HIV inhibits early signal transduction events triggered by CD16 cross-linking on NK cells, which are important for antibody-dependent cellular cytotoxicity

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ABSTRACT

Measurement of NK cell cytolytic activity in the setting of chronic viral infection is important for determining viral pathogenicity. Mobilization of LAMP-1 (CD107a) to the NK cell surface is a surrogate marker for cytotoxic granule release and hence, NK cell cytotoxicity. We have developed a convenient, rapid, whole blood flow cytometric assay for measuring CD107a mobilization in response to CD16 cross-linking, a surrogate for NK cell ADCC activity ex vivo, which can be performed using small volumes of patient whole blood. Using this assay, we show that CD107a mobilization, in response to CD16 cross-linking, is triggered in CD56dim but not CD56bright NK cells, requiring Syk/Zap70 tyrosine kinase activity, and that there is a significant correlation between CD107a mobilization and pSyk/Zap70 tyrosine kinase activity, and that there is a significant correlation between CD107a mobilization and pSyk/Zap70 in response to CD16 cross-linking. We compared whole blood from treatment-naı√ve, HIV-infected patients with age- and sex-matched HIV-uninfected control subjects and found a significant reduction in CD16-dependent pSyk/Zap70 (median=32.7% compared with 67.8%; P=0.0002) and CD107a mobilization (median=9.72% compared with 32.9%; P=0.046) in NK cells. Reduction of both correlated strongly with reduced CD16 surface expression on NK cells of HIV-infected individuals (P<0.01). These data suggest that ADCC is inhibited in NK cells from therapy-naı√ve, HIV-infected individuals at the level of early events in CD16 signal transduction, associated with low CD16R expression, and our method is a useful and reliable tool to detect pathological defects in NK cell degranulation. J. Leukoc. Biol. 89: 149–158; 2011.

Introduction

NK cells play a major role in detecting and killing tumor cells in host versus graft reactions and in antiviral immunity, independently and in association with the adaptive immune response. The “decision” to initiate killing is based on a balance of intracellular signals that the NK cell receives through activating versus inhibitory surface receptors, which recognize ligands on the target cell [1–3]. In addition to innate immune mechanisms, i.e., NC, NK cells have potent ADCC activity, which depends on their ability to bind antibody-opsonized targets using the low-affinity IgG FcγRIIIA (CD16) expressed on their surface [4]. Under pathological conditions, ADCC and NC may be impaired, as has been shown in the setting of HIV infection [5–11].

NK cells are able to kill HIV-infected cells via ADCC [12]: the levels of HIV-specific antibodies capable of promoting ADCC and of NK cells are associated with beneficial outcomes of disease progression. The presence of HIV-specific, ADCC-mediating antibodies correlates with higher and stable CD4+ T cell counts [13, 14] and lower HIV RNA in plasma [15], findings that are supported by studies in the macaque model [16]. In acute infection, HIV-specific, ADCC-mediating antibodies have the potential to control viremia in vivo and in vitro [17, 18]. In one study, elite controllers of HIV infection, although having similar levels of neutralizing antibody to viremic patients, had higher levels of NK ADCC-mediated killing [19]. The importance of FcγR-expressing effector cells was established in studies using SHIV-infected macaques, in which broadly neutralizing antibodies, engineered to lack the Fc fragment, lost their ability to protect in a vaginal challenge model [20]. Even when neutralizing antibodies are present, the loss of NK cell numbers and function is associated with a more rapid disease progression [5, 21, 22].
NK cell defects in HIV infection include reduced expression of NC receptors, the loss of several activating receptors, and also, the loss of the ability of NK cells to perform ADCC [10, 13, 23, 24]. Knowledge of how HIV impairs NK cell cytotoxicity is essential to understand why the immune system cannot control HIV infection and the immune defects in HIV-infected patients. In addition, characterizing defects in NK cell ADCC in detail might identify new therapeutic approaches and factors contributing to improved immunization strategies.

