AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 32, Number 10/11, 2016

DOI: 10.1089/aid.2016.0099

NKG2D Acts as a Co-Receptor for Natural Killer Cell-Mediated Anti-HIV-1 Antibody-Dependent Cellular Cytotoxicity

Matthew S. Parsons,[1] Jonathan Richard,[2] Wen Shi Lee,[1] Hillary Vanderven,[1] Michael D. Grant,[3] Andrés Finzi,[2] and Stephen J. Kent[1,4,5]

Abstract

The utility of antibody-dependent cellular cytotoxicity (ADCC) for eliminating HIV-1-infected cells is of much interest for the design of both prophylactic vaccines for HIV-1 prevention and therapeutics to eliminate latently infected cells following reactivation. Significant research has been conducted to understand the antibody specificities involved in anti-HIV-1 ADCC responses. Perhaps equally important as the identity of the antibodies mediating these responses are factors regulating the ability of ADCC effector cells, in particular, natural killer (NK) cells, to respond to antibody-coated target cells. Indeed, a plethora of activating and inhibitory receptors expressed on the surface of NK cells might act in conjunction with CD16 to influence ADCC. As the expression of NKG2D and its ligands has been linked to HIV-1 disease progression, we evaluated if signals through NKG2D were involved in anti-HIV-1 ADCC. Utilizing assays measuring cytolysis, we provide the first data implicating NKG2D in antibody-dependent NK cell responses against a target cell line either pulsed with gp120 or infected with HIV-1. These observations are highly significant for understanding antibody-dependent NK cell responses against HIV-1 and might be useful for optimizing prophylactics and therapeutics aiming to utilize antibodies and optimally functional NK cells to control HIV-1.

Keywords: NK cells, ADCC, NKG2D

Introduction

A prophylactic vaccine is needed to slow the global burden of new HIV-1 infections. Traditional attempts to design such a vaccine have focused heavily on the induction of cytotoxic T lymphocytes (CTL) and/or broadly neutralizing antibodies (BnAbs). Given that clinical trials of CTL-based vaccines have failed and that current vaccine protocols are unable to elicit BnAbs, novel vaccine concepts are required.[1–3] The RV144 vaccine trial in Thailand represented the first HIV-1 vaccine to offer detectable protection against HIV-1 acquisition, providing a modest 31% efficacy.[4] Post-vaccination analyses revealed that the vaccine did not induce BnAbs or robust CTL responses; instead the major correlate of protection was IgG capable of binding the V1V2 region of the HIV-1 envelope.[4–6] Further analyses revealed an association between protection and the ability of IgG to mediate antibody-dependent cellular cytotoxicity (ADCC).[5] This putative protective role for ADCC, however, was only observed when individuals exhibited low plasma levels of anti-viral IgA, which can compete with IgG for epitope binding and block ADCC.[5,7] The postvaccination analyses of the RV144 trial collectively suggest that non-neutralizing antibody-dependent effector functions, such as ADCC, might represent a novel avenue for HIV-1 vaccine research.

Designing vaccines to utilize ADCC requires not only an understanding of the characteristics of ideal antibody responses but also an understanding of the effector cells that exploit induced antibodies to mediate ADCC. Natural killer (NK) cells and monocytes are the major mediators of anti-HIV-1 ADCC, but the response can also be mediated to a lesser degree by neutrophils.[8] The role of NK cells is particularly interesting, as these cells express a plethora of activating and inhibitory receptors, in addition to the activating

[1]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Australia.
[2]Department of Microbiology, Infectiology and Immunology, Centre de Recherche du CHUM, Université de Montreal, Montreal, Canada.
[3]Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Canada.
[4]Melbourne Sexual Health Centre, Central Clinical School, Monash University, Melbourne, Australia.
[5]ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, University of Melbourne, Parkville, Australia.

CD16/FcγRIIIa receptor for the IgG constant region.[9] These additional activating and inhibitory receptors can act in conjunction with CD16 to influence the ability of the NK cell to mediate antibody-dependent functions. Indeed, ligation of inhibitory killer immunoglobulin-like receptors by their major histocompatibility complex class I (MHC-I or HLA-I) ligands can inhibit ADCC triggered by anti-HIV-1 and therapeutic monoclonal antibodies.[10,11] Others and we, however, have reported that this inhibition can be at least partially overcome upon antibody-dependent NK cell activation.[10,12–14] In addition to evidence implicating signals from inhibitory receptors, antibody-mediated NK cell receptor crosslinking data have recently been published to suggest that ligation of certain activating receptors, including NKG2D and 2B4, can synergize with CD16 ligation to enhance NK cell calcium flux.[15]

The putative role of NKG2D in enhancing CD16-mediated NK cell responses is of particular interest in the context of antibody-dependent anti-HIV-1 NK cell responses. Ligands of the NKG2D receptor are upregulated on HIV-1-infected cells by the viral Vpr protein, and NKG2D signaling has been implicated in the direct cytolysis of HIV-1-infected target cells by NK cells.[16–18] Furthermore, heightened levels of NKG2D expression on NK cells from HIV-1-infected individuals are associated with lower viral load set point and lower viral loads in long-term nonprogressors.[19] Further studies have revealed that HIV-1 may be able to evade NKG2D-mediated NK cell responses through Nef-mediated downregulation of NKG2D ligands or through Vpu-mediated downregulation of the NTB-A coactivation ligand.[20,21] In addition, matrix-metalloproteinases (MMPs) produced in HIV-1-infected cells are able to cleave NKG2D ligands from the surface of infected cells, which results in the downregulation of NK cell NKG2D on binding of soluble receptors.[22,23]

Collectively, much evidence implicates NKG2D in NK cell recognition of HIV-1-infected target cells and the disease progression status of HIV-1-infected individuals. The contribution of NKG2D to NK cell-mediated anti-HIV-1 antibody-dependent activation and/or cytolysis has not yet been determined. We hypothesized that a collaborative relationship between NKG2D and CD16 could exist that determines the potency of anti-HIV-1 antibody-dependent NK cell responses. This could be instrumental in understanding HIV-1 pathogenesis and instructive for designing an HIV-1 vaccine and/or therapeutic utilizing anti-HIV-1 ADCC. As such, we assessed the contribution of NKG2D to anti-HIV-1 envelope glycoprotein (Env) ADCC. The presented data suggest NKG2D collaborates with CD16 to enhance NK cell-mediated antibody-dependent responses against HIV-1 gp120-coated and HIV-1-infected T-cell line targets.

