells than noneducated NK cells (15, 17–19). Second, the ability of an NK cell to mediate effector functions upon encountering a putative target cell is determined by the cumulative signal received through surface-activating and inhibitory receptors (20). Target cells expressing HLA-I recognized by inhibitory receptors on the NK cell initiate inhibitory signals that can inhibit mediation of effector functions, whereas target cells lacking HLA-I recognized by inhibitory receptors and that express sufficient ligands for activating NK cell receptors stimulate the NK cell to mediate effector functions. This principle has been demonstrated in an assessment of anti-HIV-1 ADCC against autologous target cells, where blockade of inhibitory receptors that interact with HLA-C and HLA-E restored cytolyis (21). Collectively, these two tiers of regulation interact to create a scenario whereby educated NK cells are prevented from mediating autoreactive responses by the constitutive expression of HLA-I but have the potential to respond to virus-infected cells that have downregulated HLA-I (22). Although the impacts of NK cell education and target cell HLA-I expression have been studied in the context of anti-HIV-1 Ab-dependent NK cell activation against autologous targets (17), the influences that NK cell education and the divergent surface HLA-I phenotypes of allogeneic target cells have on anti-HIV-1 Ab-dependent NK cell activation have not been studied.

Given the lack of existing data on anti-HIV-1 Ab-dependent activation against allogeneic target cells, we utilized intracellular cytokine staining and cytotoxicity assays to measure and assess the factors regulating these responses. We assessed the anti-HIV-1 Ab-dependent cytolyis of primary allogeneic T cells and the CEM.NKr-CCR5 established T-cell line. Furthermore, we evaluated the impact of NK cell education on NK cell-mediated Ab-dependent activation, as well as the ability of educated NK cells to become activated in the context of matches and mismatches between the inhibitory KIR expressed on NK cells and the HLA-I profiles of different allogeneic target cells. The presented work regarding KIRs focused on NK cells expressing the inhibitory KIR3DL1 receptor, which recognizes HLA-A and HLA-B molecules carrying the HLA-Bw4 epitope to the exclusion of molecules carrying the HLA-Bw6 epitope (i.e., HLA-Bw4+/−) (23). We assessed if KIR3DL1-expressing NK cells from individuals carrying the HLA-Bw4 epitope exhibited an education-induced activation advantage over the KIR3DL1+ NK cell population. All together, we demonstrate robust Ab-dependent cytolyis of target cells and activation of NK cells by HIV-1 gp120-coated allogeneic primary T cells and CEM.NKr-CCR5 T cells. Activation data suggest that Ab-dependent activation of NK cells can result in activation in the presence of ligands for inhibitory KIR.
TABLE 1 HLA-A and HLA-B typing of primary T-cell target donors

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* HLA-Bw4* alleles are shown in bold.

TABLE 2 HLA typing of NK cell donors utilized for evaluation of control

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* HLA-Bw4* alleles are shown in bold.

MATERIALS AND METHODS

Subjects. Whole blood was collected from 29 HIV-1-uninfected healthy controls by forearm venipuncture into vacuettes containing anticoagulant. Whole blood was used as a source of effector cells and to purify primary T-cell targets for Ab-dependent NK cell activation and cytotoxicity assays. NK cells from all effector cell donors utilized for the whole blood allogeneic anti-HIV-1 Ab-dependent activation assay were shown to express KIR3DL1 by flow cytometry surface staining with the DX9 anti-KIR3DL1 Ab (BD Biosciences, which recognizes the inhibitory KIR3DL1 receptor to the exclusion of the activating KIR3DS1 receptor (24). Plasma samples were obtained from two HIV-1-infected clients of the Melbourne Sexual Health Center (Carlton, Victoria, Australia) and one HIV-1-infected donor from the HIV STAR study (25). Two of these individuals have previously been shown to have anti-HIV-1 Abs capable of activating NK cells in Ab-dependent NK cell activation assays (26, 27). These plasma samples were used as a source of Abs to activate NK cells in the experiments reported in this report. A plasma sample from an HIV-1-uninfected individual was also utilized as a negative control. Informed consent was obtained before collection of all biological samples, and the ethics committees of all participating institutions approved the described studies.

Antibodies and cell lines. Aside from the utilized plasma samples described above, several polyclonal and monoclonal Abs were implemented to assess NK cell activation and Ab-dependent cytotoxicity. These Abs included HIVIG (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH), HIV-1-IVIG (bioCSL, Parkville, Australia), and the anti-HIV-1 A32 monoclonal Ab (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH). Additionally, CEM.NKra-CCR5 cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were utilized as targets for NK cell activation and cytosis assays.

HLA-1 typing. The Victorian Transplant and Immunogenetics Service (Parkville, Australia) typed donors of effector cells and primary T-cell targets for HLA-1 alleles. Sequence-based typing resolved the HLA-A and/or HLA-B alleles of donors to two-digit resolution. The HLA types of the primary T-cell donors and effector cell donors utilized to assess NK cell education are listed in Tables 1 and 2, respectively. Several additional effector cell donors that were included in this study are not listed in Table 2. These donors (i.e., G-L and P-X) were not utilized for experiments evaluating the impact of NK cell education on anti-HIV antibody-dependent NK cell activation.

