Activation of NK Cells by ADCC Antibodies and HIV Disease Progression

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Abstract: Antibody-dependent cellular cytotoxicity (ADCC) is of considerable interest as an immune response that may facilitate the control of HIV infection. We studied ADCC responses prospectively in a cohort of 79 HIV-positive subjects followed up for a mean of 2.3 years without antiretroviral therapy. We used a novel assay of the ability of ADCC to activate natural killer (NK) cells, either from the same HIV-positive subject or from a healthy blood donor. We found that ADCC responses to either gp140 Env protein or HIV peptide pools were common in HIV-positive subjects when NK cells from the HIV-positive subject were used but did not correlate with markers of HIV disease progression. In contrast, ADCC responses to whole gp140 Env protein were strongly associated with a slower decline in CD4 T-cell loss when healthy donor NK cells were used as effectors. Our data had implications for induction of the most effective ADCC responses by HIV vaccines.

Key Words: HIV, ADCC, NK Cells, CD4 T cells, viral load

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INTRODUCTION

There is an urgent need to identify effective immunity against HIV.[1–3] The recent Thai RV 144 HIV vaccine trial led to modest but significant protective efficacy.[4] There is intense interest in the correlates of immunity in this study. $CD8^+$ cytotoxic T lymphocyte responses were infrequently induced, and neutralizing antibody responses were typically narrowly directed toward the vaccine strains only. Antibody-dependent cellular cytotoxicity (ADCC) antibodies were, however, induced by this regimen and could be responsible, in part, for the efficacy observed.[5]

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ADCC antibodies mediate their function by bringing innate immune cells, such as natural killer (NK) cells, into contact with adaptive antibody responses to control virus-infected cells.[6] The impact of ADCC on HIV disease progression is controversial. ADCC antibodies are often present at very high titers in HIV-infected subjects, and there is a considerable body of mainly older literature with many, but not all, correlating ADCC responses with slower progression of HIV infection (reviewed in Chung et al[7]). Several cross-sectional human cohort studies have demonstrated that ADCC antibodies correlate with slower progression of HIV-1 infection.[8–11] However, these studies have used healthy donor peripheral blood mononuclear cells as effector cells to evaluate the efficacy of ADCC activity, some of the cohorts studied have been small, and most studies have been cross-sectional. No previous studies have compared autologous NK cells with healthy donor NK cells for their efficiency in mediating ADCC effector functions. In addition, ADCC activity has primarily been measured through target cell killing or viral inhibition of Env-protein–coated cells; no correlations with other ADCC antibody-mediated effector mechanisms, such as cytokine secretion or degranulation, have been analyzed. Given the renewed interest in ADCC as a mechanism of control of HIV, we felt it was important to readdress HIV-specific ADCC immune responses in a new prospective cohort.

Newer assays have been developed to measure HIV-specific ADCC responses, including the recently described ADCC intracellular cytokine staining (ICS) assay. This assay measures NK cell activation (both cytokine expression and degranulation markers) in response to HIV antibodies and HIV proteins or peptides.[12] The activation of NK cells to express the degranulation marker CD107a correlates with ADCC responses as measured by standard cytotoxicity assays.[6] The responses in the assay are mediated by IgG in the plasma and is not dependent on free antigen–antibody complexes in the supernatant.[6,12]

We recruited a prospective cohort consisting of 79 antiretroviral therapy–naive subjects to investigate the role of ADCC immune responses on HIV disease progression. ADCC activity, as measured by either healthy donor or autologous NK cell activation, was correlated with markers of HIV disease progression during longitudinal follow-up.

SUBJECTS AND METHODS

Subject Cohort

HIV-infected adults (n = 79) not on antiretroviral therapy were recruited to donate blood samples between February

2004 and July 2008 (see **Table, Supplemental Digital Content 1**, http://links.lww.com/QAI/A201). The relevant human research ethics committee approved all studies, and all subjects provided informed consent. Subjects provided sodium–heparin anticoagulated blood for fresh whole-blood ADCC assays using the ICS method; plasma was stored for assays using the same healthy HIV-negative donor cells. Follow-up was censored if the patient commenced ART or at closure of the study in 2010. To facilitate analysis, all relevant data from the cohort was placed in a specifically designed database (Microsoft Access).

