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Anti-HIV Antibody–Dependent Activation of NK Cells Impairs NKp46 Expression

Matthew S. Parsons,* Chi-Chang Tang,* Sinthujan Jegaskanda,* Robert J. Center,* Andrew G. Brooks,* Ivan Stratov,*† and Stephen J. Kent*†

There is much interest in the potential of Ab-dependent cellular cytotoxicity (ADCC) to slow disease progression following HIV infection. Despite several studies demonstrating a positive association between ADCC and slower disease progression, it is possible that continued stimulation of NK cells by ADCC during chronic HIV infection could render these cells dysfunctional. Indeed, activation of NK cells by ADCC results in matrix metalloproteinase–induced reductions in CD16 expression and activation refractory periods. In addition, ex vivo analyses of NK cells from HIV-infected individuals revealed other alterations in phenotype, such as decreased expression of the activating NKp46 receptor that is essential for NK-mediated antitumor responses and immunity from infection. Because NKp46 shares a signaling pathway with CD16, we hypothesized that activation-induced downregulation of both receptors could be controlled by a common mechanism. We found that activation of NK cells by anti-HIV or anti-CD16 Abs resulted in NKp46 downregulation. The addition of a matrix metalloproteinase inhibitor attenuated NKp46 downregulation following NK cell activation by anti-HIV Abs. Consequently, these results suggest that continued stimulation through CD16 has the potential to impair natural cytotoxicity via attenuation of NKp46-dependent signals. The Journal of Immunology, 2014, 192: 308–315.
cell line was shown to reduce the expression of CD16 and reduce the ability of NK cells to respond to further stimulation (27, 28). Activation of NK cells with ADCC target cells decreases CD16 and CD56 expression and reduces the potential of the NK cell to respond to further stimulation (26, 29). The decrease in CD16 expression after activation of NK cells for ADCC is similar to the decreases in other activating receptors, such as NKp46, after NK cell activation through direct ligation of activating receptors (30). However, this raises the conundrum of how NKp46 expression is decreased on NK cells during HIV infection. Indeed, the expression of NKp46 ligands is not induced in CD4+ T lymphocytes upon HIV infection (31). As such, the direct activation of NK cells through NKp46 cannot explain the decreased expression of NKp46 observed during chronic HIV infection. These observations suggest that the downregulation of NKp46 that occurs during HIV infection is driven by an indirect mechanism(s).

Because the expression of activating receptors is typically decreased through direct ligation (30, 32), the involvement of mechanisms elicited through their signaling pathways may be involved. Because NKp46 expression is decreased in the absence of direct ligation during HIV infection (13, 15, 20, 31) and given that NKp46 shares a signaling pathway with CD16 (23), we hypothesized that decreases in NKp46 expression could be induced by the activation of NK cells through CD16 ligation. Furthermore, we hypothesized that the CD16 ligation-dependent upregulation of matrix metalloproteinases (MMPs), which are responsible for decreased CD16 expression (26), contribute to decreased NKp46 expression. In this study, we demonstrate that activation of NK cells ex vivo by either anti-HIV or anti-CD16 Abs decreases the expression of NKp46 and that the presence of an MMP inhibitor can maintain the expression of NKp46 on activated NK cells. The chronic activation of NK cells and loss of NKp46 have implications for broad defects in innate immunity during chronic HIV infection.

Materials and Methods

Study population

Whole blood was collected from 16 healthy controls, who were not infected with HIV, by forearm venipuncture into vacutainers containing sodium heparin anticoagulant. As a source of HIV-specific Abs, plasma was prepared from whole blood samples from two HIV-infected clients of the Melbourne Sexual Health Center. These two individuals previously were demonstrated to have Abs capable of robustly activating NK cells through CD16 (33, 34). Individual statistical tests reported throughout the article consist of assays completed with only one of the two plasma donors. Informed consent was obtained before collection of all biological samples, and the ethics committees of the University of Melbourne and Alfred Health approved the described studies.