Isolation of primary T-cell targets. Whole blood was obtained from four donors for isolation of primary T-cell targets for the allogeneic anti-HIV-1 Ab-dependent activation assay. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from whole blood through Ficoll Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation. According to the manufacturer’s instructions, CD3+ T lymphocytes were isolated from the PBMC using the EasySep human CD3-positive selection kit (Stemcell). Prior to use in the Ab-dependent NK cell activation assay, CD3+ T cells were cultured overnight at 1 × 10^6 cells/ml at 37°C in RPMI medium (Life Technologies) supplemented with 10% fetal calf serum (FCS) (DKSH), penicillin-streptomycin-glutamine (PSG) (Gibco), and 20 U/ml interleukin 2 (IL-2) (Peprotech).

Similarly, CD4+ T cells were obtained from cryopreserved PBMC by negative selection using the Easysep human CD4+ enrichment kit (Stemcell). CD4+ T cells were then activated with phytohemagglutinin L (10 μg/ml) for 48 h and maintained in RPMI medium supplemented with recombinant IL-2 (100 U/ml) (NIH AIDS Reagent Repository). These cells were utilized for HIV-1 infections and assessment of the relative gp120 expression on infected and gp120-coated cells and served as infected or coated targets for the flow cytometry-based cytosis assay.

Labeling of primary T cells and CEM.NKra-CCR5 with gp120. Primary T cells and CEM.NKra-CCR5 cells were coated with a range of concentrations of HIV-1 Southern gp120 (NIH AIDS Reagent Repository) or HIV-1 Southern gp120, expressed and purified as described in reference 28, by incubation together for 90 min at 4°C. The concentrations/amounts of gp120 used for coating are listed for each assay in Results. Uncoated cells, which were used as controls in the NK cell activation assay, were incubated for 90 min at 4°C in the absence of gp120. Prior to use in the Ab-dependent NK cell activation assay, labeled and unlabeled cells were washed with RPMI medium.

Infection of primary T cells. As previously described (29, 30), vesicular stomatitis virus G (VSVG)-pseudotyped NL4.3 green fluorescent protein (GFP)-expressing ADA-based viruses, either wild type or defective for the nef and vpu genes, were used to infect primary CD4+ T cells. Infection was achieved by spin inoculation at 300 × g for 1 h in 96-well plates at 37°C. Infected cells were used as target cells in the flow cytometry-based antibody-dependent cytotoxicity assay 48 h postinfection.

Determination of amount of gp120 on infected primary T cells. The amount of gp120 expressed on HIV-1-infected primary CD4+ T cells was determined by treating cells infected with Vpu- and Nef-defective HIV-1 or uninfected primary T cells coated with a range of concentrations of HIV-1 Southern gp120 (25, 50, 100, and 200 ng/ml for 7.5 × 10^5 target cells) with the anti-HIV-1 2G12 monoclonal antibody (AB002; Polymun). Relative binding of 2G12 to the cells was assessed with an AF-647-conjugated anti-human secondary antibody (Invitrogen), and mean fluorescence intensities (MFIs) were compared, as previously described (29, 30).

Ab-dependent NK cell activation assays. A whole blood intracellular cytokine staining assay was used to assess NK cell activation by Ab-coated allogeneic primary T cells. This assay is a modification of a previously reported whole blood autologous anti-HIV-1 Ab-dependent NK cell activation assay (17, 26, 27). Briefly, 150 μl of whole blood from HIV-1-uninfected healthy controls was incubated for 5 h in the presence of 50 μl of plasma from an HIV-1+ donor, brefeldin A (Sigma) (5 μg/ml), monensin (BD Biosciences) (6 μg/ml), allopurinol (APC-H)-H7-conjugated anti-CD107a Ab (BD Biosciences; clone HA3), and 5 × 10^4 to 3 × 10^5 allogeneic primary T cells coated with gp120. Control incubations were conducted with plasma from an HIV-1+ donor or allogeneic primary T cells not coated with gp120. After incubation, cells were surface stained with peridinin chlorophyll protein (PerCp)-conjugated anti-CD3 (BioLegend; clone SK7), phycoerythrin (PE)-conjugated anti-KIR3DL1 (BD Biosciences; clone DX9), and PE-Cy7-conjugated anti-CD56 (BD Biosciences; clone NCAM16) Abs. Next, the combinations of whole blood and
target cells were treated with lysing solution (BD Biosciences) to remove red blood cells. The remaining white blood cells were then treated with permeabilization solution (BD Biosciences) and stained with Alexa-Fluor 700-conjugated anti-interferon gamma (IFN-γ) Ab (BD Biosciences; clone B27). Flow cytometry data were collected with a BD FACS Canto II flow cytometer (BD Biosciences) and were analyzed using FlowJo version 9.2 software (Tree Star).