HIV-1 Antigens

All subjects were tested for ADCC activity against HIV-1 protein peptide pools containing 15-mers, overlapping by 11 amino acids, spanning all 9 HIV-1 proteins (HIV-1 consensus subtype B Gag, Pol, Env, Rev/Tat/Vpu (RTV), and Nef/Vif/Vpr (NVV), supplied by the National Institutes of Health AIDS Research and Reference Reagent Program). The large pool spanning the Pol enzyme was split into 2, termed Pol1 (peptides 1-125) and Pol2 (peptides 125-249). Subjects were also tested for ADCC responses to recombinant gp140 HIV-1_{AD8} Env protein.

NK Cell Activation Assay

The ICS-based ADCC assay was used as previously described.[12] In brief, for fresh whole-blood assays, 200 μl of blood was incubated with all the abovementioned antigens (1 μg/mL), or control dimethyl sulfoxide, for 5 hours in the presence of brefeldin A and monensin. At the end of the incubation and staining, $CD56^+CD3^-$ or $CD2^+CD3^-$ NK lymphocytes were studied for the expression of intracellular interferon gamma (IFNγ) and surface staining for CD107a. Antibodies (all from BD Biosciences, San Jose, CA) utilized in the assay were CD3 (PerCP, catalog #347344), CD2 (FITC, catalog #556611), CD56 (PE, catalog #555516), CD107a (custom order, APC), and IFNγ (Alexa700, catalog #557995). All assays were conducted using fresh whole blood to minimize manipulation of responding NK cells. The assays were conducted using both whole blood from the subjects and using 50 μLof subject's plasma incubated with 150 μL of healthy HIV-negative donor blood specifically testing with Env peptides or gp140 HIV-1_{AD8} Env protein antigens. We used healthy donor blood from a single subject, a white man in his 40s, similar to the demographics of the cohort tested. Responses were subtracted of background unstimulated responses, which were generally $<0.1\%$. The cut-off for a positive response to each antigen was the mean + 2 SD of responses in 10 HIV-negative healthy donors, which were generally $<0.1\%$.

Analysis and Statistical Methods

All analyses were conducted in accordance with a predetermined analysis plan on the database locked on 18 June, 2010. No imputations were made for missing data. All correlations were analyzed using Spearman correlation, as ADCC responses were not generally normally distributed. Associations between total HIV-specific ADCC response and initiation of ART were assessed using Wilcoxon signed rank sum test.

ADCC activity was measured by monitoring NK cell activation in response to antibody and peptide (or whole protein) antigen stimulation. ADCC responses were identified by (1) intracellular IFNγ expression, (2) $CD107a^+$ surface expression, or (3) dual expression of both IFNγ and CD107a. ADCC responses were directed to the antigens HIV-1 consensus subtype B Gag, Pol, Env, RTV, and NVV peptide pools or recombinant gp140 Env protein. ADCC responses were assayed using fresh whole blood from the subject or using stored subject plasma with healthy donor cells. No corrections were made for multiple comparisons.

RESULTS

NK Cell-Mediated ADCC Immune in ART-Naive HIV-Positive Cohort

We recruited 79 HIV-positive subjects not on ART to donate blood samples to measure ADCC (see **Table, Supplemental Digital Content 1**, http://links.lww.com/QAI/A201). The subjects had a mean CD4 T-cell count of 527 cells per microliter at enrollment to the study and were followed up for an average of 2.3 years. At completion of the study, the mean CD4 T-cell count had declined to 431 cells per microliter. During follow-up, 45 of the 79 subjects commenced ART.

All 79 subjects were tested for NK cell-mediated ADCC immune responses to all HIV-1 peptide pool antigens using the whole-blood antibody-dependent NK cell activation assay. ADCC responses were observed within 84% (66 of 79 subjects) of the cohort (Fig. 1A). The commonest and strongest ADCC responses were to the Env peptide pool, with 54 (68%)of the 79 responders targeting this antigen (Fig. 1B). ADCC responses to other overlapping peptide pools of other HIV-1 proteins were also detected, including pools comprising the regulatory/accessory pools, RTV (24%, 19 of 79 subjects) and NVV (26%, 21 of 79 subjects).

No Correlation Between Progression and Autologous Blood ADCC Responses

To investigate ADCC responses relative to disease progression, $CD4^+$ T-cell decline over time was correlated with ADCC responses as measured by our NK cell activation assay. $CD4^+$ T-cell decline was first correlated with ADCC responses detected using subjects' own whole blood, including the sum of ADCC response across all HIV-1 overlapping peptide pools, Gag, Pol, Env, RTV, NVV (Fig. 2A), and responses to specific protein peptide pools for Env (Fig. 2B) and accessory proteins Rev, Tat, Vpu, Nef, Vif, and Vpr (Fig. 2C). There were no statistically significant correlations between $CD4^+$ T-cell decline and ADCC responses, as measured using patient whole blood, to any specific HIV peptide pool or the sum of all responses.

