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NK Cell Lysis of HIV-1-Infected Autologous CD4 Primary T Cells: Requirement for IFN-Mediated NK Activation by Plasmacytoid Dendritic Cells

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In vivo, several mechanisms have been postulated to protect HIV-1-infected cells from NK surveillance. In vitro, previous research indicates HIV-1-infected autologous CD4+ primary T cells are resistant to NK lysis. We hypothesized that NK lysis of HIV-1-infected target cells would be augmented by the presence of accessory cells and/or accessory cell factors. In this study, we show that stimulation of plasmacytoid dendritic cells (PDC) with the TLR9 agonist, CpG ODN 2216, triggered NK lysis of HIV-1-infected autologous CD4+ primary T cells. PDC-stimulated NK lysis was dependent upon MHC class I (MHC-I) down-regulation on infected cells, and primary HIV-1 isolates that exhibited enhanced MHC-I down-regulation were more susceptible to NK-mediated lysis. PDC-stimulated NK lysis of HIV-1-infected autologous CD4+ primary T cells was blocked by neutralizing Abs to type 1 IFN and was perforin/granzyme dependent. Overall, our data suggest that HIV-infected cells are not innately resistant to NK lysis, and that exogenous NK stimulation derived from PDC can trigger NK cytotoxicity against HIV-1-infected autologous CD4+ primary T cells. The Journal of Immunology, 2007, 179: 2097–2104.
Materials and Methods

**PBMC purification and cell culture**

Blood was drawn from a panel of 50 healthy, HIV-1-seronegative donors from the Wistar Institute Blood Donor Program. Institutional Review Board approval (from the Wistar Institute) and informed consent were obtained before blood donation. PBMC were separated by ficoll-paque (Amersham Biosciences) density gradient separation and were cultured at 2.5 × 10^6/ml in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 15% FBS and antibiotics. CD4^+ primary T cells were isolated from PBMCs by positive selection using anti-CD4 magnetic beads (Miltenyi Biotec), and purified NK cells were isolated from whole blood by negative selection using NK rosette mixture (StemCell Technologies), according to the manufacturer’s instructions. CD4^+ T cell and NK cell purity was tested by flow cytometry using Abs to the cell surface markers CD4/CD3 and CD56/CD65/CD16, respectively, and routinely found to be >95% PDC were enriched to >98% purity from PBMCs using PDC enrichment mixture (Diamond Isolation Kit; Miltenyi Biotec), according to the manufacturer’s instructions. NK-depleted PBMCs (<1.5% NK positive) were prepared using anti-CD56^+ magnetic beads in conjunction with LD depletion column (Miltenyi Biotec).

**HIV-1 infection**

PBMC were stimulated for 3 days with 10 μg/ml PHA-p (Sigma-Aldrich) and 100 U/ml human IL-2 (BD Pharmingen) before CD4^+ primary T cell purification and infected according to the following spinfection protocol: 5 × 10^6 CD4^+ primary T cells were infected with 150 ng of p24 containing HIV-1 supernatant in the presence of 8 μg/ml polybrene (Sigma-Aldrich) for 2 h at 1800 rpm. Uninfected CD4^+ primary T cells were exposed to polybrene and spun alongside infected cells. At 4 days postinfection, HIV-1-infected and uninfected CD4^+ primary T cells were passaged through CD4-negative selection magnetic column (Miltenyi Biotec) before use in flow cytometric or NK-mediated cytotoxicity assays to remove non-CD4-containing cells. Nonviable cells were subsequently removed by ficoll density gradient separation. The purity and viability of infected and uninfected CD4^+ primary T cells were >95% in all reported experiments.

The HIV-1 isolate TYBE (X4 tropic, from CNS) was isolated at the University of Pennsylvania Centers for AIDS Research Viral Core Facility, and the HIV-1 isolates NL-4-3 (X4), IIIB (X4), and 96USHP59 (R5/X4) were obtained from the National Institutes of Health AIDS Health and Reference Reagent Program (National Institutes of Health). All isolates were expanded and titered by the University of Pennsylvania Centers for AIDS Research Viral Core Facility.