Similarly, anti-HIV-1 Ab-dependent NK cell activation was assessed against gp120 (5 μg/1 × 10^6 cells)-coated CEM.NKr-CCR5 cells. Briefly, PBMC effector cells were obtained from whole blood donors by Ficoll-Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation. Isolated PBMC (1 × 10^6) were combined with gp120-coated CEM.NKr-CCR5 cells (1 × 10^6) at a 10:1 ratio in the presence of a 1:2,000 dilution of HIV-1-1 plasma, APC-conjugated anti-CD107a (BD Biosciences; clone HA3), brefeldin A (Sigma) (5 μg/ml), and monensin (BD Biosciences) (6 μg/ml) for 5 h at 37°C. Alternatively, NK cell activation was induced by incubating PBMC at a 10:1 ratio with gp120-labeled CEM.NKr-CCR5 cells that had been coated with the anti-gp120 monoclonal Ab, A32 (5 μg/million cells), for 1 h at 4°C. Control conditions included incubation of PBMC alone or incubation of PBMC with uncoated CEM.NKr-CCR5 cells in the presence of HIV-1-1 plasma or A32. After incubation, the cells were surface stained with PerCP-conjugated anti-CD3 (BioLegend; clone SK7), PE-Cy7-conjugated anti-CD56 (BD Biosciences; clone NCAM16) and PE-conjugated anti-KIR3DL1 (BD Biosciences; clone DX9) Abs. Next, cells were fixed in formaldehyde (Polysciences Inc.) and then treated with permeabilization buffer (BD Biosciences) and stained with Alexa Fluor 700-conjugated anti-IFN-γ Ab (BD Biosciences; clone B27). Flow cytometry data were collected with a BD FACS Canto II flow cytometer (BD Biosciences) and were analyzed using FlowJo version 9.2 software (Tree Star).

**LDH cytotoxicity assay.** The CytoTox 96 nonradioactive cytotoxicity assay kit (Promega) was used to measure ADCC via lactate dehydrogenase (LDH) release. CEM.NKr-CCR5 target cells were prepared by coating with gp120 (5 μg/1 × 10^6) for 90 min at 4°C. For some tests, gp120-coated CEM.NKr-CCR5 cells were additionally coated with the anti-HIV-1 monoclonal Ab A32 (5 μg/1 × 10^6 cells) for 60 min at 4°C. LDH assays were conducted in 96-well round-bottom tissue culture plates (Greiner Bio-One) with freshly isolated PBMC as effector cells. All conditions were conducted in triplicate. The background spontaneous LDH release of target and effector cells was assessed by incubation of each cell population alone. Maximum LDH release from target cells was assessed by adding the number of particles (range), statistics within Results are presented as median (range) versus median in-group differences were compared using Wilcoxon matched-pair tests. Data analyses were performed using GraphPad Prism version 4.0 software. All data were analyzed with nonparametric tests. Within-group differences were compared using Wilcoxon matched-pair tests. Statistics within Results are presented as median (range) versus median (range), P value of statistical test.

**RESULTS**

Ab-dependent activation of NK cells against allogeneic target cells. As much previous research on anti-HIV-1 Ab-dependent NK cell responses has utilized the CEM.NKr cell line as a target cell (30–33), we initiated our assessment of anti-HIV-1 Ab-dependent NK cell responses against allogeneic target cells using the CEM.NKr-CCR5 cell line as targets. The gating strategy used to analyze these data is exhibited in Fig. 1A. As depicted in Fig. 1B, CEM.NKr-CCR5 cells that were coated with gp120 (5 μg/million cells) robustly activated NK cells to produce IFN-γ and express CD107a in the presence of plasma from an HIV-1-infected donor, HIVIG, or the A32 monoclonal Ab. Alternatively, NK cells were not activated to produce IFN-γ or express CD107a when stimulated with uncoated CEM.NKr-CCR5 cells in the presence of HIV-1-infected plasma, HIVIG, or the A32 monoclonal Ab. These data demonstrate that allogeneic immortalized T-cell line targets can activate NK cells in an anti-HIV-1 Ab-dependent fashion, providing a rationale for assessing anti-HIV-1 Ab-dependent NK cell activation against primary allogeneic T-cell targets.