Assay to measure CD16-mediated activation of NK cells by anti-HIV Abs

A whole-blood intracellular cytokine-staining assay was used to measure activation of NK cells through CD16 by anti-HIV Abs, as previously described (35). Briefly, 150 μl whole blood from uninfected controls and 50 μl plasma from an HIV-infected individual were mixed together and incubated at 37°C for 5 h in the presence of 1 μg/ml HIV-1 gp140 from the subtype B AD8 strain (obtained as previously described) (36), 5 μg/ml brefeldin A (Sigma), and 6 μg/ml monensin (BD). Control wells contained all components of the activation wells with the exception of HIV-1 gp140. After incubation, cells were surface stained with combinations of the following Abs: PerCP-conjugated anti-CD3 (BioLegend), PE-Cy7–conjugated anti-CD56 (BioLegend), PE-conjugated anti-NKp46 (BD), FITC-conjugated anti-CD16 (BD), and allophycocyanin-conjugated anti-CD107a (BD). Next, the whole blood was treated with lysing solution (BD) to remove RBCs. The remaining WBCs were treated with permeabilization solution and stained with Alexa Fluor 700–conjugated anti–IFN-γ Ab (both from BD). Flow cytometric data were collected using a BD FACSCanto II flow cytometer and analyzed with the FlowJo version 9.2 software (TreeStar). Values for activation markers reported were obtained after subtraction of background activation observed in control wells.

Anti-NKp46–blocking studies

For some of the anti-HIV Ab–dependent CD NK cell–activation assays, a saturating concentration of PE-conjugated anti-NKp46 Ab (clone 9E2) or an equivalent concentration of a PE-conjugated isotype-control Ab was added to the wells for the duration of the 5-h incubation. The 9E2 anti-NKp46 clone was shown previously to block NK cell activation through NKp46 (37).

CD16 cross-linking NK cell–activation assay

Briefly, 200 μl whole blood from healthy controls, who were not infected with HIV, was mixed with FITC-conjugated 3G8 clone anti-CD16 Ab (BD) for 5 h at 37°C with 5 μg/ml brefeldin A (Sigma) and 6 μg/ml monensin (BD). Control wells were incubated in the absence of the anti-CD16 Ab. Following incubation, cells were surface stained with the following Abs: Per-CP–conjugated anti-CD3 (BioLegend), PE-Cy7–conjugated anti-CD56 (BioLegend), and allophycocyanin-conjugated anti-CD107a (BD). Control wells that did not receive FITC-conjugated anti-CD16 Ab prior to the 5-h incubation were stained for CD16 at this time. Next, whole blood was treated with a lysing solution (BD) to remove RBCs. The remaining WBCs were treated with a permeabilization solution (BD) and stained with Alexa Fluor 700–conjugated anti-IFN-γ Ab (BD). Flow cytometry data were collected using a BD FACSCanto II flow cytometer and analyzed with FlowJo version 9.2 software (TreeStar). Values for activation markers reported were obtained after subtraction of background activation observed in control wells.

MMP inhibition

To test the effect of activation-induced MMP production on NK cell activation–induced phenotype changes, anti-HIV Ab-dependent CD16 NK cell–activation assays were conducted in the presence of various concentrations (50, 5, 0.5 μM) of MMP inhibitor GM6001 dissolved in DMSO (both from Sigma) or in the presence of an equivalent amount of DMSO vehicle alone.

Statistics

Data sets were tested for their conformity to a normal distribution with the Kolmogorov–Smirnov test, as well as the D’Agostino and Pearson omnibus and Shapiro–Wilks normality tests. Data sets that were not shown to violate the Gaussian distribution were analyzed with paired t tests, whereas data sets that were suggested to not fall within the Gaussian distribution were analyzed with Wilcoxon matched-pairs tests. All statistical analyses were conducted using GraphPad Prism 4.0 software.