**Flow cytometry**

All cell surface Abs and isotype controls were used at the recommended dilution of 0.25 μg of Ab/million cells in PBSA (PBS with 0.09% sodium azide). The Abs used in this study include CD3 (SK7), CD4 (SK3), CD16 (3GB), CD56 (B159), CD69 (FN50), CD107α (H4A3), MHC class I A2 (BB7.2), MHC class I A,B,C (G46.2.6), CD161 (DX12), and NKGD2 (ID-11) from BD Pharmingen; NKp30 (Z444), NKp44 (Z231), NKp46 (BAB281), NK2CA (Z199), KIR2DL1/S1 (EB6B), KIR2DL2/S2 (GL183), and LIR/ILT2 (HP-F1) from Beckman Coulter; and BDCA-4 (neuropilin-1) (ADS-17F6) from Miltenyi Biotec. Cells were permeabilized for intracellular staining with the Cytofix/Cytoperm kit (BD Pharmingen), as described by the manufacturer. Intracellular p24 staining to identify HIV-1-infected cells was performed with a PE-conjugated mouse mAb against the HIV-1 p24 gag protein (clone KC7; Beckman Coulter) used at a 1/1000 dilution. A minimum of 100,000 events was collected on a FACScalibur flow cytometer, and samples were subsequently analyzed with FlowJo software (Tree Star). Before analysis, all samples were gated by forward and side scatter to exclude dead cells.

**NK cytotoxicity assay**

A total of 1 × 10^5 HIV-1-infected or uninfected CD4 T cells was labeled with 100 μCi of Na_2^35ClO_4 for 3 h in complete medium supplemented with 100 U/ml IL-2, washed twice, and cocultured with autologous PBMCs or purified NK cells at various E:T ratios. Percentage cytotoxicity was calculated as described previously (16). The percent specific lysis adjusted for infectivity (%SL/%p24) was calculated by subtracting nonspecific lysis of uninfected CD4^+ primary T cells from HIV-1-specific lysis and adjusting for the percentage of infected p24-positive cells within each experiment (see depiction of methodology in Fig. 3A).

In the indicated experiments, PBMC or purified PDC were treated for 18 h with 10 μg/ml CpG ODN 2216 (InvivoGen). When enriched PDC were added to purified NK cells for CpG ODN 2216 stimulation experiments, the optimal NK to PDC ratio was determined individually for each donor based upon the ratio of NK to PDC present within PBMCs (typically between 5:1 and 25:1). When indicated, purified NK cells or PBMCs were incubated directly with 1000 U/ml rIFN-α 2a (Roferon/Roche). In all cases, the supernatant was removed following overnight stimulation and effector cells were washed twice before culturing with autologous labeled target cells. Of note, no change in spontaneous or experimental target cell lysis was observed in the presence of supernatants from 18-h stimulation of effectors with CpG-2216 or IFN-α 2a. During IFN-blocking experiments, neutralizing sera against human IFN-α/β (sheep polyclonal) and anti-human IFN-α/β receptor 2 (mouse, monoclonal IgG1) were added 30 min before and during CpG ODN 2216 stimulation of PBMC at 10 μg/ml (R&D Systems). As controls, normal sheep serum and mouse monoclonal IgG1 isotype controls were used at 10 μg/ml. During concanamycin A experiments, CpG ODN 2216-stimulated or unstimulated PBMCs were pretreated for 3 h before and during the chromium lysis assay with 100 ng/ml concanamycin A (Sigma-Aldrich) in DMSO or DMSO alone.