Materials and Methods

Subjects

Blood was obtained from 22 HIV-1-uninfected donors by forearm venipuncture into vacuettes containing sodium heparin anticoagulant. Peripheral blood mononuclear cells (PBMC) were enriched from blood via the Ficoll–Hypaque density gradient. These PBMC were used as effector cells in cytolysis assays or to enrich primary NK effector cells for some renditions of the lactate dehydrogenase (LDH) release assay. All donors provided informed consent before sample collection, and all participating institutions approved the described studies.

Anti-HIV-1 antibodies and cell lines

The CEM.NKr-CCR5 cell line (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) was used as target cells in the anti-HIV-1 ADCC LDH release and flow cytometry-based cytolysis assays. The A32 monoclonal antibody and the polyclonal HIV immunoglobulin (HIVIG) (both from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were used to induce anti-HIV-1 ADCC in the flow cytometry-based and LDH release cytolysis assays, respectively.

Enrichment of primary NK cells from PBMC

Enrichment of NK cells was performed, according to the manufacturer's protocol, using an NK cell enrichment kit (StemCell Technologies). Purity of enriched NK cells was assessed by flow cytometry. Pre- and post-enrichment cell populations were stained with anti-CD3 (Clone: SK7; Per-CP conjugated; BD) and anti-CD56 (Clone: NCAM 16.2; PE-Cy7 conjugated; BD) antibodies. After staining, cells were fixed with 1% formaldehyde and acquired using an LSR Fortessa flow cytometer (BD). Data were analyzed with FlowJo software (Treestar).

Lactate dehydrogenase release cytolysis assay

The LDH release cytolysis assay for ADCC detection was performed as previously described.[12] To measure anti-HIV-1 ADCC by LDH release, we utilized the Cytotox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega). The CEM.NKr-CCR5 target cells were prepared by pulsing with HIV-1_{Bal} gp120 (3 μg/ml) (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) for 90 min at 4°C. Effector cells consisted of freshly isolated PBMC or enriched NK cells. All assay conditions were conducted in triplicate in 96-well round-bottomed plates (Greiner Bio-One). The maximum LDH release of target cells was determined by the addition of lysis solution (Promega) to wells containing only target cells for the last 45 min of the assay. The spontaneous release of LDH from both targets and effectors was assessed using wells containing either cell population alone. For experimental and control conditions, effector and target cells were combined at 25:1 (5×10^5 effectors $+2\times10^4$ targets) and 10:1 (2×10^5 effectors $+2\times10^4$ targets) ratios. The experimental conditions measured the LDH released on coincubation of PBMC, or enriched NK cells, with gp120-pulsed CEM.NKr-CCR5 and a 1:2,000 dilution of HIVIG. Control conditions measured LDH released on incubations of PBMC and gp120-pulsed CEM.NKr-CCR5 cells in the absence of antibody. To assess the role of NKG2D in the anti-HIV-1 ADCC observed, additional experimental conditions were conducted containing 5 μg/ml of an anti-NKG2D antibody capable of blocking NK cell activation through NKG2D (Clone: 1D11; BioLegend), or 5 μg/ml of an isotype control antibody (Clone: MOPC-21; BioLegend).[24] A series of additional control wells containing media only were also set up. Next, plates were spun for 4 min at $250\times g$ and incubated for 4 h at 37°C. After 3 h and 15 min, the lysis solution was added to the maximum target release wells and plates were

incubated for a further 45 min at 37°C. After the complete 4-h incubation, plates were spun for 4 min at $250 \times g$. Next, fifty microliters of supernatant was removed from each well and transferred to an ELISA plate. Following transfer of supernatants to the ELISA plates, 50 μl of substrate (Promega) was added to each well containing a supernatant and incubated for 30 min at room temperature in the dark. Finally, 50 μl of stop solution was added to each well and absorbance was read at 492 nm. The optical density values for wells containing media only were subtracted from all other wells. The remaining values were used to calculate the percent cytotoxicity using the following formula: % cytotoxicity = [(experimental − effector spontaneous − target spontaneous)/(target maximum − target spontaneous)] × 100.

HIV-1-infection of CEM.NKr-CCR5 cells

CEM.NKr-CCR5 cells were infected with vesicular stomatitis virus G (VSVG)-pseudotyped NL4.3 green fluorescent protein (GFP) expressing ADA Env-based viruses that carried wild-type or defective *Vpu* and *Nef* genes (U-N-). Infection was accomplished by spin inoculation at $800 \times g$ for 1 h in 96-well plates at 25°C. After a 48-h period postinfection, these cells were utilized as target cells in the flow cytometry-based cytolysis assay and for detection of NKG2D ligand expression. An average of 11.8% and 10.7% of cells were infected with the wild-type and U-N- viruses, respectively.

Detection of NKG2D ligand expression on CEM.NKr-CCR5 cells

To assess the expression of NKG2D ligands on HIV-1-uninfected or HIV-1-infected CEM.NKr-CCR5 cells, we treated cells with a NKG2D-Fc fusion protein (R&D Systems) and detected binding with an Alexa-Fluor-647-conjugated goat anti-human IgG antibody (Invitrogen). Data were collected on an LSR II flow cytometer (BD) and analyzed on FlowJo software (Treestar).