Results

NK cell activation by anti-HIV Abs alters the phenotype of NK cells

Activation of NK cells through CD16 triggers NK cells to degranulate, as measured by CD107a expression, and produce cytokines, as measured by IFN-γ production. Such activation alters the phenotype of the activated NK cells, inducing downregulation of CD16, which is mediated by the activation-dependent production of MMPs (26). The anti-HIV Ab–dependent activation assay was demonstrated to involve the binding of HIV-1 gp140 to CD4 on the surface of CD4+ T cells and the binding of anti-HIV Abs to bound gp140. These bound Abs subsequently activate NK cells (35). To confirm that this assay was appropriate to examine activation-induced alterations in NK cell phenotype, we determined whether NK cell activation, as indicated by degranulation and IFN-γ production, coincided with MMP-induced downregulation of CD16. The assay activates NK cells for both degranulation and cytokine production, and the observed activation is dependent upon the presence of both HIV-1 gp140 and anti-HIV Abs (Fig. 1A). Furthermore, the activation coincides with CD16 downregulation (Fig. 1B), and CD16 down-regulation can be prevented in a dose-dependent manner by the addition of the GM6001 MMP inhibitor (Fig. 1C). These results demonstrate that the assay used efficiently measures CD16-mediated NK cell activation and detects the activation-induced NK cell phenotype alterations mediated through MMPs that were reported previously (26).
Activation of NK cells by anti-HIV Abs reduces surface expression of NKp46

The NKp46 and CD16 receptors are subjected to similar regulations, as exemplified by their sharing of a common signaling pathway (23). Therefore, we hypothesized that the mechanisms involved in the activation-induced downregulation of these receptors also could be similar. This infers that activation of NK cells through one of these receptors could induce downregulation factors that would affect both receptors, even if one of them was not involved in the activation. To test this possibility, we activated NK cells through CD16 using the anti-HIVAb-mediated NK cell activation assay and assessed its impact on NKp46 expression. To accurately gate on NKp46+ NK cells, we used the fluorescence minus one (FMO) gating strategy, in which a gate defining negative and positive cells is determined by staining cells with all Abs from the panel, with the exception of the anti-NKp46 clone (Fig. 2A). Activation by anti-HIV Abs resulted in a decreased expression of NKp46 on the surface of NK cells (Fig. 2B). This activation-induced decrease in NKp46 expression was consistently observed across all 13 independent NK cell donors tested (mean ± SD: 76.2 ± 12.5% versus 68.3 ± 14.2%; p < 0.0001, paired t test) (Fig. 2B). Indeed, NK cells activated to express the CD107a degranulation marker (mean ± SD = 75.6 ± 17.1% versus 24.4 ± 17.1%) or produce IFN-γ (mean ± SD = 87.1 ± 16.2% versus 12.9 ± 16.2%) were preferentially observed in the NKp46− gate compared with the NKp46+ gate (Fig. 2C). These results demonstrate that the activation of NK cells via CD16 engagement can contribute to the downregulation of NKp46. This is consistent with a hypothesis that NKp46 downregulation can be initiated upon activation through CD16.

Anti-HIV Ab–dependent activation of NK cells does not require NKp46 ligation

The results presented demonstrate that activation of NK cells via a CD16-dependent pathway results in the downregulation of NKp46 and suggest that NKp46 downregulation is a byproduct of CD16 activation. However, it is possible that the NKp46 receptor acts as an activating coreceptor in the used anti-HIV Ab-dependent activation assay and that its downregulation is driven by its own ligation. To test this possibility, we carried out the NK cell acti-
vation assay in the presence of a saturating concentration of the blocking-competent 9E2 anti-NKp46 Ab clone or an equivalent concentration of mouse IgG1 isotype control. The 9E2 Ab clone was shown to block activation through NKp46 (37). As depicted in Fig. 3, the anti-NKp46 Ab did not mediate any consistent observable effect on activation-induced NK cell CD107a expression (median: 7.2 versus 7.1; \( p = 0.91 \), Wilcoxon matched-pairs test) or IFN-\( \gamma \) production (mean \( \pm \) SD: 3.7 \( \pm \) 2.1\% versus 3.1 \( \pm \) 1.8\%, \( p = 0.38 \), paired \( t \) test) compared with an isotype control across nine independent NK cell donors. This observation suggests that the activation of NK cells in this assay occurs independently of NKp46 ligation.