NK cells are generally defined as CD3-CD56+ lymphocytes with two main subpopulations: CD56dim and the minor subset of CD56bright NK cells [25]. A minor CD56-negative subpopulation has also been described under certain pathological situations [26]. These subsets exhibit phenotypic and functional differences [25, 27, 28], and the most characteristic is expression of CD16, which is expressed solely on the CD56dim subset [29]. Functionally, the CD56dim16+ cells are more efficient at killing. The CD56bright16– subpopulation produces higher killing. The CD56bright16+ cell ADCC in clinical samples and explore its use by comparing CD16-dependent CD107a mobilization in NK cells from HIV-infected and -uninfected individuals. We show that NK cells from treatment-naïve, HIV-infected individuals have defective early signaling events in ADCC.

Measurement of receptor-specific signaling and CD16-dependent CD107a mobilization

To measure FcyR-specific ITAM signaling, activation of pSyk/Zap70, and mobilization of CD107a, 100 μl whole blood collected in sodium heparin tubes was incubated on ice in polypropylene tubes (Falcon, BectonDickson, San Diego, CA, USA). To measure pSyk/Zap70 following CD16 cross-linking, 5 μl (5.5 μg) anti-CD16 (clone 3G8, kindly donated by Mark Hogarth, Burnet Institute, Melbourne, Australia) was added and incubated on ice for 10 min. Cells were washed once with ice-cold PBS and then centrifuged (600 g, 7 min, 4°C), supernatant discarded, and the cell pellet resuspended and incubated with 8 μl goat anti-mouse F(ab’)2 fragment (55487, ICN Cappel, Costa Mesa, CA, USA) for a further 5 min before transferred to a 37°C water bath to initiate signaling. The reaction was stopped after the indicated times by adding Lyse/Fix buffer (558049, BD Biosciences, San Jose, CA, USA) for 10 min at 37°C. To measure NC-dependent pSyk/Zap70, the blood was incubated with 2 × 106 K562 cells, followed by trans-
fer to a 37°C water bath. Cells were fixed after the indicated times as above.

Lyse/Fix-buffered cells were centrifuged (600 g, 7 min, 22°C) and resuspended in 1 ml Perm/Wash solution 1 (BD Biosciences) for 10 min at 22°C, washed in Perm/Wash, and then blocked in 100 µl Perm/Wash solution containing 10% mouse serum (M9905, Sigma Chemical Co., St. Louis, MO, USA) for 10 min at 22°C. Cells were incubated with a cocktail containing anti-pSyk/Zap70-PE (5 µl; BD Biosciences; 554656), and CD16-APC (IM2474, Beckman Coulter, Fullerton, CA, USA) for 10 min at 22°C. RBCs were lysed with Becton Dickinson FACS lysing solution (1.5% formaldehyde in PBS–) (10 min, 22°C) and resuspended in 1 ml Perm/Wash solution 1 (BD Biosciences) for 10 min at 22°C, washed in Perm/Wash, and then blocked in 100 µl stain buffer (BD Biosciences; 554656), and stored at 4°C until data acquisition.

To measure mobilization of CD107a to the cell surface in response to CD16 cross-linking, 100 µl aliquots whole blood were treated as above for the measurement of pSyk/Zap70 with the following amendments: following cross-linking of CD16 or incubation with K562 cells, cells present in whole blood were incubated for 10 min on ice, washed, and blocked in 100 µl ice-cold PBS containing 10% mouse serum (Sigma Chemical Co.; M9905) for 10 min at 4°C (only CD16 cross-linked samples) and then treated with 10 µl anti-CD107a antibody (BD Biosciences; 555801) and cultured at 37°C for 1 h. Brefeldin A (final concentration, 10 µg/ml) and monensin (final concentration, 5 µM) were added, and the cells were incubated for 2–5 h [48]. Cells were then fixed and processed for flow cytometry as above. Samples were analyzed by flow cytometry using a FACScalibur analyzer (BD Biosciences).