Flow cytometry-based cytolysis assay

The flow cytometry-based ADCC assay was conducted as previously described.[25,26] Briefly, CEM.NKr-CCR5 targets infected with wild type or U-N- HIV-1 were stained with the AquaVivid viability (Invitrogen) and cellular marker eFluor670 (eBiosciences) dyes. The PBMC effectors were stained with the cellular marker eFluor 450 dye (eBiosciences). Next, effectors and targets were combined at a 10:1 ratio in V-bottomed 96-well plates (Corning) in the presence or absence of the A32 anti-HIV-1 monoclonal antibody (5 μg/ml). To assess the role of NKG2D in the A32-mediated ADCC of HIV-1-infected $CD4^+$ T cells, conditions were conducted in which an anti-NKG2D antibody (10 μg/ml, Clone: 1D11; BioLegend) or an isotype control antibody (Clone: MOPC-21; BioLegend) was added. These combinations of effectors, targets, and antibodies were incubated at room temperature for 15 min, which was followed by a 1-min spin at $300 \times g$ and a 6-h incubation at 37°C. After the incubation, cells were fixed with a 2% formaldehyde solution containing flow cytometry particles (5×10^4/ml) (AccuCount Blank Particles, 5.3 μm; Spherotech). An LSRII flow cytometer was utilized to collect samples, which were capped at a constant number of particles (1×10^3) to normalize the number of viable HIV-1-infected (GFP^+) target cells. Data were analyzed using FlowJo software (Treestar). Percent cytolysis was calculated with the following formula: %ADCC = [(number of viable target cells in Effectors + Targets) − (number of viable target cells in Effectors + Targets + A32)]/[number of viable target cells in Effectors + Targets] × 100.

Statistics

The significance of differences between paired and unpaired data sets was analyzed with the Wilcoxon matched-pairs signed rank and Mann–Whitney tests, respectively. The significance of differences between three paired data sets was analyzed using the Friedman test, followed by Dunn's *post hoc* tests. Statistical analyses were performed using GraphPad Prism Version 4.0 software. Data throughout the article are presented in the following format: [Median (Range) vs. Median (Range), *p*-value of statistical test].

Results

Role of NKG2D in HIV-1-specific ADCC of gp120-pulsed CEM.NKr-CCR5 target cells

To assess the role of NKG2D in anti-HIV-1 ADCC, we investigated if ligation of NKG2D was involved in the ADCC of gp120-pulsed CEM.NKr-CCR5 target cells. To measure anti-HIV-1 ADCC, we implemented the LDH release cytolysis assay, as previously described.[12] The LDH release assay readily detects anti-HIV-1 ADCC of gp120-pulsed CEM.NKr-CCR5 target cells by effector cells within PBMC in the presence of HIVIG (Fig. 1). To assess the contribution of NKG2D to anti-HIV-1 ADCC, we implemented conditions containing anti-NKG2D blocking or isotype control antibodies in addition to PBMC effectors, gp120-pulsed CEM.NKr-CCR5 target cells, and HIVIG. As depicted in Figure 1, at both 25:1 and 10:1 effector to target cell ratios, the anti-NKG2D antibody reduced observed ADCC [25:1—29.2% (9.7%–55.1%); 10:1—16.3% (6.0%–20.1%)] compared to that observed in the presence of the isotype control antibody [25:1—39.7% (22.3%–65.1%) $p < .05$; 10:1—23.5% (12.9%–37.3%) $p < .05$] or no murine antibody [25:1—43.6% (21.5%–71.2%) $p < .05$; 10:1—24.2% (10.5%–44.2%) $p < .05$] (Friedman's test with Dunn's post hoc tests). No difference in ADCC was observed between conditions containing the isotype control antibody or no murine antibody ($p > .05$). These data imply that ligation of NKG2D contributes to the anti-HIV-1 ADCC of gp120-pulsed CEM.NKr-CCR5 target cells, as the background lysis of CEM.NKr-CCR5 targets in the absence of HIVIG (25:1—median: 2.6%; 10:1—median: 0.5%) was too small to account for the observed effect of NKG2D blocking.

Role of NKG2D in anti-HIV-1 ADCC of gp120-pulsed CEM.NKr-CCR5 target cells by enriched primary NK cells

To confirm that antibody-mediated blockade of NKG2D was directly inhibiting NK cells, we next enriched NK cells from the PBMC of seven donors. Before enrichment, NK cells comprised 13.8% (7.3%–21.3%) of lymphocytes within PBMC. After enrichment, NK cells comprised 92.1% (82.9%–97.6%) of lymphocytes. Figure 2A depicts a purity check performed by flow cytometry. Following enrichment, NK cells were used as effector cells against gp120-pulsed