Previous research from our group has demonstrated that NK cells within whole blood can be stimulated to exhibit anti-HIV-1 Ab-dependent activation against autologous targets that bind and present HIV-1 proteins or peptides (17, 26, 27, 34). These cell-associated antigens subsequently become bound by antiviral Abs that interact with the NK cell CD16 receptor, triggering degranulation and the release of cytokines. Although much has been es-
established about the regulation of Ab-dependent NK cell activation against autologous targets (17), little has been established about the potential of divergent allogeneic target cells to differentially influence Ab-dependent NK cell activation. To provide a basis to assess NK cell-mediated Ab-dependent activation against allogeneic T cells, we modified the previously described autologous whole blood Ab-dependent activation assay to assess NK cell responses to allogeneic targets. In the modified assay, whole blood containing NK cell effectors was obtained from a series of donors and individually combined with isolated gp120-labeled primary T cells, from a common donor, in the presence of plasma from an HIV-1-infected individual (Fig. 2A). The assay also contains two
control conditions: (i) whole blood in combination with gp120-labeled primary T cells in the presence of HIV-1-uninfected plasma and (ii) whole blood in combination with nonlabeled primary T cells in the presence of HIV-1-infected plasma. Reflecting the ability of the assay to detect Ab-dependent activation of NK cells, NK cell activation required the presence of HIV-1 gp120-coated cells and anti-HIV-1 Abs. Indeed, assessments of the different assay conditions using NK cells from three independent donors against primary T cells from target cell donor 4 revealed that Ab-dependent activation of NK cells to produce IFN-γ or express CD107a was most robust when the NK cells in the whole blood were stimulated with gp120-coated (5 μg/1 × 10⁶) primary T cells in the presence of HIV-1-infected plasma (Fig. 2B).

Following the demonstration that the modified allogeneic target Ab-dependent NK cell activation assay was capable of detecting anti-HIV-1 Ab-dependent NK cell activation, we proceeded to optimize the assay for detection of responses. First, we assessed the impact that the number of target cells used had on the NK cell activation readout. Whole blood from three donors was independently combined with 3.0 × 10⁵, 1.0 × 10⁵, or 5.0 × 10⁴ gp120-coated (5 μg/1 × 10⁶) primary T-cell targets from target cell donor 3. As depicted in Fig. 2C, the percentage of NK cells activated to produce IFN-γ was most robust when the NK cells in the whole blood were stimulated with gp120-coated (5 μg/1 × 10⁶) primary T cells in the presence of HIV-1-infected plasma (Fig. 2B).
produce IFN-γ or express CD107a was proportional to the number of target cells that were added to the assay, with the highest percentage of activated NK cells being observed across all three donors when \(3 \times 10^5\) gp120-coated target cells were added. Lastly, we assessed the impact of the amount of gp120 used to coat the primary T-cell targets on the Ab-dependent NK cell activation observed. Primary T cells from donor 4 were coated with 5.0, 2.5, 1.3, 0.6, 0.3, or 0.16 μg of gp120 per 1.0 \(\times 10^6\) cells. We then independently combined 3.0 \(\times 10^6\) of each set of coated cells with whole blood from three donors for the anti-HIV-1 Ab-dependent stimulation. Given the results depicted in Fig. 2C and D, the remaining experiments presented in this report, except when stated otherwise, were conducted by stimulating whole blood with 3.0 \(\times 10^6\) primary T-cell targets coated with 5 μg of gp120 per 1.0 \(\times 10^6\) cells per stimulation condition.

Collectively, the results presented in Fig. 1 and 2 highlight the ability to measure anti-HIV-1 Ab-dependent activation of NK cells by an allogeneic T-cell line and primary allogeneic T cells. The capability to derive and utilize primary T-cell targets from different donors makes this assay ideal for answering questions about the potential of different target cells to influence Ab-dependent NK cell activation, as well as the factors regulating these responses.

Anti-HIV-1 Ab-dependent responses of educated NK cells against allogeneic T cells. Several research groups, including ours, have previously evaluated the impact of NK cell education on the capacity of NK cells to mediate Ab-dependent activation against anti-HIV-1 and non-HIV-1 Ab-coated targets (15, 17–19). NK cells educated through the interaction of inhibitory KIR and self-HLA-I are primed to mediate more robust Ab-dependent effector functions. There remains controversy, however, regarding whether the presence, on the target cell, of the HLA-I ligand of the educating KIR can lead to inhibition of the educated cells, rendering the noneducated NK cell population more functional against certain targets. Indeed, noneducated NK cells were recently shown to be more functional than educated NK cells in an ADCC study that implemented a therapeutic monoclonal Ab for in vitro assays against neuroblastoma cell lines expressing HLA-I ligands for educating KIR (19). Meanwhile, other groups have presented evidence that Ab-dependent activation of NK cells can overcome the inhibitory influence of the presence of HLA-I ligands for self-KIR (17, 35). Indeed, our group has previously shown that educated KIR3DL1+ NK cells overcome the presence of their HLA-Bw4 ligand to be the most responsive subset to anti-HIV-1 Ab-dependent activation against autologous target cells (17). Given this previous observation, we investigated whether the KIR3DL1+ NK cells from HLA-Bw4 carriers were more responsive than the KIR3DL1− subset, which contains noneducated NK cells and NK cells educated by other HLA/KIR combinations, against the CEM.NKr-CCR5 cell line and allogeneic primary T cells that express or lack HLA-Bw4.