Measurement of CD16 surface expression
Surface antigen staining was performed using 100 µl whole blood, collected in EDTA-containing tubes, incubated with CD55-FITC (BD Biosciences; 555332), CD16-PE-Cy7 (BD Biosciences; 557744), and CD56-APC (IM2474, Beckman Coulter; IM2474) at saturating concentrations in the dark at 22°C for 30 min. RBCs were lysed with Becton Dickinson FACS lysing solution (10 min, 22°C), and cells were washed once with cold PBS–. Cells were resuspended in 200 µl 1.5% formaldehyde in PBS– (10 min, 22°C) and stored in the dark at 4°C until analysis by flow cytometry. Surface staining was measured within 6 h using a Becton Dickinson FACSAria cell sorter/analyzer.

Analysis of NK cell signaling (pSyk/Zap70) and degranulation (CD107a) was performed on the CD3−CD56dim subset, unless stated otherwise. To minimize inter assay variability, the instruments were calibrated using Sphero Rainbow beads (BD Biosciences; 556291) before each analysis, and uncompensated data were recorded. Postacquisition compensation and analysis were performed with FlowJo (Version 8.8.4, Tree Star Inc., Ashland, OR, USA). A population comparison tool (based on the Overton’s cumulative subtraction [54]) was used to estimate percent-positive cells after stimulation in comparison with a Time 0 or untreated control.

Statistical analysis
Statistical significance between uninfected and HIV-1-infected groups was calculated using the nonparametric Wilcoxon signed rank sum test. Statistical significance was assumed when probability values were <0.05. Spearman’s rank test for nonparametric data was used to determine associations, and an association was assumed to be significant when probability values were <0.05. Statistical analyses were carried out using STATA for Macintosh software (V10.1, StataCorp, College Station, TX, USA) or GraphPad Prism for Mac OS X (V5.0a, GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Syk/Zap70 is phosphorylated in NK cells following CD16 cross-linking but not incubation with K562 cells
To measure ITAM-dependent signal transduction associated with ADCC or NC, cells present in whole blood were stimulated for 0–60 min by addition of anti-CD16 followed by cross-linking with goat anti-mouse F(ab′)2 or by addition of K562 cells, respectively. NK cells were identified by the gating strategy shown in Fig. 1A. CD16 cross-linking led to a rapid pSyk/Zap70 between 1 and 3 min in the CD56+ NK cell population, which returned to baseline levels after 5 min (Fig. 1C). In contrast, there was no pSyk/Zap70 in response to CD16 cross-linking evident at any time-point in the CD3−CD56dim NK cell population (Fig. 1B), which is consistent with the lack of CD16 expression on these cells. Incubation of whole blood with the NC target cell line K562 did not result in pSyk/Zap70 within either NK cell subset. The kinetics of pSyk/Zap70 in response to the various stimuli for independent experiments from three independent donors is summarized in Fig. 2. pSyk/Zap70 was induced rapidly following CD16 cross-linking, measured as percent-positive cells or mean fluorescence within 1.5 min after activation, and declined quickly, returning to baseline levels within 1 h. No pSyk/Zap70 was observed in response to addition of K562 cells at any time within 60 min of adding target cells.

We next compared the kinetics of CD107a mobilization on NK cells present in whole blood in response to CD16 cross-linking or exposure to K562 cells. In CD56dimCD16+ NK cells, CD107a was mobilized after CD16 cross-linking, whereas in CD56brightCD16− NK cells, there was no change in CD107a (Fig. 3A), suggesting that CG degranulation is stimulated by CD16-mediated signal transduction. CD107a was mobilized rapidly in response to CD16 cross-linking within 3 h and reached a value of 50%–positive cells at 5 h compared with 75% following stimulation with 40 ng/ml phorbol ester and 1 µg/ml ionomycin as a positive control (Fig. 3B). CD107a

Table 1. Description of HIV-Infected Patients and Control Subjects Enrolled in this Study: For All Variables, Median and Range Are Given