In addition to the peptide pools, all cohort subjects were also tested for ADCC responses to whole gp140 HIV-1_{AD8} Env protein using fresh autologous whole blood in the NK cell activation assay. Whole gp140 protein can detect conformational ADCC epitopes and linear epitopes and may provide a broader analysis of Env-specific ADCC activity. Gp140-specific ADCC responses were then correlated with $CD4^+$

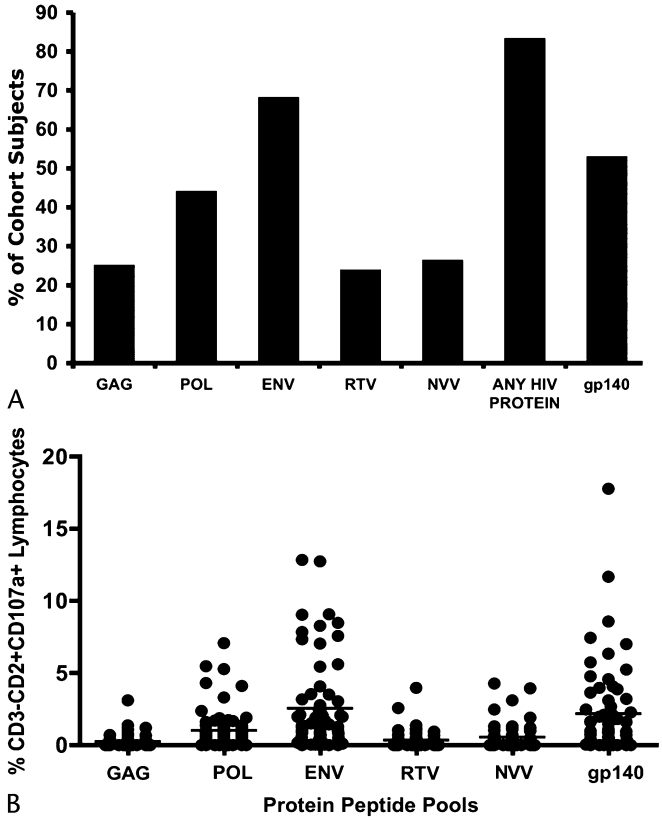

FIGURE 1. NK cell–mediated ADCC responses within the ADCC cohort. All 79 subjects within the cohort were assayed for ADCC responses to HIV-1 protein peptide pools, Gag Pol, Env, and the accessory proteins RTV (Rev, Tat, Vpu) and VVN (Vif, Vpr, Nef) using the ADCC ICS assay with fresh autologous whole blood. A, Number of subjects with ADCC responses to protein peptide pools along with whole gp140 protein. ''Any HIV protein'' refers to the number of subjects that had ADCC responded to at least 1 of the HIV protein peptide pool. B, Strength of ADCC responses as measured by %$CD3^-CD2^+CD107a^+$ lymphocytes.

decline over time. No statistically significant correlations were observed for analysis of total IFNγ expression, total CD107a expression, or dual IFNγ and CD107a expression (Figs. 2D–F).

Total ADCC responses were also correlated with HIV viral load at enrollment and the slope of the HIV viral load over time (Figs. 2G, H). No correlation was observed for either of these analyses. Similarly, there was no correlation with baseline CD4 T-cell count at enrollment (Fig. 2I).

All 79 subjects were ART-naive when recruited to the cohort; however, 45 subjects initiated ART during follow-up. We studied the association between time before commencement of antiretroviral therapy (years since recruitment) and total HIV peptide-specific ADCC responses. No correlation was observed for either IFNγ expression or CD107a expression from NK cells ($P = 0.79$ and 0.62, respectively).