To evaluate whether the educated KIR3DL1+ NK cell subset was more functional than the KIR3DL1− subset, we first measured the percentage of IFN-γ-producing or CD107a-expressing NK cells in each subset after stimulation with gp120-coated (5 μg/1 \(\times 10^6\)) CEM.NKr-CCR5 targets in the presence of HIV-1-infected plasma or A32 monoclonal Ab. Figure 3A shows the gating procedure utilized to identify KIR3DL1+/− NK cell subsets and assess their functionality. As depicted in Fig. 3B, educated KIR3DL1+ NK cells from six HLA-Bw4+ carrying donors exhibited higher levels of NK cell IFN-γ production (48.2% [12.2 to 54.9%] versus 28.6% [5.9 to 37.7%], \(P = 0.03\), Wilcoxon matched-pairs test) and CD107a expression (60.0% [25.9 to 66.4%] versus 46.9% [15.3 to 53.5%], \(P = 0.03\), Wilcoxon matched-pairs test) than the KIR3DL1− NK cell subset, which contains noneducated NK cells and those educated through other KIR/HLA combinations, when stimulated with coated CEM.NKr-CCR5 targets in the presence of HIV-1-infected plasma. Similarly, stimulation of PBMC with CEM.NKr-CCR5 targets coated with both gp120 and A32 resulted in a preferential activation of educated KIR3DL1+ NK cells, compared to KIR3DL1− NK cells, to produce IFN-γ (4.4% [1.6 to 24.6%] versus 1.7% [0.1 to 8.8%], \(P = 0.03\), Wilcoxon matched-pairs test) and express CD107a (4.8% [3.4 to 21.4%] versus 2.9% [1.0 to 11.3%], \(P = 0.03\), Wilcoxon matched-pairs test) (Fig. 3C).

Given that the previously reported activation advantage of KIR3DL1 educated NK cells for Ab-dependent stimulation was recaptured in the activation assay utilizing the CEM.NKr-CCR5 cell line, we next conducted assays utilizing different combinations of nine HLA-Bw4+ NK cell donors independently stimulated with anti-HIV-1 Ab-coated primary T-cell targets from two HLA-Bw4+ and two HLA-Bw4− donors. Appraisal of these responses revealed that higher percentages of the educated KIR3DL1+ NK cell subset produced IFN-γ and expressed CD107a than the KIR3DL1− subset across all four target cells tested (Fig. 3D). Wilcoxon matched-pairs tests demonstrated the higher percentages of IFN-γ-producing or CD107a-expressing cells to be statistically significant against target cell donors 1, 3, and 4, while statistical significance was approached against target cell donor 2 (Table 3). We obtained further evidence of a role for NK cell education in the activation of KIR3DL1+ NK cells by assessing the activation of KIR3DL1+ NK cells from three HLA-Bw4+ whole blood donors against target cells from donor 3. Indeed, we observed a 3-fold-higher median IFN-γ production in KIR3DL1+ NK cells from HLA-Bw4+ donors than in KIR3DL1− NK cells from HLA-Bw4− donors (20.6% versus 6.5%). These results corroborate previously published data demonstrating higher Ab-dependent stimulation in educated NK cells (15, 17–19). Additionally, these data suggest that KIR3DL1+ NK cells can at least partially overcome inhibition in the presence of its ligand through Ab-dependent stimulation.

Anti-HIV-1 Ab-dependent cytolysis of allogeneic target cells. The data presented thus far demonstrate that allogeneic T-cell target cells can activate NK cells. Although the noncytolytic activation of NK cells can inhibit HIV-1 replication (36), cytolytic activity might be necessary for elimination of infected allogeneic Trojan horse lymphocytes upon exposure. As such, we next evaluated the anti-HIV-1 ADCC cytolytic activity directed toward both the CEM.NKr-CCR5 cell line and primary CD4+ T cells.