|                      | HIV− | HIV+ | Wilcoxon rank sum P value |
|----------------------|------|------|---------------------------|
| Number (n)           | 9    | 17   |                          |
| Age                  | 51 (28–64) | 40.5 (26–63) | 0.29                      |
| CD4 count            | not determined | 429 (41–907) |                        |
| % CD4                | 60.45 (48.8–75.4) | 31.95 (4.57–52.5) | <0.001                    |
| Viral load           | not determined | 21,500 (<50–100,000) |                        |
| % CD3−CD56+ (% of lymphocytes) | 8.3 (2.28–19.5) | 3.3 (1.44–12.6) | 0.0076                      |
| % CD56bright (% of CD56+ NK cells) | 5.88 (4.36–34.6) | 9.72 (3.57–24.8) | 0.27                        |
| % CD56dim (of CD56− NK cells) | 94.1 (64.7–95.6) | 90.3 (74–96.4) | 0.26                        |
surface expression was specific for CD16 cross-linking, as whole blood incubated with goat anti-mouse F(ab')2 alone did not result in CD107a labeling in NK cells. However, we observed a gradual increase in nonspecific CD107a mobilization in untreated whole blood after 3 h of incubation (Fig. 3B). Experiments using the K562 cell line to induce CD107a mobilization in whole blood needed a longer incubation period (5 h or longer) to obtain measurable changes in CD107a expression; however, results varied highly from 5% to 20% (n=5) between different blood donors after 5 h (data not shown).

CD107a mobilization in response to CD16 cross-linking is a Syk/Zap70-dependent process

To further investigate the signaling requirements for CD107a degranulation downstream of CD16, whole blood was preincubated for 30 min with increasing concentrations (1–10 μM) of Syk inhibitor 1, followed by CD16 cross-linking and measurement of CD107a mobilization as above. Syk inhibitor 1 reduced CD107a mobilization in a dose-dependent manner with a near-complete inhibition at 10 μM (Fig. 4). However, Syk inhibitor 1 at these concentrations did not inhibit tyrosine pSyk or pZap70, suggesting that phosphorylation at Y352 (or Y319, respectively) in NK cells is not predominantly a result of autophosphorylation but likely, of transphosphorylation by Src kinases.

HIV infection impairs pSyk and CD107a mobilization following CD16 cross-linking and is correlated with CD16 surface expression

CD107a mobilization has been used as a surrogate measure of NK cell killing. Having shown that CD107a mobilization can be measured conveniently and reliably following CD16 cross-linking in small aliquots of whole blood and hence, may be
used as a surrogate measure for ADCC activity in NK cells, we used this assay to compare NK cell ADCC activity and CD16-dependent pSyk/Zap70 in HIV-infected patients and HIV-uninfected control subjects whose blood was analyzed within 3 h of collection. In a study group of treatment-naive, HIV-infected individuals (Table 1; \( n = 17 \)), CD16-dependent pSyk measured at its peak (1.5 min) was reduced significantly in CD3\(^{-}\)CD56\(^{\text{dim}}\) NK cells compared with HIV-uninfected individuals (\( n = 9 \); median 32.7% compared with 67.8%; \( P = 0.0002 \); Fig. 5A, left panel) with defective CD16-dependent signal transduction in NK cells from these individuals. Levels of pSyk/Zap70 did not correlate with CD4 count or viral load (in this cohort of therapy-naive individuals; Spearman’s rho: \(-0.014, P = 0.59, \) and \(0.31, P = 0.22, \) respectively) but strongly correlated with the percentage of NK cells in total lymphocytes (Spearman’s rho: 0.48, \( P = 0.013 \); Fig. 5B, left panel).

In a subset of 11 HIV-infected individuals and seven uninfected controls, for which these data were collected, we observed that in blood from therapy-naive, HIV-infected patients, there was a significant reduction in CD107a mobilization in CD3\(^{-}\)CD56\(^{\text{dim}}\) NK cells (median 9.72% compared with 32.9%; \( P = 0.046 \); Fig. 5A, right panel). CD107a mobilization did not correlate with CD4 count or viral load in this cohort of therapy-naive individuals (Spearman’s rho: \(-0.018, P = 0.96, \) and \(0.20, P = 0.57, \) respectively) but did strongly correlate with NK cell proportion of lymphocytes (Spearman’s rho: 0.77, \( P < 0.01 \); Fig. 5B, right panel). These data suggest that following CD16 cross-linking, pSyk/Zap70 and degranulation measured by

![Figure 2. Time course of pSyk/Zap70 in CD56\(^{\text{dim}}\) NK cells.](image)

![Figure 3. CD107a mobilization in NK cells.](image)
CD107a surface expression are impaired significantly in CD56dimCD16⁺ NK cells during HIV infection. There was a strong correlation between CD107a mobilization and pSyk/Zap70 measured using this assay (Spearman’s rho: −0.8246, \( P<0.01 \)), suggesting that early signal transduction events upstream of Syk/Zap70 activation are rate-determining for NK cell degranulation (Fig. 5C).