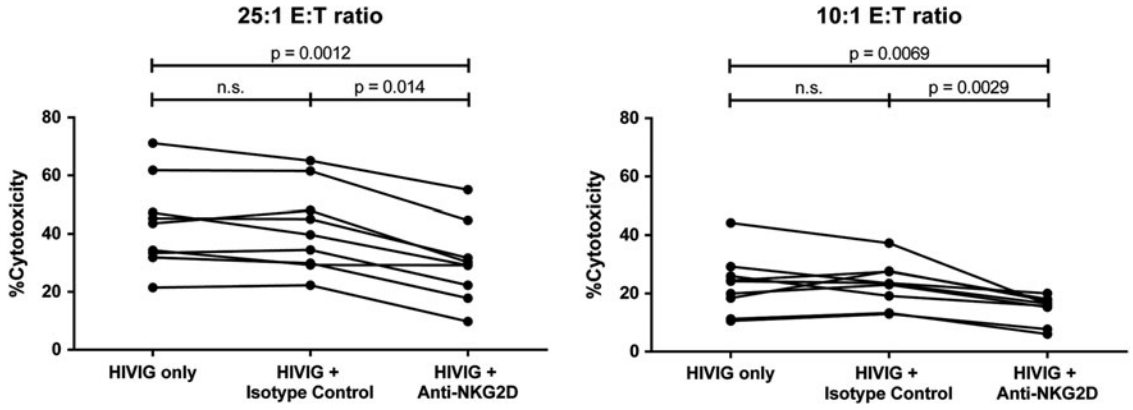

FIG. 1. Role for NKG2D in anti-HIV-1 ADCC of gp120-coated CEM.NKr-CCR5 cell line targets. The ability of PBMC effectors to mediate antibody-dependent cytolysis of gp120-coated CEM.NKr-CCR5 target cells was measured using the lactate dehydrogenase release assay. The graphs depict the relative levels of anti-HIV-1 ADCC observed across PBMC from nine donors in the 25:1 (*left*) and 10:1 (*right*) effector to target cell ratios in the presence of HIVIG alone, or upon the addition of an anti-NKG2D blocking antibody or a murine isotype control antibody. ADCC, antibody-dependent cellular cytotoxicity; HIVIG, HIV immunoglobulin; NK, natural killer; PBMC, peripheral blood mononuclear cells.

CEM.NKr-CCR5 target cells, in the presence of HIVIG, in an LDH release assay. As shown in Figure 2B, NK cells robustly killed gp120-pulsed CEM.NKr-CCR5 cells at a 10:1 effector to target cell ratio in the presence of HIVIG [32.2% (15.2%–45.0%)]. Furthermore, this killing was significantly inhibited across all seven donors in the presence of anti-NKG2D antibody [22.9% (4.2%–28.8%) $p < .05$], but not in the presence of an isotype control antibody [32.7% (13.9%–46.4%) $p > .05$] (Friedman's test with Dunn's post hoc tests). Again, these data imply ligation of NKG2D contributes to anti-HIV-1 ADCC of gp120-pulsed CEM.NKr-CCR5 target cells, as background lysis of CEM.NKr-CCR5 target cells in the absence of HIVIG (median: 0.7%) was too small to account for the observed effect of NKG2D blockade.

Role of NKG2D in anti-HIV-1 ADCC of HIV-1-infected CEM.NKr-CCR5 target cells

As we had observed a role for NKG2D in the anti-HIV-1 ADCC of gp120-pulsed CEM.NKr-CCR5 target cells, we next investigated if NKG2D contributed to the elimination of HIV-1-infected CEM.NKr-CCR5 cells by ADCC. Previous research has demonstrated that HIV-1 Vpu and Nef downregulate cell surface CD4 on HIV-1-infected cells, which prevents viral envelope from entering into the CD4-bound conformation.[26] This phenomenon allows HIV-1-infected cells to evade anti-HIV-1 Env ADCC, as ADCC-competent anti-HIV-1 antibodies in patients primarily target epitopes revealed in the CD4-bound envelope conformation.[26,27] As such, to investigate the potential role of NKG2D in the anti-HIV-1 ADCC of infected T cells, we utilized CEM.NKr-CCR5 target cells infected with either wild-type HIV-1 or U-N- HIV-1 and assessed the ability of PBMC effectors to kill these targets through utilization of the A32 anti-HIV-1 monoclonal antibody, which is an ADCC-mediating monoclonal antibody that best recognizes the CD4-bound conformation of envelope.[26,28,29]

Before measuring anti-HIV-1 ADCC, we assessed target cells infected with wild-type or U-N- HIV-1 for expression of NKG2D ligands and binding of the A32 antibody. Utilizing the NKG2D-Fc fusion protein, we detected expression of NKG2D ligands on cells not infected with HIV-1 or infected with either wild-type or U-N- viruses (Fig. 3A). Similar to previously reported data suggesting Nef can downregulate NKG2D ligands on HIV-1-infected T-cell lines, expression of NKG2D ligands was lower on T cells infected with wild-type HIV-1, compared to cells infected with U-N- HIV-1 ($p = .0006$) (Mann–Whitney test) (Fig. 3A).[20] Also, reflective of previously reported data, we primarily detected A32 binding on cells infected with U-N- virus ($p = .0006$) (Mann–Whitney test) (Fig. 3B).[26,30]

To measure anti-HIV-1 ADCC of HIV-1-infected targets, we implemented a flow cytometry-based cytolysis assay that measures the loss of viable HIV-1-infected target cells, as previous described.[25,26] As expected from the A32 binding data, A32-mediated ADCC was consistently observed against cells infected with U-N-deficient HIV-1, but not against those infected with wild-type HIV-1 (Fig. 3C Middle graph vs. Left graph). To determine if NKG2D signals were contributing to the observed ADCC, we implemented conditions in which target cells, PBMC effectors, and A32 monoclonal antibodies were coincubated with the anti-NKG2D antibody or an isotype control. As depicted in Figure 3C (Middle and Right graphs), the anti-NKG2D antibody reduced anti-HIV-1 ADCC of targets infected with U-N- HIV-1 [19.8% (9.3%–34.1%)], compared to conditions containing no murine antibody [28.8% (16.1%–53.9%), $p = .0485$] or the isotype control antibody [24.2% (20.9%–58.4%), $p = .0485$] (Friedman's test with Dunn's post hoc tests). No significant difference was observed between the ADCC observed in the no antibody and isotype control conditions. Furthermore, the NKG2D blockade effect cannot be accounted for by background lysis of infected CEM.NKr-CCR5 target cells, as the utilized percent cytolysis formula measures ADCC corrected for background lysis. These data imply that NKG2D ligation contributes not only to the ADCC of gp120-pulsed $CD4^+$ T-cell lines but also to the ADCC of HIV-1-infected T-cell lines.