**CD107a degranulation assay**

PBMC or purified NK cells were cocultured alone (no target control) or with HIV-1-infected or uninfected autologous CD4 primary T cells at a 5:1 E:T ratio in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) and 20 μl of anti-CD107a mAb for 4 h, as previously described (37). NK cells were then harvested, and intracellular cytokine expression was measured with flow cytometry.
were gated by CD56^+/CD3^- staining, and CD107a expression was determined based on background levels of staining exhibited by no target control cells.

**IFN-α ELISA**

Supernatants from overnight stimulation of PBMC with CpG ODN 2216 were collected and frozen at ~80°C. Supernatants were thawed and tested in duplicate for IFN-α secretion using the IFN-α 2a ELISA kit (PBL Biomedical Laboratories), according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analyses were performed with JMP Software. Wilcoxon matched pair, nonparametric t-tests were used for paired analysis. Linear regression analysis was performed with normally distributed and independent data. In all cases, p values were two sided with significance <0.05.

**Results**

CpG stimulation of PBMC augments NK-dependent lysis of aHIV^+CD4

We hypothesized that NK lysis of aHIV^+CD4 would be augmented by the presence of accessory cell and/or accessory cell factors. We began by testing both laboratory-adapted (IIIB, NL4-3) and primary strains (96USHIPS9, TYBE) of HIV-1 to control for viral strain-specific differences in NK susceptibility. As
shown in Fig. 1, all four isolates possessed a similar capacity to infect CD4 primary T cells, as determined by intracellular p24 levels (Fig. 1A, insets) and down-regulation of CD4 from the cell surface (data not shown). However, we observed that the primary HIV-1 strains TYBE and 96USHIPS9 (SHIP) exhibited a significantly greater capacity to down-regulate MHC-I when compared with the laboratory-adapted strain IIIB or NL4-3 (Fig. 1B). As a result, we postulated that the increased capacity of SHIP and TYBE to induce MHC-I down-regulation would lead to an enhanced susceptibility of these isolates to NK lysis. Nevertheless, in support of previous findings (33), we observed that aHIV/H11001 CD4 were resistant to lysis by either purified NK cells or total PBMCs, regardless of the HIV-1 strain used for infection (Fig. 2, A and B).

We next tested whether preactivation of dendritic cells could overcome the observed block to NK lysis of aHIV/H11001 CD4. As a stimuli, we choose the TLR9 ligand, CpG ODN 2216 (CpG-2216), to activate dendritic cells. Our results showed that CpG-2216-activated dendritic cells could trigger NK-dependent lysis of HIV-1-infected autologous CD4 primary T cells (Fig. 3). A representative depiction of the method for normalization of a donor’s NK lysis activity based on the subtraction of background lysis against uninfected target cells (specific lysis, on left) and adjustment for the frequency of p24 Ag-positive cells (%SL/%p24, on right). B, %SL/%p24-normalized lysis data from 10 autologous donors tested for both unstimulated and CpG-2216-stimulated PBMC lysis of NL4-3- and SHIP-infected CD4 T cells at a 100:1 E:T cell ratio. C, Regression analysis of %SL/%p24 vs MHC-I expression following CpG-2216 stimulation of PBMC lysis at a 100:1 E:T cell ratio. The %SL/%p24 results with NL4-3, IIIB, SHIP, and TYBE are included.