Activation of NK cells by direct Ab-dependent ligation of CD16 decreases NK cell surface expression of NKp46

Because the activation-induced downregulation of NKp46 was independent of NKp46 ligation, we next tested whether CD16 ligation alone could induce the decreased surface expression. Whole blood was incubated in the absence or presence of an anti-CD16 Ab, 3G8. This was a modified version of a previous assay that demonstrated the 3G8 Ab is capable of cross-linking CD16 and inducing NK cell activation (38). As expected, it was observed that 3G8 induced NK cell degranulation and IFN-\( \gamma \) production (Fig. 4A). The activation of NK cells solely through CD16 was sufficient to induce decreased surface expression of NKp46 (Fig. 4B), an effect reproducible across all 11 NK cell donors tested (mean \( \pm \) SD: 65.0 \( \pm \) 14.9\% versus 35.1 \( \pm \) 17.0\%, \( p < 0.0001 \), paired \( t \) test). These results confirm that NKp46 downregulation can be induced solely through the ligation of CD16.

Inhibition of MMPs maintains NKp46 expression on NK cells activated through CD16

The observation that the ligation of CD16 resulted in decreased surface expression of NKp46, as well as the fact that CD16 and NKp46 share a common signaling pathway through CD3\( \zeta \) and FcεRI\( \gamma \) (23), raises the hypothesis that NKp46 downregulation is mediated by the same mechanism as activation-induced CD16 downregulation. As demonstrated by several independent groups and replicated in Fig. 1C, activation-induced CD16 downregulation is driven by the activation-induced expression of MMPs (26, 28). As such, we next tested whether inhibition of MMP during the activation of NK cells through CD16 would prevent or attenuate the downregulation of NKp46 surface expression. Indeed, when NK cells were incubated in the presence of the GM6001 MMP inhibitor for the duration of the anti-HIV AdCC--dependent NK cell--activation assay, NKp46 expression was maintained at levels more similar to those observed in nonactivated NK cells (Fig. 5A). Across all 10 donors tested, NKp46 was expressed on a higher percentage of NK

**FIGURE 2.** Activation of NK cells by anti-HIV Abs induces downregulation of NKp46 expression. (A) Establishment of a gate for NKp46 expression using FMO control. FMO control for a representative donor (left panel). Application of the FMO gate to an NK cell population stained with PE-conjugated anti-NKp46 Ab (right panel). (B) Anti-HIV Ab-dependent activation of NK cells induces a downregulation in NKp46 expression on NK cells. Dot plots represent the expression of NKp46 on NK cells not stimulated for anti-HIV AdCC (upper left panel) and the decreased expression after activation with anti-HIV ADCC Abs (upper right panel). NKp46 downregulation was observed across 13 independent donors upon NK cell stimulation (lower panel). (C) The downregulation of NKp46 occurs primarily in activated NK cells. Indeed, the majority of NK cells expressing CD107a (left panel) or producing IFN-\( \gamma \) (right panel) are observed in the NKp46\(^{-} \) gate compared with the NKp46\(^{+} \) gate. The horizontal lines on the graphs represent the mean % of activated NK cells in the NKp46\(^{+} \) and NKp46\(^{-} \) gates.
cells when incubated with the MMP inhibitor than when incubated in the absence of the inhibitor (mean ± SD: 79.6 ± 10.7% versus 71.7 ± 12.4%, *p* = 0.0003, paired *t* test). This effect on NKp46 expression was not observed when NK cells were incubated for the duration of the assay with the DMSO carrier (Fig. 5A). Adding further credence to this observation, the effect of the GM6001 MMP inhibitor was observed to be dose dependent (Fig. 5B). Thus, CD16-dependent downregulation of NKp46 is driven by the MMPs produced upon NK cell activation.