Discussion

Previous research utilizing antibody-mediated cross-linking of NK cell receptors has demonstrated that coligation

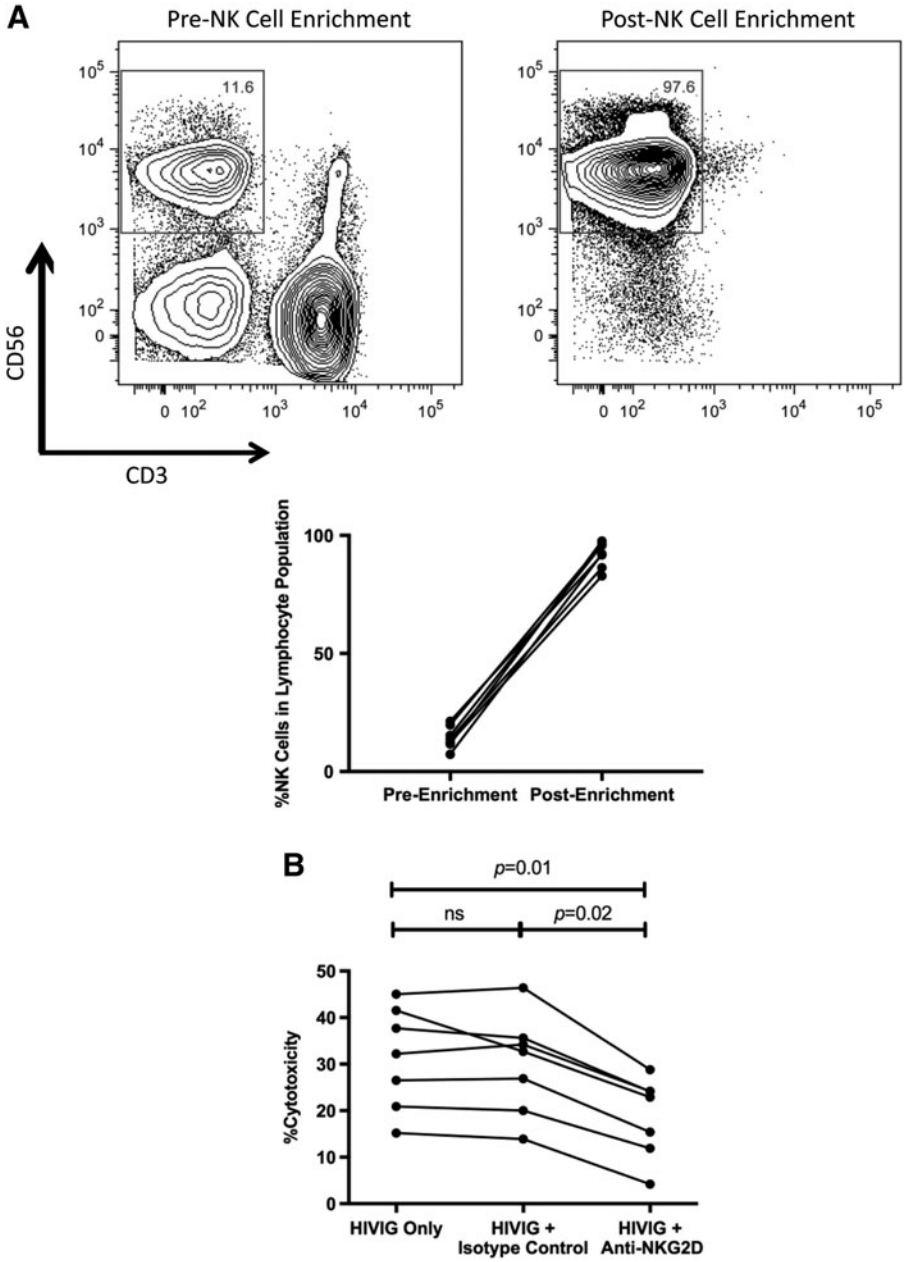

FIG. 2. Effect of antibody-mediated blockade of NKG2D on anti-HIV-1 ADCC mediated by enriched NK cells. NK cells were enriched from PBMC via magnetic bead selection and used as effector cells in the lactate dehydrogenase release assay to measure anti-HIV-1 ADCC. **(A)** FACs plots depict the percentage of $CD3^{-}CD56^{+}$ NK cells in pre- and post-enrichment PBMC samples from one of seven donors. The graph depicts the percentages of NK cells in pre- and post-enrichment PBMC samples from all seven donors. **(B)** The graph depicts the level of anti-HIV-1 ADCC mediated by NK cells against gp120-pulsed CEM.NKr-CCR5 from seven donors at a 10:1 effector to target cell ratio in the presence of HIVIG alone, or upon addition of an anti-NKG2D blocking antibody or a murine isotype control antibody.

of several activating NK cell receptors, including NKG2D, can act synergistically with signals through CD16 to enhance NK cell calcium flux.[15] The data in the present article build on this observation through demonstrating that ligation of NKG2D contributes to anti-HIV-1 ADCC. A role for NKG2D was observed for anti-HIV-1 ADCC against both gp120-pulsed and HIV-1-infected CEM.NKr-CCR5 targets. These observations have potential ramifications for optimizing vaccinations and/or therapeutics utilizing ADCC and/or other antibody-dependent NK cell-mediated effector functions.

Although the data presented in the current article demonstrate a role for NKG2D in the anti-HIV-1 ADCC of target cells infected with HIV-1, it should be noted that due to the inability to detect ADCC against cells infected with wild-type HIV-1, a role for NKG2D could only be demonstrated against cells infected with U-N- HIV-1. As such, the role NKG2D might play in ADCC against cells infected with wild-type HIV-1 remains unknown. It has previously been reported that Nef downregulates NKG2D ligands from the surface of HIV-1-infected T-cell lines.[20] Importantly, the capacity of Nef to downregulate NKG2D ligands was not observed in primary T cells infected with HIV-1.[31] The reason(s) for these conflicting results should be addressed in future studies. The data in the current article, however, corroborate the observation that Nef downregulates NKG2D ligands in HIV-1-infected T-cell lines, as we consistently observed lower mean fluorescence intensity for the binding of NKG2D-Fc fusion protein to CEM.NKr-CCR5 cells infected with wild-type HIV-1, compared to those infected with U-N-HIV-1. It should be noted, however, that the level of NKG2D ligands on T cells infected with wild-type HIV-1 was similar to the level of these ligands expressed on noninfected T cells. Given that activated noninfected T cells have previously been