FIGURE 3. MHC-I down-regulation is associated with susceptibility of HIV-1-infected autologous CD4 primary T cells to CpG-2216-stimulated PBMC lysis. A, A representative depiction of the method for normalization of a donor’s NK lysis activity based on the subtraction of background lysis against uninfected target cells (specific lysis, on left) and adjustment for the frequency of p24 Ag-positive cells (%SL/%p24, on right). B, %SL/%p24-normalized lysis data from 10 autologous donors tested for both unstimulated and CpG-2216-stimulated PBMC lysis of NL4-3- and SHIP-infected CD4 T cells at a 100:1 E:T cell ratio. C, Regression analysis of %SL/%p24 vs MHC-I expression following CpG-2216 stimulation of PBMC lysis at a 100:1 E:T cell ratio. The %SL/%p24 results with NL4-3, IIIB, SHIP, and TYBE are included.
which can activate PDC to secrete IFN-α and activate NK (1–4). As shown in Fig. 2B, we found that pretreatment of PBMC with CpG-2216 triggered a strong HIV-1-specific lysis against TYBE-infected aHIV+CD4. However, lysis of aHIV+CD4 infected with the IIIB isolate, which lacked the ability to down-regulate MHC-I, was similar to uninfected cell background. An increase in background lysis against uninfected CD4 T cells was also noted, although it was substantially lower than lysis against TYBE-infected aHIV+CD4. These results were further supported by CD107a flow-based degranulation assay, which showed that prestimulation of PBMCs with CpG-2216 triggered degranulation of CD56+/CD3+–gated NK cells in response to TYBE-infected aHIV+CD4, but not lab-adapted aHIV+CD4 (Fig. 2C and D).

In subsequent experiments, we separated the HIV-specific increase in lysis of aHIV+CD4 from background activity against uninfected CD4 primary T cells by subtraction of uninfected cell lysis from HIV-1-specific lysis following CpG-2216 stimulation of PBMCs. We then adjusted for differences in efficiency of HIV-1 infection between experiments by normalizing each experiment by intracellular p24 expression (see Fig. 3A). Using this approach, we found a statistically significant increase in PBMC lysis of SHIP-infected (p < 0.05, n = 10), but not NL4-3-infected aHIV+CD4 following CpG stimulation (Fig. 3B), in support of our previous results with TYBE and IIIB. For all viral isolates tested, MHC-I down-regulation in aHIV+CD4 was found to positively regress with NK lysis following CpG induction of PBMC, confirming the role of MHC-I down-regulation in target cell susceptibility to NK lysis (Fig. 3C).

Next, we confirmed that CpG-dependent PBMC lysis of aHIV+CD4 was mediated through PDC stimulation of NK cells. First, depletion of NK cells from PBMC led to a substantial reduction in CpG-2216-stimulated PBMC lysis of aHIV+CD4, as shown in Fig. 4A. Second, direct incubation of purified NK with PDC in the presence of CpG-2216 triggered NK activation (as evidenced by CD69 up-regulation) and lysis of aHIV+CD4 (Fig. 4B and C). In contrast, direct stimulation of NK with CpG-2216, or incubation of NK with PDC in the absence of CpG-2216, failed to trigger NK CD69 expression or lysis of aHIV+CD4 (Fig. 4B and C).

Together these results strongly indicated that NK lysis of aHIV+CD4 was induced by the presence of PDC accessory cell help, and is dependent on the extent of MHC-I down-regulation in infected target cells.

CpG-2216 stimulation of PDC triggers lysis of aHIV+CD4 through type 1 IFN-mediated NK activation and perforin granule exocytosis

In addition to confirming that CpG-2216 stimulation of PBMC triggers the production of IFN-α (Fig. 5A), we directly tested the role of type 1 IFN in PDC stimulation of NK activity through the use of blocking Abs. As shown in Fig. 5B, C, and D, we observed a complete reduction in CD69 up-regulation on NK cells as well as...
a reduction in CpG-2216-stimulated NK lysis of aHIV + CD4 in the presence of neutralizing Abs against IFN-α/β and the IFN-α/β receptor, IFN-R2. Conversely, the ability to increase both NK and PBMC cytotoxicity through the addition of exogenous IFN-α 2α alone suggests that secretion of IFN-α by PDC can stimulate NK lysis of aHIV + CD4 (Fig. 5D).