ADCC Responses to gp140 Correlate With $CD4^+$ T-Cell Decline Using Healthy Donor Cells

To assess the ADCC activity of subjects' plasma alone without also assessing the function of autologous NK cells from the HIV-positive donor, heparinized plasma was collected from all subjects and tested with the same healthy donor PBMCs in the NK cell activation assay. We stimulated with either the Env overlapping peptide pool or gp140 HIV-1_{AD8} Env protein antigens. No correlation was detected between $CD4^+$ decline and Env peptide pools (Figs. 2J, K). However, a significant correlation was observed between $CD4^+$ decline and ADCC responses to gp140 when assaying with autologous plasma and healthy donor cells (for IFNγ expression, $P = 0.003$, Fig. 2L; for CD107a expression, $P = 0.058$, Fig. 2M; and for dual $IFN\gamma^+CD107a^+$ NK cells, $P = 0.004$, Fig. 2N).

DISCUSSION

We prospectively followed up 79 ART-naive HIV-infected subjects to analyze the relationship between HIV disease progression and ADCC responses using an assay that detects antigen-specific NK cell activation. Using autologous blood as the source of the NK cell effectors, no association between HIV-specific ADCC activity and HIV disease progression markers was observed. A strong association was, however, observed between ADCC activity to HIV-1 gp140 Env protein and slower progression when NK cells were derived from a healthy donor.

Our finding that ADCC responses to Env protein using healthy donor effector cells correlates with slower CD4 T-cell decline is consistent with other studies using killing-based ADCC assays.[8] The lack of correlation using autologous blood may reflect dysfunctional NK cells present in HIV-1–infected subjects.[13] NK cell dysfunction is most marked in HIV-positive subjects with viremia[14]—our untreated cohort had a mean viral load of approximately 50,000 copies per milliliter. We previously showed that NK cells from individuals with advanced HIV responded poorly to ADCC antibodies form other HIV-positive subjects.[12] This study on the response of healthy NK cells to HIV-specific ADCC antibodies used blood from a single healthy donor tested against all the plasma samples. There are differences in the responsiveness of NK cells between different donors, although in our experience the patterns of relative ADCC responses between different subjects are similar (data not shown). Future work could focus on testing NK cells from larger panels of both HIV-negative and HIV-positive subjects for ADCC reactivity.

Although a correlation between gp140-specific ADCC responses and CD4 T-cell decline was observed when donor NK cells were studied, no correlation was observed with Env overlapping peptide pool responses. Responses focused on conformational gp140 epitopes may be more effective than pooled responses to linear Env epitopes or to other HIV proteins that may not be presented efficiently to ADCC antibodies. ADCC assays to whole proteins or peptide pools measure the combined effect of several ADCC antibodies; particular ADCC responses to specific epitopes may be responsible for protective effects, as observed with cytotoxic T lymphocyte responses.[15] Recent work has shown that ADCC antibodies frequently target the C1 region of Env[16]; however, ADCC antibodies to Env can frequently select for immune escape variants.[17] Isolating specific ADCC monoclonal antibodies associated with control of HIV viremia should permit a more definitive assessment of their utility.[18]