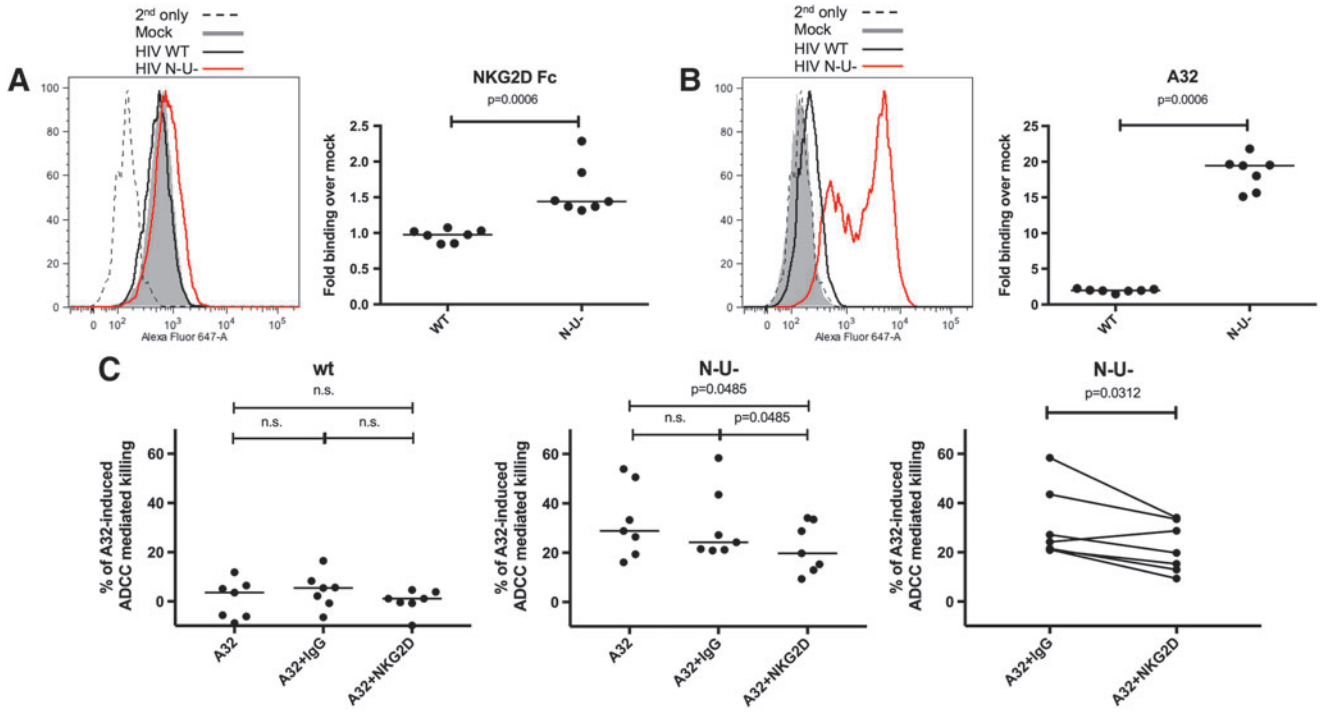

FIG. 3. Role for NKG2D in anti-HIV-1 ADCC of HIV-1-infected CEM.NKr-CCR5 target cells. CEM.NKr-CCR5 T cells were infected with wild-type HIV-1 or HIV-1 with defective Vpu and Nef (U-N-). **(A)** CEM.NKr-CCR5 cells that were uninfected, infected with wild-type HIV-1, or infected with U-N- HIV-1 were treated with NKG2D-Fc fusion protein, and binding of the fusion protein to cells was detected with Alexa-Fluor 647-conjugated anti-human IgG secondary antibody. The histogram depicts the relative detection of NKG2D ligands on uninfected (i.e., mock), wild-type HIV-1-infected (i.e., HIV WT), and U-N- HIV-1-infected (i.e., HIV U-N-) CEM.NKr-CCR5 cells, compared to CEM.NKr-CCR5 cells treated with the secondary antibody only (i.e., 2nd only) for a representative experiment. The graph shows the fold binding over uninfected CEM.NKr-CCR5 cells of the NKG2D-Fc across seven experiments. **(B)** The relative binding of A32 to CEM.NKr-CCR5 cells that were uninfected or infected with either wild-type or U-N- HIV-1 was assessed. Infected cells were treated with A32 and binding was assessed with an Alexa-Fluor-647-conjugated anti-human IgG secondary antibody. The histogram depicts the relative binding of A32 to CEM.NKr-CCR5 cells that were uninfected (i.e., mock) or infected with wild-type (i.e., HIV WT) or U-N- HIV-1 (i.e., HIV U-N-), compared to cells treated with the secondary antibody only (i.e., 2nd only) for a representative experiment. The graph shows the fold binding over uninfected CEM.NKr-CCR5 cells of the A32 monoclonal antibody across seven experiments. **(C)** Finally, the flow cytometry-based ADCC assay was utilized to measure A32-mediated anti-HIV-1 ADCC against CEM.NKr-CCR5 cells infected with wild-type or U-N- HIV-1. The graphs on the *left* and *middle* depict the relative levels of ADCC observed against CEM.NKr-CCR5 cells infected with wild-type (*left*) and U-N- HIV-1 (*middle*) in the presence of A32 alone, A32+IgG (i.e., murine IgG1 isotype control), or A32+NKG2D (i.e., murine anti-human NKG2D IgG1). The graph on the *right* depicts the paired relative A32-mediated anti-HIV-1 ADCC observed against CEM.NKr-CCR5 cells infected with U-N- HIV-1 in the presence of an isotype control or an anti-NKG2D antibody.