To investigate the mechanism through which CpG-2216 stimulation of PDC augments NK lysis of HIV-1-infected targets, we tested the effect of CpG-2216 pretreatment on the expression of NK-inhibitory and activating receptors. As shown in Fig. 6A, stimulation of PBMCs with CpG-2216 did not alter the expression of any of the measured inhibitory or activating receptors on CD56 +/CD3 - gated NK cells, including CD16, NKp2D, NKp30, NKp44, NKp46, KIR2DL1/S1, KIR2DL2/3/S2, CD161, KIR2A, and ILT2. However, CpG-2216 stimulation did result in the robust up-regulation of CD69, an activation marker associated with NK activation via IFN-α, as shown in Fig. 5B. Analysis of CD56 +/CD3 - gated NK cells following a 4-h incubation of CpG-2216-stimulated PBMCs with aHIV + CD4 also showed no change in the expression of activating or inhibitory NK receptors on CD107a-degranulated NK cells (data not shown), as has been described following receptor/ligand interaction in other NK/target systems (38–40).

We next investigated the mechanism of killing used by NK cells in lysing aHIV + CD4 following PDC stimulation using the perforin inhibitor, concanamycin A. As previously reported (41), we observed that pretreatment of purified NK cells (data not shown) or PBMCs (Fig. 6, B and C) with concanamycin A decreased intracellular perforin content in CD56 + NK cells. Indicative of a role of the perforin/granzyme pathway, pretreatment with concanamycin A also led to inhibition of PBMC-mediated lysis of aHIV + CD4 following CpG-2216 stimulation (Fig. 6D). As expected, pretreatment with concanamycin A did not alter CD107a degranulation of CpG-2216-stimulated PBMCs following coculture with aHIV + CD4 (data not shown).

Together, these results establish that CpG-2216 stimulation of PBMCs results in PDC secretion of IFN-α and activation of NK to lyse aHIV + CD4 in a perforin/granzyme-dependent mechanism without modulation of major inhibitory or activating NK receptors.

**Discussion**

In this study, we document the first evidence that NK responses are able to recognize and lyse aHIV + CD4 in the presence of CpG-2216-activated PDC or IFN-α. Although PDC enhancement of NK lysis has been described against nonautologous virally infected targets or transformed tumor cells, we now establish that viral evasion mechanisms exhibited by HIV-1 to guard against NK lysis do not prevent NK cytotoxicity if PDC stimulation is present. Specifically, we found that CpG-2216 stimulation of PDC led to a significant increase in NK-dependent lysis against aHIV + CD4 when MHC-I was down-regulated following infection (Figs. 2 and 4), and that the stimulatory effect of CpG-2216 was mediated through activation of NK via type 1 IFN (Fig. 5). Our data highlight the ability of the NK cytotoxic response to lyse HIV-1-infected autologous CD4 primary T cell targets in the presence of PDC function. Conversely, in the absence of PDC stimulation, our data support previous reports of an inefficient lysis of aHIV + CD4 by NK cells despite MHC-1 down-regulation (33).

It is of relevance to note that our findings concerning CpG-2216-stimulated PBMC lysis of aHIV + CD4 are most likely an underestimate of the total potential effect due to the inefficiency of HIV-1 infection achieved within CD4 + primary T cells (typically 35–55% infected; see Fig. 1A). By comparison, virally infected heterologous targets such as SupT1 (in which close to 100% of cells are infected) or MHC-devoid cell lines such as K562 cells are highly susceptible to NK lysis. As depicted in Fig. 3A, we addressed this limitation by correcting PBMC lysis by the percentage of p24-positive cells in each experiment and subtracting nonspecific lysis of uninfected cells.