**Discussion**

Several independent studies (13, 15, 20) using clinical samples from HIV-infected individuals demonstrated that the surface expression of the activating NKp46 receptor is downregulated on NK cells directly stained ex vivo. This downregulation has been linked to the state of disease progression, being associated with viremia (20). Furthermore, the expression level of NKp46 is partially restored after successful antiretroviral therapy (20). However, despite these observations, the mechanism underlying this downregulation has remained elusive. Reductions in NKp46 expression in HIV-unrelated in vitro experiments was demonstrated to occur upon activation and, in some cases, after direct ligation of the receptor (30, 39). However, infection of CD4+ T lymphocytes with HIV does not induce the expression of NKp46 ligands (31). As such, ligation-induced downregulation is unlikely to explain the altered patterns of NKp46 expression observed on NK cells from HIV-infected individuals. The signaling pathway used upon NKp46 ligation is shared with CD16 (23), suggesting that activation-induced regulation of receptor expression may be shared. We now show that activation-induced MMP production decreases NKp46 expression.
expression on NK cells. We note that it will be useful to corroborate our findings of NKp46 downregulation on NK cells activated through CD16 with studies analyzing NKp46 expression on NK cells activated by Ab-coated HIV-infected cells (40–42). However, the currently presented observation raises several questions about the role of anti-HIV ADCC, and antiviral NK cell responses in general, in slowing the progression of chronic HIV infection toward AIDS.

Potent ADCC Ab levels are associated with slower progression from HIV infection to AIDS (4). Indeed, some studies (4) demonstrated that HIV-infected elite controllers have heightened levels of Abs capable of mediating anti-HIV ADCC. Furthermore, SPs have ADCC responses against a broader array of HIV Ags than do non-SPs (5). Several groups also published data suggesting the ability of NK cells to mediate ADCC decreases during HIV infection (11, 14, 17, 22). The association of ADCC with slower disease progression was observed in the rhesus macaque SIV model: maintenance of ADCC activity was observed in slow progressing macaques, and lower plasma ADCC activity was observed in macaques with more rapidly progressing infections (6). Cumulatively, these studies suggest that ADCC could play a pivotal role in slowing disease progression. Because of the ramifications of the activation of NK cells for ADCC on phenotype and functionality, we now question the notion that the continued stimulation of NK cells for ADCC during chronic HIV infection is directly involved in slowing disease progression.

Several groups demonstrated that the activation of NK cells by ADCC Abs results in decreased surface expression of CD16 and makes NK cells refractory to further stimulation (26, 29). The data presented in this article corroborate the previous finding of de-

**FIGURE 5.** Downregulation of NKp46 by anti-HIV Ab-dependent NK cell activation involves MMPs. (A) Dot plots assessing the effect of pharmacological MMP blocking on activation-induced NKp46 downregulation. Dot plots represent data from 2 of 10 tested donors. Inclusion of the GM6001 MMP inhibitor in the anti-HIV Ab-dependent activation assay maintained NKp46 expression on NK cells (right panels) at higher levels than that observed on NK cells stimulated in the absence or presence of the DMSO vehicle (middle panels) and approaching that observed on nonstimulated NK cells (left panels). A consistent effect was observed across all 10 donors tested (bottom panel). (B) Dot plots demonstrate that maintenance of NKp46 expression in the presence of the GM6001 MMP inhibitor was dose dependent.
creased CD16 expression and demonstrate that CD16-mediated activation reduces the expression of the key activating NK cell NKp46 receptor. The NKp46 receptor was demonstrated to be essential for the in vitro functionality of NK cells (15) and, consequently, was suggested to be crucial to the in vivo immunity conferred by NK cells against tumors and viruses (24, 25). The level of NKp46 expressed on the NK cell surface is associated with the magnitude of the response elicited upon ligation (15). Chronic HIV infection that induces ongoing activation of NK cells due to ADCC would be expected to elicit in vivo phenotypic and functional changes in NK cells similar to those observed after in vitro activation (i.e., reduced CD16 expression, hyposresponsiveness, and reduced NKp46 expression) (26–29, 39). Indeed, HIV infection results in a set of dysfunctional NK cell changes similar to that observed after CD16-mediated activation in vitro (11–22). Thus, we speculate that continued activation of NK cells as a result of ADCC during untreated chronic viral infections could explain the reduced functionality of NK cells observed during HIV infection.