shown to be susceptible to direct NK cell-mediated lysis via NKG2D signaling, we predict that the levels of NKG2D on the surface of T cells infected with wild-type HIV-1 will be sufficient to contribute to anti-HIV-1 ADCC.[32] Future experiments to investigate this issue should be possible, given the recent demonstration that certain CD4 mimetics can open cell surface HIV-1 envelope into the CD4-bound conformation and make ADCC epitopes accessible to antibodies.[30] These CD4 mimetics have great therapeutic potential for ''shock and kill'' strategies that wish to utilize ADCC to eliminate HIV-1-infected cells, and an extensive knowledge of other NK cell receptors that could contribute to anti-HIV-1 ADCC might be important for optimization of such anti-latency approaches.[33,34]

The observation that NKG2D contributes to ADCC mediated by NK cells also draws attention to previous studies demonstrating the MMP-induced release of soluble NKG2D ligands by HIV-1-infected target cells.[23] These soluble ligands bind to NK cell or T-cell surface NKG2D and induce downregulation of the activating receptor, leading to poorer antibody-independent responses. Given our observation that NKG2D not only contributes to direct NK cell functions but also plays a role in antibody-dependent functions, we predict that soluble NKG2D ligands might also impede the ability of NK cells to mediate robust antibody-dependent responses. Future studies should investigate this possibility by assessing NK cell-mediated ADCC against HIV-1-infected cells that release soluble NKG2D ligands. In addition to cleaving NKG2D ligands from the surface of target cells, MMPs are involved in cleaving CD16 from the surface of NK cells post-activation.[35–38] Furthermore, inhibition of MMPs in cultures of NK cells and antibody-coated target cells improves the ability of NK cells to function.[39] Future research should further investigate the prospect of specifically

targeting MMPs to improve the ability of NK cells to utilize therapeutic antibodies.

Future research should also investigate the role of NKG2D in ADCC mediated by NK cells obtained from HIV-1-infected donors. Indeed, viremic HIV-1 infection is characterized by alterations to the NK cell population, including the development of a hypofunctional $CD56^{-}CD16^{+}$ subset.[40] HIV-1-infected donors who are coinfected with CMV also exhibit an exaggerated development of a $CD57^{+}NKG2C^{+}$ NK cell subset, which normally arises during CMV infection.[41] In CMV-infected donors, these NK cells exhibit more robust CD16-dependent stimulation than $NKG2C^{-}$ NK cells.[42] The relative utilization of NKG2D or other ADCC coreceptors by NK cells from HIV-1-infected donors represents important information for optimizing the application of therapeutic antibodies in these individuals.

There is much interest in utilizing non-neutralizing antibody effector functions, such as ADCC, to target HIV-1 via vaccination or to eliminate latently infected cells that have been reactivated.[43,44] The data in the present article reveal that these NK cell-mediated antibody-dependent responses are not only solely mediated through the CD16 Fc receptor but also depend on ligation of an antibody-independent activating receptor, NKG2D. Future experiments assessing how the role of NKG2D in anti-HIV-1 antibody-dependent NK cell functions can be utilized to augment these responses will be helpful in improving the efficacy of antibody-based vaccines and/or therapeutics.

Acknowledgments

This work was supported by program grant 1052979 from the National Health and Medical Research Council of Australia, as well as CIHR operating grants #119334 and #134117. A.F. is the recipient of a Canada Research Chair on Retroviral Entry. MSP and JR are recipients of CIHR Fellowship Awards.

Author Disclosure Statement

No competing financial interests exist.

References

1. Buchbinder SP, Mehrotra DV, Duerr A, *et al.*: Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): A double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 2008;372:1881–1893.
2. Hammer SM, Sobieszczyk ME, Janes H, *et al.*: Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. N Engl J Med 2013;369:2083–2092.
3. Haynes BF, McElrath MJ: Progress in HIV-1 vaccine development. Curr Opin HIV AIDS 2013;8:326–332.
4. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, *et al.*: Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 2009;361: 2209–2220.
5. Haynes BF, Gilbert PB, McElrath MJ, *et al.*: Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 2012;366:1275–1286.
6. Montefiori DC, Karnasuta C, Huang Y, *et al.*: Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials. J Infect Dis 2012;206:431–441.
7. Tomaras GD, Ferrari G, Shen X, *et al.*: Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proc Natl Acad Sci U S A 2013;110:9019–9024.
8. Smalls-Mantey A, Connors M, Sattentau QJ: Comparative efficiency of HIV-1-infected T cell killing by NK cells, monocytes and neutrophils. PLoS One 2013;8:e74858.
9. Lanier LL: NK cell recognition. Annu Rev Immunol 2005; 23:225–274.
10. Terszowski G, Klein C, Stern M: KIR/HLA interactions negatively affect rituximab- but not GA101 (obinutuzumab)-induced antibody-dependent cellular cytotoxicity. J Immunol 2014;192:5618–5624.
11. Ward JP, Bonaparte MI, Barker E: HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts. AIDS 2004;18:1769–1779.
12. Gooneratne SL, Richard J, Lee WS, Finzi A, Kent SJ, Parsons MS: Slaying the Trojan horse: Natural killer cells exhibit robust anti-HIV-1 antibody-dependent activation and cytolysis against allogeneic T cells. J Virol 2015;89:97–109.
13. Lang P, Pfeiffer M, Handgretinger R, *et al.*: Clinical scale isolation of T cell-depleted CD56+ donor lymphocytes in children. Bone Marrow Transplant 2002;29:497–502.
14. Parsons MS, Wren L, Isitman G, *et al.*: HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity. J Virol 2012;86:4488–4495.
15. Bryceson YT, March ME, Ljunggren HG, Long EO: Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood 2006;107:159–166.
16. Fogli M, Mavilio D, Brunetta E, *et al.*: Lysis of endogenously infected CD4+ T cell blasts by rIL-2 activated autologous natural killer cells from HIV-infected viremic individuals. PLoS Pathog 2008;4:e1000101.
17. Richard J, Sindhu S, Pham TN, Belzile JP, Cohen EA: HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. Blood 2010;115:1354–1363.
18. Ward J, Bonaparte M, Sacks J, *et al.*: HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. Blood 2007;110:1207–1214.
19. Kulkarni AG, Paranjape RS, Thakar MR: higher expression of activating receptors on cytotoxic NK cells is associated with early control on HIV-1C multiplication. Front Immunol 2014;5:222.
20. Cerboni C, Neri F, Casartelli N, *et al.*: Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. J Gen Virol 2007;88(Pt 1):242–250.
21. Shah AH, Sowrirajan B, Davis ZB, *et al.*: Degranulation of natural killer cells following interaction with HIV-1-infected cells is hindered by downmodulation of NTB-A by Vpu. Cell Host Microbe 2010;8:397–409.
22. Nolting A, Dugast AS, Rihn S, *et al.*: MHC class I chain-related protein A shedding in chronic HIV-1 infection is associated with profound NK cell dysfunction. Virology 2010;406:12–20.
23. Matusali G, Tchidjou HK, Pontrelli G, *et al.*: Soluble ligands for the NKG2D receptor are released during HIV-1