We established a role for PDC and type 1 IFN in mediating CpG-2216-stimulated PBMC lysis of aHIV + CD4 through the addition of activated PDC directly to isolated NK cells (Fig. 4C), as well as by neutralizing type 1 IFN activity by blocking IFN-α/β and the IFN-α/β receptor, IFN-R2 (Fig. 5C). Taken together with the observations that exogenous IFN-α can mediate similar anti-HIV activity (Fig. 5D), and recent data identifying stimulated PDC products with anti-HIV activity independent of IFN-α (42), our...
data add support to the interpretation that activation of PDC may promote innate antiviral mechanisms of control against HIV-1. Of note, we also observed an increase in the background level of PBMC lysis against uninfected CD4+ primary T cells following CpG-2216 stimulation. We postulate that this increase is due to a lowering of the threshold of NK lysis toward all targets because no change in the major inhibitory or activating receptors on NK cells other than CD69 was noted (Fig. 6A). Importantly, the PDC-stimulated NK lysis of aHIV+CD4 was always greater than the background level of lysis against uninfected CD4+ primary T cells, allowing us to interpret the presence of HIV-1-specific lysis against aHIV+CD4.

Previously, research from several groups has indicated that enriched PDC cultures can respond directly to HIV-1–infected CD4+ primary T cells through TLR7-mediated production of IFN-α (28, 29). It is of relevance to stress that in our experiments shown in this study, a maximum of 4-h incubation was present between unstimulated PBMC and aHIV+CD4, which may not be long enough to allow for PDC recognition of infected target cells and NK-dependent lysis. It remains to be determined whether TLR7-mediated IFN-α from prolonged incubations with aHIV+CD4 can result in NK-mediated lysis, as suggested by our data.

In terms of a mechanism for CpG-2216-stimulated NK cytotoxic function, previous research has shown that HIV-1 gp41 can induce the expression of a NKp44 ligand on CD4+ primary T cells and sensitize infected targets to lysis by IL-2–activated NK cells in vitro (43, 44). However, we found that NKp44 was not expressed by NK cells in our system either before or following CpG-2216 stimulation of PBMCs (Fig. 6A), suggesting that alternative mechanisms of NK recognition may be responsible for the observed lysis of autologous HIV-1–infected targets following PDC stimulation. In terms of NK-inhibitory receptors, previous research has shown that the removal of NK cells expressing inhibitory KIR augments NK lysis of aHIV+CD4 (32). Our results showing a significant correlation between the degree of MHC-I down-regulation on aHIV+CD4 and the extent of PDC-stimulated NK lysis (Fig. 3C) further suggest a dominant role for MHC-I/KIR interactions in regulating NK lysis of autologous targets.

In vivo, the correlation between PDC/NK responses, IFN-α, and HIV-1 control is supported by several important observations, as follows: 1) long-term nonprogressors have retained PDC and NK function in the presence of low viral loads (7); 2) PDC frequency has been implicated as a correlate of viral rebound following therapy interruption (12, 45); 3) increased NK activity has been proposed as a correlate of protection in HIV-1–exposed, but uninfected Vietnamese i.v. drug users (46); 4) exogenous IFN-α has been shown to result in a drop of >0.5 log in viral load in vivo when introduced in the absence of antiretroviral therapy (47); and 5) the NK-activating receptor allele, KIR3DS1, in combination with HLA-B Bw4, is associated with delayed progression to AIDS in individuals infected with HIV-1 (48). Based on our observations supporting the ability of NK to lyse HIV-infected autologous targets following PDC accessory cell stimulation, it remains to be investigated whether NK cytotoxicity contributes to mechanisms of HIV-1 control in vivo. It is also of interest that despite PDC dysfunction in chronic HIV-1 infection (8–12), a positive correlation between IFN-α serum levels and disease progression has been described (11). It remains to be determined what biological activity circulating IFN-α has in activating NK cytotoxicity when compared with de novo production by PDC following in vitro stimulation with CpG-2216.

Activation of MHC-I-dependent mechanisms of NK lysis upon acute infection or upon anti-retroviral therapy-mediated immune reconstitution may represent powerful correlates of vaccine protection and viral control, respectively, when in the presence of functional NK and PDC compartments. The cooperative role between NK cells and PDC in control of HIV-1 supports further consideration of this mechanism of antiviral activity in advancement of immune-based therapies targeting functional activation/reconstitution of NK or PDC compartments during HIV-1 infection.

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Disclosures
The authors have no financial conflict of interest.

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