Using an LDH release cytotoxicity assay that measures the release of LDH from lysed cells, we observed robust anti-HIV-1 Ab-dependent lysis of gp120-coated CEM.NKr-CCR5 cells by freshly isolated PBMC (Fig. 4A). Both polyclonal HIVIG and the A32 monoclonal Ab triggered anti-HIV-1 ADCC, and no cytolytic activity was observed when IVIG (pooled from HIV-1-negative donors)
FIG 3 Influence of NK cell education through KIR3DL1 on anti-HIV-1 Ab-dependent activation against the allogeneic CEM.NK-R-CCR5 cell line and allogeneic primary T cells coated with gp120. (A to C) PBMC effectors were cultured with CEM.NK-R-CCR5 target cells coated or not coated with gp120 (5 μg/ml × 10^6 cells) at a 10:1 ratio in the presence of plasma from an HIV-1-infected donor or the A32 monoclonal Ab. After incubation, the relative abilities of KIR3DL1^+ and KIR3DL1^− NK cells to become activated to produce IFN-γ and/or express CD107a was assessed by flow cytometry. (A) The FACS plot depicts representative gating upon KIR3DL1^+ and KIR3DL1^− NK cells. (B) Graphs portray the relative total IFN-γ production (left) and CD107a expression (right) of KIR3DL1^+ and KIR3DL1^− NK cells stimulated with gp120-coated CEM.NK-R-CCR5 target cells in the presence of plasma from an HIV-1-infected donor. Depicted values were obtained after subtraction of background stimulation of NK cells following incubation with uncoated CEM.NK-R-CCR5 targets in the presence of plasma from an HIV-1-uninfected donor. (C) Graphs depict the relative total IFN-γ production (left) and CD107a expression (right) of KIR3DL1^+ and KIR3DL1^− NK cells stimulated with gp120-coated CEM.NK-R-CCR5 target cells in the presence of the A32 monoclonal Ab. Depicted values were obtained after subtraction of background stimulation of NK cells following incubation with uncoated CEM.NK-R-CCR5 targets in the presence of the A32 monoclonal Ab. (D) NK cells from a series of donors were stimulated for anti-HIV-1 Ab-dependent activation, as described in Fig. 2, against primary allogeneic T cells from four donors. The relative total IFN-γ production (top) and CD107a expression (bottom) of KIR3DL1^+ and KIR3DL1^− NK cells are illustrated in the graphs. The effector cell donors assessed for activation against each of the target cell donors are listed in the legend on the right side of the top graphs.
TABLE 3 Activation of KIR3DL1⁺ and KIR3DL1⁻ NK cells against a series of allogeneic primary T-cell target cells

<table>
<thead>
<tr>
<th>Target donor ID (activation marker)</th>
<th>Median % (range) of NK cell activation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KIR3DL1⁺</td>
<td>KIR3DL1⁻</td>
</tr>
<tr>
<td>1 (IFN-γ)</td>
<td>21.5 (4.3–28.4)</td>
<td>9.8 (2.6–14.6)</td>
</tr>
<tr>
<td>1 (CD107a)</td>
<td>21.2 (1.5–28.8)</td>
<td>7.4 (1.6–11.5)</td>
</tr>
<tr>
<td>2 (IFN-γ)</td>
<td>27.6 (6.8–39.5)</td>
<td>16.3 (5.8–18.4)</td>
</tr>
<tr>
<td>2 (CD107a)</td>
<td>21.9 (1.9–28.7)</td>
<td>10.3 (0.9–13.3)</td>
</tr>
<tr>
<td>3 (IFN-γ)</td>
<td>20.6 (3.4–38)</td>
<td>9.2 (2.1–15.4)</td>
</tr>
<tr>
<td>3 (CD107a)</td>
<td>27.4 (1.9–40.7)</td>
<td>11.6 (1.0–12.9)</td>
</tr>
<tr>
<td>4 (IFN-γ)</td>
<td>19.6 (5.6–51.6)</td>
<td>6.9 (2.6–22.1)</td>
</tr>
<tr>
<td>4 (CD107a)</td>
<td>18.6 (1.8–46.6)</td>
<td>5.5 (0.4–16.8)</td>
</tr>
</tbody>
</table>

was utilized as an Ab source (Fig. 4A to C). As the A32 monoclonal Ab triggered cytolysis of the CEM.Nkr-CCR5 transformed targets, we utilized the flow cytometry-based cytotoxicity assay to assess A32-mediated ADCC of allogeneic primary T cells. We utilized this assay, which has been reported on in detail in two recent studies (29, 30), to measure A32 ADCC mediated by PBMC against autologous primary CD4⁺ T cells that were infected with either wild-type HIV-1 or Vpu- and Nef-defective HIV-1, as well as autologous targets coated with amounts of gp120 reflective of the level of gp120 observed on cells infected with Vpu- and Nef-defective HIV-1 (Fig. 4D). We demonstrated that A32-mediated
ADCC against autologous target cells is observed only against target cells infected with Vpu- and Nef-defective HIV-1 or targets coated with similar amounts of gp120 (Fig. 4E). This phenomenon is related to the necessity for interactions between CD4 and gp120, which are abrogated by the downregulation of CD4 by Vpu and Nef and by the Vpu-mediated BST-2 antagonism, for A32 binding to gp120 (30). As the amount of gp120 on the surface of infected autologous cells was sufficient to trigger ADCC, we next assessed if this amount of gp120 would be sufficient to observe ADCC of primary allogeneic target cells. Indeed, we observed A32-mediated ADCC of allogeneic T-cell target cells using PBMC effector cells from four independent donors (Fig. 4E). Lastly, we coated primary CD3+ T cells with the 100-ng/ml concentration of gp120, which obtained levels of gp120 on coated CD4+ primary T cells similar to those observed on CD4+ T cells infected with Vpu- and Nef-defective virus (Fig. 4D) and utilized them in the allogeneic whole blood anti-HIV-1 Ab-dependent NK cell activation assay. As depicted by the example in Fig. S1 in the supplemental material, educated KIR3DL1+ NK cells from two independent donors still exhibited higher frequencies of activated cells than KIR3DL1− NK cells against HLA-Bw4+ target cells. This observation suggests KIR3DL1+ NK cells can at least partially overcome inhibition to exhibit anti-HIV-1 Ab-dependent activation, even against target cells presenting levels of gp120 similar to those found on cells infected with Vpu- and Nef-defective virus. Overall, these data suggest that primary allogeneic Trojan horse lymphocytes should be targetable by ADCC.