Although the idea that chronic activation of NK cells for ADCC would induce phenotypic alterations, and functional anergy fits with the presently available data on in vitro NK cell activation, it raises the question of how ADCC responses are associated with slower disease progression in HIV-infected individuals (4). We hypothesize that the primary usefulness of HIV-specific ADCC in natural HIV infection is related to the presence of ADCC responses during early HIV infection. As such, it is possible that the trend toward increased ex vivo functional potential of NK cells from HIV-infected SPs (43), compared with those from individuals with more rapidly progressing infections, is a result of a lower level of chronic Ag-induced in vivo activation. Indeed, because SPs generally exhibit lower viral loads than do progressors, it is possible that the higher functionality and more intact phenotype of SPs’ NK cells is due to lower Ag burden rather than the ADCC itself. It is feasible that the presence of ADCC during acute HIV infection (44) can reduce set point viral load, efficiently reducing Ag burden during chronic viral infection and minimizing the chronic activation of NK cells by anti-HIV ADCC. This possibility is concordant with the epidemiological observation that SPs have higher frequencies of inhibitory KIR3DL1/HLA-Bw4 ligand combinations than do individuals with more rapidly progressing infections (45). It was demonstrated that these KIR/HLA combinations increase the functional potential of NK cells during the ontological process of NK cell education (46, 47) and that this education results in higher anti-HIV ADCC against autologous targets (48). Furthermore, an additional study (49) demonstrated that NK cells carrying KIR3DL1 are expanded in primary HIV infection. If the activation of KIR3DL1-expressing NK cells for anti-HIV ADCC occurs primarily during acute HIV infection to reduce viral load set point in SPs, it would reduce the requirement for chronic activation of this NK cell subset and explain how NK cells from SPs maintain higher functionality than do those from more rapid progressors throughout chronic infection. It should be noted that ADCC Abs induced by vaccination prior to HIV infection may well have a protective role, as suggested by the RV144 trial and macaque studies (7–9), because NK cells in uninfected individuals should exhibit maximal NK cell functional potential.

The concept that chronic in vivo ADCC responses correlate with HIV disease progression is supported by two recent independent studies. Two groups separately demonstrated that HIV-infected subjects carry a CD16 polymorphism, which increases the IgG binding of the receptor and enhances the NK cell ADCC potential (50), exhibit faster disease progression (51, 52). It should be noted that an additional study by Forthal et al. (53) did not observe an effect of CD16 polymorphisms on HIV disease progression. However, studies revealing a potential role for CD16 polymorphisms in HIV disease progression, in combination with the current study and other studies showing alterations in NK cell phenotype and functional potential as a result of activation through CD16 (26, 29), suggest that a critical reassessment of the role of ADCC in chronic HIV infection is essential.

In combination with other studies (26, 29) demonstrating that the activation of NK cells through CD16 can alter cellular phenotypes and reduce functional potential, as well as investigations (51, 52) linking a more potent CD16 polymorphism with enhanced HIV disease progression, the data presented in this article fortify the necessity for future studies reassessing the role of ADCC in the progression of HIV infection to AIDS. Future studies should focus on the longitudinal evaluation of the ability of autologous combinations of NK cells, Abs, and HIV-infected CD4+ T lymphocytes to induce ADCC in groups of patients with different stages of disease progression. Such a study would allow the unique NK cell CD16 genotypes (51, 52), potentially protective KIR/HLA-I combinations (45), and Ab-glycosylation patterns (54) to be taken into consideration and would clarify the role of ADCC in HIV infection.

In summary, we provide novel and important information about the scope of the effects of Ab-dependent activation on NK cell phenotype. That NK cell activation by anti-HIV ADCC Abs can result in alterations in the NK cell phenotype, which reflect phenotypic alterations associated with disease-related NK cell dysfunction, suggests that a critical reassessment of the role of ADCC in slowing HIV disease progression is necessary.

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Disclosures

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