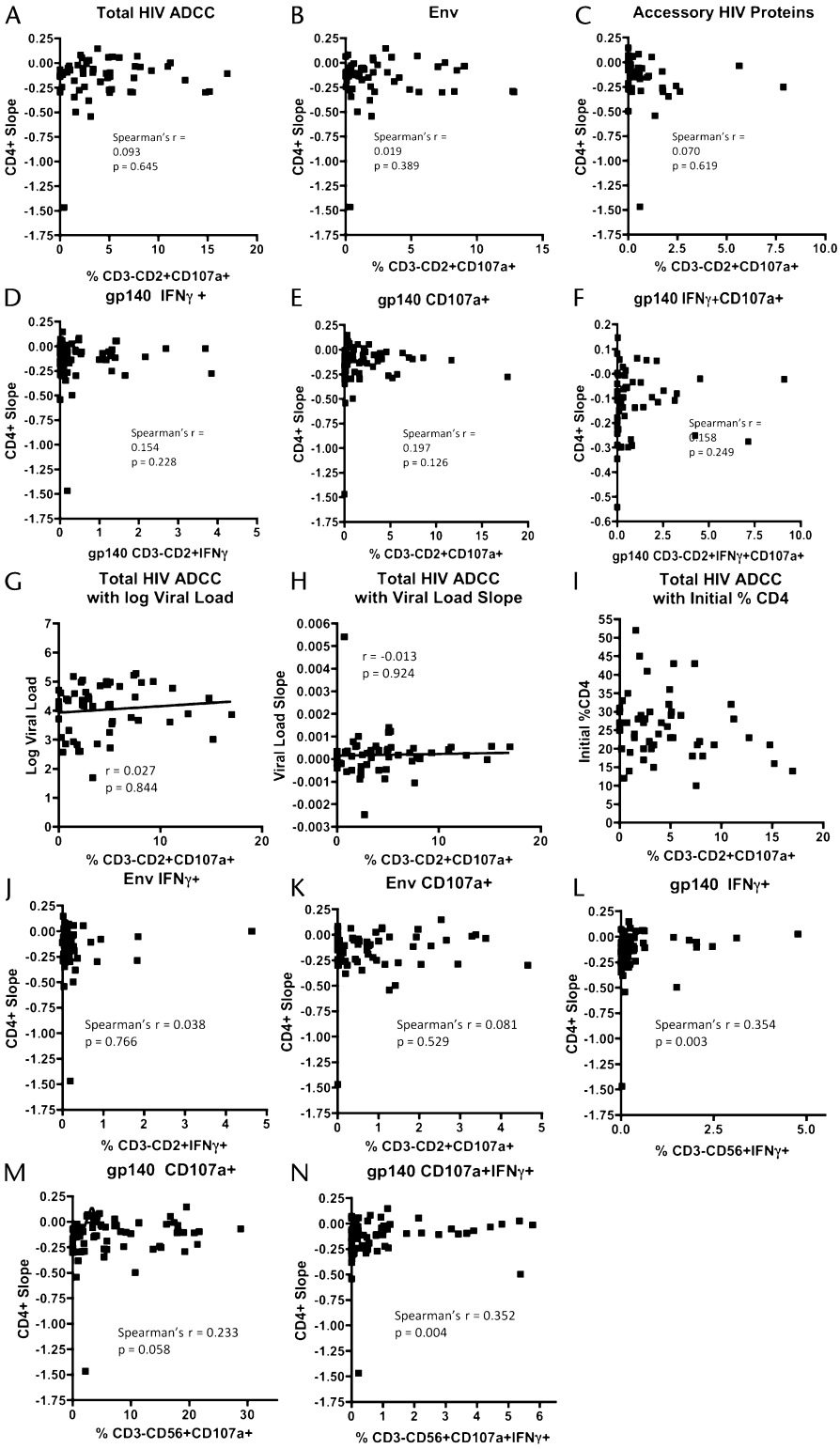

FIGURE 2. Correlation between HIV-specific ADCC activity and disease progression using autologous and healthy donor NK cells. A–F, Correlation with HIV-specific ADCC activity (%$CD3^{-}CD56^{+}$ NK cell $CD107a^{+}$ or IFNγ expression by ICS) in a cohort of 79 ART-naive subjects and as measured using autologous whole blood with slope of CD4 decline. A–C, Responses to peptide pools. D–F, Response to gp140 protein shown in D-F. A, Total HIV ADCC responses was calculated by addition of ADCC responses to all HIV protein peptide pools Gag, Pol, Env, and the accessory proteins (Rev, Tat, Vpu, Vif, Vpr and Nef). B, Consensus subtype B Env peptide pool ADCC responses. C, Consensus subtype B accessory HIV proteins peptide pools (Rev, Tat, Vpu, Vif, Vpr, and Nef). Correlation between CD4 decline and gp140-specific ADCC activity as measured by whole-blood ADCC ICS assay is shown as measured by (D) %IFNγ expression, (E) $\%CD107a^{+}$ expression, and (F) % double-positive $IFNg^{+}CD107a^{+}$ expression. Correlations of total ADCC activity using autologous whole blood and (G) baseline viral load, (H) viral load increase during follow-up, and (I) initiation of ART. Correlation between $CD4^{+}$ decline and ADCC activity as measured by ADCC ICS assay using the HIV-positive subject's plasma (50 μL) with healthy donor blood (150 μL). J–K, Response to Env peptide pool. L–N, Responses to gp140 protein. J, %IFNγ expression by NK lymphocytes to Env peptide pool. K, %CD107a expression by NK lymphocytes to Env peptide pool. L, %IFNγ expression by NK lymphocytes to gp140 protein. M, %CD107a expression by NK lymphocytes to gp140 protein. N, %IFNγ/CD107a double-positive expression by NK lymphocytes to gp140 protein.

In summary, this prospective study observed a correlation between total HIV-specific ADCC responses mediated by healthy donor NK cells and disease progression. We speculate that if ADCC responses were induced by vaccination, before HIV infection and consequently dysfunctional NK cell activity, these ADCC responses may assist in preventing infection.[4,5] HIV vaccine strategies that induce robust ADCC responses should be actively explored.

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