**DISCUSSION**

The Trojan horse hypothesis states that HIV-1-infected lymphocytes within the genital secretions of HIV-1-infected individuals can transport, protect, and transmit virus to noninfected sexual partners (8). Much evidence has been provided for this hypothesis in animal studies, as well as through assessments of human transmission partners (8–11, 13). Prophylactic human vaccines may need to prevent the transmission of HIV-1-infected allogeneic lymphocytes, as well as free virions. Although some broadly neutralizing Abs, such as PGT121, efficiently block cell-to-cell transmission of HIV-1, other HIV-1-specific neutralizing antibodies fail to efficiently do so (37). One potential vaccine-inducible effector mechanism for eliminating HIV-1-infected allogeneic lymphocytes is ADCC. This effector function was linked to the partial protection conferred by the RV144 vaccine regimen and is associated with the protection provided to exposed uninfected infants through breast milk Abs (5, 38). Given the potential utility of anti-HIV-1 ADCC responses against allogeneic primary T cells, we measured anti-HIV-1 Ab-dependent NK cell activation against allogeneic primary T-cell targets. As reported here, HIV-1-specific Abs can recognize antigen on both allogeneic primary T cells and T-cell lines and robustly activate peripheral blood NK cells and trigger the cytolyis of target cells. Induction of mucosal HIV-1-specific Abs, with the potential to activate NK cells, might therefore provide a line of defense against transmission of HIV-1 from infected cells.

The allogeneic cell Ab-dependent activation assay allows for interchangeable combinations of effector and target cell donors to be assessed. We capitalized on this feature to demonstrate that NK cells educated through KIR3DL1 exhibited more robust anti-HIV-1 Ab-dependent activation against allogeneic targets, regardless of whether those targets express HLA-Bw4 or not. These results suggest that stimulation of NK cells for Ab-dependent responses can at least partially overcome inhibitory signals mediated through combinations of inhibitory KIR and HLA-I. Although we have hypothesized that the ontological process of education is the driving factor behind the higher anti-HIV-1 Ab-dependent activation observed in the KIR3DL1+ NK cell subset, it remains possible that other factors could be driving this NK cell subset to be more robustly activated upon Ab-dependent stimulation. For example, CD56bright NK cells, which mediate poor ADCC, lack KIR expression. Inclusion of these cells within the NK cell gate could generate a biased observation of higher antibody-dependent NK cell activation within the KIR+ NK cell subset. This is unlikely the case with the presented data, as reanalysis with extremely tight gates to ensure removal of CD56bright NK cells still revealed higher antibody-dependent NK cell activation in the CD56dimKIR3DL1+ than in the CD56brightKIR3DL1− NK cell subset (Data not shown). Another potential confounding variable for understanding the differences in antibody-dependent NK cell activation between KIR3DL1+ and KIR3DL1− NK cells in HLA-Bw4+ donors is the level of CD16 expression on each subset. Indeed, we have observed a general trend toward higher CD16 expression on KIR3DL1+ NK cells than on KIR3DL1− NK cells (C. C. Tang, G. Isitman, J. Bruneau, C. Tremblay, N. F. Bernard, S. J. Kent, and M. S. Parsons, unpublished data). However, the preferential Ab-dependent activation of KIR3DL1+ NK cells is unlikely explained by this observation, as preferential activation of KIR3DL1+ NK cells is observed even in individuals with similar CD16 expression on their KIR3DL1+ and KIR3DL1− NK cell subsets (39). Other potential variables that could contribute to the preferential Ab-dependent activation of KIR3DL1+ NK cells are factors within plasma other than IgG. Indeed, we have previously demonstrated that non-IgG factors within plasma can alter Ab-dependent NK cell activation profiles (40). Although it is possible that such factors could lead to the preferential activation of KIR3DL1+ NK cells, this seems unlikely given the data demonstrating preferential activation of KIR3DL1+ NK cells against gp120-coated CEM.NKr-CCR5 target cells in the presence of A32 monoclonal Ab (Fig. 3C).