When CD16 surface expression was measured, a significant reduction on CD3−CD56dim NK cells from HIV-infected subjects was observed (median MFI=1194 compared with 4763; \( P<0.001 \); Fig. 6A). There was a strong correlation between CD16 surface expression and CD107a mobilization (Spearman’s rho: −0.769, \( P<0.001 \); Fig. 6B, right) and pSyk/Zap70 in response to CD16 cross-linking (Spearman’s rho: −0.836, \( P<0.001 \); Fig. 6B, left), suggesting that CD16R expression may be rate-determining for early signal transduction and the ability of the cell to degranulate.

**DISCUSSION**

We have developed a rapid, convenient, and reliable assay to measure CD16-dependent mobilization of CD107a, requiring no target cell line and only small volumes of whole blood, which can be used as a measure of early events in antibody-dependent cellular cytotoxicity by NK cells under pathological conditions. As the assay does not require purification of NK cells or separation of these cells from autologous plasma, the assay can be used with a limited amount of patient sample (100 μl/test tube) and also likely reflects the activity of NK cells Syk Inhibitor I (μM) pSyk/Zap70 CD107a

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cells in vivo, in contrast to assays using isolated peripheral blood cells or purified NK cells [49–51, 53, 55–57]. We have demonstrated the potential use of this assay in a limited, cross-sectional clinical study, demonstrating for the first time reduced pSyk/Zap70 and CD107a mobilization in response to CD16 cross-linking on CD56dim NK cells from therapy-naive, HIV-infected individuals. Furthermore, we show a close relationship between CD16 surface expression and the magnitude of CD16-mediated function. These results suggest that ADCC is inhibited in NK cells from ART naïve, HIV-infected individuals through the modulation of CD16R surface expression.

Usually, CD107a mobilization is assessed in isolated PBMC or PBL [49–51, 53, 55–57]. Whole blood measurements of CD107a mobilization have been used before to study HIV-specific responses of NK cells using HIV antigen peptides to trigger degranulation, indicating an underlying ADCC mechanism [58]. However, to our knowledge, the direct link between CD16 stimulation and CD107a mobilization and characterization of intracellular signaling events required for CD107a mobilization in ADCC has not been demonstrated. Our data show that CD16 cross-linking swiftly leads to robust pSyk/Zap70, which was not observed following incubation with the NC target cell line K562. This result indicates that CD16 stimulation specifically leads to activation of an ADCC-specific pathway, which is not necessarily involved in NC. Evidence that degranulation of CG in NK cells during ADCC and NC requires separate signaling pathways has been described in experiments showing differential sensitivity to the PI3K inhibitor wortmannin [59–61].

We observed an increase in CD107a mobilization in untreated whole blood after 3 h in culture, possibly caused by increased cell death, RBC lysis, or bystander effects. In our hands, long-term whole blood culture (>5 h) resulted in unspecific increases in background signal during the measurement of NC, causing varying results for K562 stimulation (5–20% CD107a-positive cells in comparison with untreated cells; n=5; data not shown), which could be overcome potentially by preculturing the cells for 12–16 h [62].

We demonstrated that inhibition of Syk using a specific pharmacological inhibitor of Syk tyrosine kinase activity leads to inhibition of downstream CD107a mobilization, however did not observe an effect on pSyk/Zap70 at Y352/319, which is phosphorylated via the activity of an upstream Src kinase [63, 64]. It is unclear whether NC requires Syk or Zap70 activity or neither [65, 66], although involvement of Syk/Zap70 in NC signaling is plausible, as NC receptors such as NKp46 and NKp30 possess an ITAM motif and therefore, potential binding sites for Syk/Zap70 kinases. The question of which kinase was activated during NC in the present study could not be answered using the Phosflow method, as the antibody used to detect phosphorylation of the Y352/319 residue does not distinguish between the two kinases. However, we observed a decrease in CD107a mobilization following K562 incubation in response to the Syk inhibitor (data not shown), suggesting a requirement for Syk or Zap70 activity.