infection and impair NKG2D expression and cytotoxicity of NK cells. FASEB J 2013;27:2440–2450.
24. Holmes TD, El-Sherbiny YM, Davison A, Clough SL, Blair GE, Cook GP: A human NK cell activation/inhibition threshold allows small changes in the target cell surface phenotype to dramatically alter susceptibility to NK cells. J Immunol 2011;186:1538–1545.
25. Richard J, Veillette M, Batraville LA, *et al.*: Flow cytometry-based assay to study HIV-1 gp120 specific antibody-dependent cellular cytotoxicity responses. J Virol Methods 2014;208:107–114.
26. Veillette M, Desormeaux A, Medjahed H, *et al.*: Interaction with cellular CD4 exposes HIV-1 envelope epitopes targeted by antibody-dependent cell-mediated cytotoxicity. J Virol 2014;88:2633–2644.
27. Veillette M, Coutu M, Richard J, *et al.*: The HIV-1 gp120 CD4-bound conformation is preferentially targeted by antibody-dependent cellular cytotoxicity-mediating antibodies in sera from HIV-1-infected individuals. J Virol 2015; 89:545–551.
28. Acharya P, Tolbert WD, Gohain N, *et al.*: Structural definition of an antibody-dependent cellular cytotoxicity response implicated in reduced risk for HIV-1 infection. J Virol 2014;88:12895–12906.
29. Ferrari G, Pollara J, Kozink D, *et al.*: An HIV-1 gp120 envelope human monoclonal antibody that recognizes a C1 conformational epitope mediates potent antibody-dependent cellular cytotoxicity (ADCC) activity and defines a common ADCC epitope in human HIV-1 serum. J Virol 2011;85:7029–7036.
30. Richard J, Veillette M, Brassard N, *et al.*: CD4 mimetics sensitize HIV-1-infected cells to ADCC. Proc Natl Acad Sci U S A 2015;112:E2687–E2694.
31. Ward J, Davis Z, DeHart J, *et al.*: HIV-1 Vpr triggers natural killer cell-mediated lysis of infected cells through activation of the ATR-mediated DNA damage response. PLoS Pathog 2009;5:e1000613.
32. Nielsen N, Odum N, Urso B, Lanier LL, Spee P: Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PLoS One 2012;7:e31959.
33. Deeks SG: HIV: Shock and kill. Nature 2012;487:439–440.
34. Lee WS, Richard J, Lichtfuss M, *et al.*: Antibody-dependent cellular cytotoxicity against reactivated HIV-1-infected cells. J Virol 2015;90:2021–2030.
35. Grzywacz B, Kataria N, Verneris MR: CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. Leukemia 2007;21: 356–359, author reply 359.
36. Parsons MS, Tang CC, Jegaskanda S, *et al.*: Anti-HIV antibody-dependent activation of NK cells impairs NKp46 expression. J Immunol 2014;192:308–315.
37. Romee R, Foley B, Lenvik T, *et al.*: NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). Blood 2013;121:3599–3608.
38. Tang CC, Isitman G, Bruneau J, *et al.*: Phenotypic and functional profiles of NK cells exhibiting MMP-mediated CD16 cleavage after anti-HIV antibody-dependent activation. Clin Exp Immunol 2015;181:275–285.
39. Zhou Q, Gil-Krzewska A, Peruzzi G, Borrego F: Matrix metalloproteinases inhibition promotes the polyfunctionality of human natural killer cells in therapeutic antibody-based anti-tumour immunotherapy. Clin Exp Immunol 2013;173: 131–139.
40. Mavilio D, Lombardo G, Benjamin J, *et al.*: Characterization of CD56-/CD16+ natural killer (NK) cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals. Proc Natl Acad Sci U S A 2005;102:2886–2891.
41. Heath J, Newhook N, Comeau E, Gallant M, Fudge N, Grant M: NKG2C(+)CD57(+) natural killer cell expansion parallels cytomegalovirus-specific CD8(+) T cell evolution towards senescence. J Immunol Res 2016;2016:7470124.
42. Wu Z, Sinzger C, Frascaroli G, *et al.*: Human cytomegalovirus-induced NKG2C(hi) CD57(hi) natural killer cells are effectors dependent on humoral antiviral immunity. J Virol 2013;87:7717–7725.
43. Wren LH, Stratov I, Kent SJ, Parsons MS: Obstacles to ideal anti-HIV antibody-dependent cellular cytotoxicity responses. Vaccine 2013;31:5506–5517.
44. Kent SJ, Reece JC, Petravic J, *et al.*: The search for an HIV cure: Tackling latent infection. Lancet Infect Dis 2013;13: 614–621.

Address correspondence to:
Matthew S. Parsons
Department of Microbiology and Immunology
Peter Doherty Institute for Infection and Immunity
University of Melbourne
Melbourne
Victoria 3000
Australia

E-mail: mattp@unimelb.edu.au