The observation that KIR3DL1+ NK cells retain the capacity to mediate Ab-dependent responses against HLA-Bw4+ target cells is both consistent and in contrast to previously published research. Consistent with the reported observation is a study by Lang et al. that demonstrated NK cells were able to overcome inhibition through HLA-I/KIR interactions to mediate ADCC against anti-CD19-coated leukemic blasts (35). Similarly, we have previously reported that KIR3DL1+ NK cells are more activated than KIR3DL1− NK cells when stimulated for anti-HIV-1 Ab-dependent responses with autologous HIV-1-infected T cells (21). One of the reasons for the different results obtained in the current study and those published by Ward et al. could be the Abs utilized to trigger ADCC. We provide data that demonstrate NK cell activation and ADCC are induced by plasma from HIV-1-infected donors or the A32 monoclonal Ab, whereas Ward et al. attempted to trigger ADCC using a cocktail of monoclonal Abs (i.e., b12, F105, 17b, and 654-30D). However, monoclonal Abs alone or in cocktails are poor inducers of ADCC in in vitro assays and are outperformed by polyclonal IgG purified from patient
sera (41). Furthermore, all monoclonal Abs are not equal in terms of their ability to trigger ADCC. Indeed, the A32 Ab has been shown to mediate superior in vitro ADCC compared to that of two of the monoclonal Abs implemented in the Ward et al. cocktail (21, 42).

A second reason that the results of the current study may differ is due to the effects of the viral Nef protein, which can downregulate CD4, HLA-A, and HLA-B molecules from the surfaces of infected cells (22, 43, 44). Ward et al. attempted to measure ADCC against targets infected with Nef-competent HIV-1 (21), whereas we observed NK activation and ADCC only against cells coated with gp120 or infected with Vpu- and Nef-defective virus. It is unlikely that the ability of Nef to alter HLA-A and HLA-B expression would impede NK cell Ab-dependent activation. Indeed, we provide data illustrating that KIR3DL1+ NK cells exhibit activation against HLA-Bw4+ target cells coated with levels of gp120 similar to those on Vpu- and Nef-defective HIV-1-infected cells. These targets express higher levels of HLA-Bw4 containing HLA-I molecules than would be present on targets infected with HIV-1 containing Nef. In fact, the downregulation of HLA-A and HLA-B by Nef would most likely decrease any inhibitory potential these ligands are exerting on anti-HIV-1 Ab-dependent activation. The role of Vpu and Nef in downregulating CD4 is most likely contributing to differences between our study and that of Ward et al. (21). Indeed, the gp120 on coated allogeneic T cells and T cells infected with Vpu- and Nef-defective viruses is able to obtain CD4-bound conformations, which reveal ADCC epitopes (30). As such, the Abs implemented in our system were able to bind their epitopes and efficiently activate NK cells and trigger ADCC. Indeed, the observations that polyclonal Abs were sufficient to trigger NK cell activation against targets coated with levels of gp120 similar to those on Vpu- and Nef-defective HIV-1-infected cells and that A32 was sufficient to trigger ADCC of targets coated with these levels of gp120 might be promising for vaccine design. Future research might be able to identify Abs capable of binding to native HIV-1 envelope trimers that elicit ADCC levels comparable to A32. As T cells infected with Vpu- and Nef-competent viruses express even lower levels of surface viral envelope than cells infected with Vpu- and Nef-deficient HIV-1, utilization of ADCC-competent native trimer binding Abs will resolve issues regarding the susceptibility of cells infected with Vpu- and Nef-competent HIV-1 to ADCC. Such Abs may serve as ideal targets to induce by vaccination to target Trojan horse lymphocytes for elimination. While initiatives to elucidate native trimer binding ADCC-competent Abs are important, it should be remembered that the RV144 trial elicited A32-like ADCC-competent Abs that recognize CD4-induced envelope epitopes, and these responses might have played a role in protection (5, 30, 45). It is possible that Trojan horse lymphocytes or autologous lymphocytes bearing transmitter/founder viruses might exhibit incomplete CD4 downregulation, making these cells susceptible to ADCC by Abs with CD4-induced specificities. Indeed, antibodies against CD4-induced epitopes appear to be important contributors to anti-HIV-1 ADCC responses, as allogeneic CEM.NKr-CCR5 target cells pulsed with gp120 or infected with Nef- and Vpu-defective virus are targeted for ADCC by antibodies directed against CD4-induced epitopes (29). Furthermore, we have recently observed that preabsorption of anti-HIV-1 antibodies from HIV-1-infected plasma samples with soluble gp120 lacking the V1V2V3 and V5 regions abrogates almost all anti-HIV-1 ADCC activity, suggesting that these epitopes do not include the HIV-1 variable loops (46).

In summary, we provide data measuring anti-HIV-1 Ab-dependent NK cell activation and cytolysis of allogeneic T-cell targets. Importantly, our data demonstrate that NK cells expressing inhibitory KIR3DL1 can overcome inhibition to exhibit robust anti-HIV-1 Ab-dependent activation against allogeneic primary T-cell targets expressing HLA-Bw4. Future research will be essential to understand the factors necessary for NK cells to overcome inhibitory signaling and mediate robust functionality against target cells expressing HLA-I ligands for inhibitory NK cell receptors. The results of this research will be helpful for designing vaccines and immunotherapies that utilize Ab-dependent NK cell responses.

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We have no conflicts of interest to declare.

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