In contrast, our data show a clear relationship among CD16 cross-linking, pSyk/Zap70, and NK cell degranulation measured by CD107a mobilization. To our knowledge, our data showing inhibition of CD107a mobilization by Syk inhibitor 1 include the first demonstration that Syk tyrosine kinase activity is a required event in CD107a mobilization in NK cells. This also shows that CD107a labeling in our assay is dependent on CD16 cross-linking without the involvement of other receptors/pathways, supporting the idea that this assay measures early events in ADCC signaling. Our clinical data further support this conclusion: the strong correlation of CD16 surface expression, pSyk/Zap70, and CD107a mobilization indicates that degranulation depends on CD16-mediated pSyk in NK cells of HIV-infected and -uninfected individuals. Furthermore, the strong correlation among CD16 expression, Syk/Zap70 signal transduction, and degranulation validates CD16-dependent CD107a mobilization as a read-out for a functional signal-
ing pathway between the receptor and effector function, which may be used in conjunction with measurement of actual killing of target cells by Cr⁵¹ release: thus, although this assay informs about intracellular pathways initiating CG release, the Cr⁵¹ release assay quantifies the cytotoxic potency of the cell.

NK cell ADCC is clinically relevant, given that NK cells are able to eliminate HIV-infected cells directly [12], and is associated with slower disease progression [5, 21, 67]. However, the titers of ADCC-mediating antibodies and ADCC competence of NK cells decrease during HIV disease progression [13, 22, 68]. Our data support earlier studies showing a reduced proportion of total CD3–CD56⁺NK cells decrease during HIV disease progression [13, 22, 68]. Further, in the present study, we show reduced ADCC in NK cells from infected individuals, confirming previous findings [9, 10, 13]. NK cell function strongly correlated with total NK cell numbers, emphasizing the importance of monitoring NK cell populations during HIV disease progression [70].

NK cell NC capacity (measured by CD107a mobilization by NKs present in PBMCs) was reported to be higher during active HIV replication compared with uninfected controls, despite lower overall NK numbers [55, 71], potentially suggesting a priming for or skewing toward NC as a result of HIV infection [72, 73]. Further, a general mechanism has been described, leading to CD16 down-regulation by NKs present in PBMCs) was reported to be higher during active HIV replication compared with uninfected controls, despite lower overall NK numbers [55, 71], potentially suggesting a priming for or skewing toward NC as a result of HIV infection [72, 73].

Our finding that CD16 surface expression, CD16 signal transduction, and CD107a mobilization are impaired in NK cells in the context of HIV infection represents novel data about underlying causes for impaired NK cell function in HIV disease. It is possible that decreased CD16 expression is a consequence of the loss of its chaperone FcRγ [75]. Depending on which components are rate-determining for pSyk, it is uncertain whether loss of signal transduction is a result of decreased levels of CD16 or of other components of early signaling such as FcRγ and TCRγ chains [75, 76]. Future studies will have to address this question further.

AUTHORSHIP

This study was conceived and designed by A.J. and G.F.L. Experiments were performed by G.F.L., A.C.M., and W.J.C. with substantial technical advice by P.U.C. Results were interpreted and the manuscript prepared by A.J. and G.F.L. Constructive discussion, reading, and contribution to the manuscript were done by S.R.L. and S.M.C.

ACKNOWLEDGMENTS

This study was funded through the National Health and Medical Research Council of Australia (NHMRC), project grant 543137. We thank Prof. Mark Hogarth for donating the anti-CD16 antibody, Cath Downs and the clinical research nursing staff of the Infectious Diseases Unit, The Alfred Hospital, for patient recruitment, and all participants and blood donors for the HiACT study. Further, we thank Dr. Marjon Navis, University of Melbourne, for constructive and critical discussion of the results.

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KEY WORDS:
CD107a • ADCC • signaling