

Rapid Degranulation of NK Cells following Activation by HIV-Specific Antibodies¹

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Ab-dependent cellular cytotoxicity (ADCC) Abs stimulate NK cell effector functions and play a role in protecting from and controlling viral infections. We characterized ADCC Abs in a cross-sectional cohort of 80 HIV-infected subjects not on antiretroviral therapy. We analyzed ADCC response by killing fluorescently labeled target cells, as well as expression of IFN- γ and the degranulation marker CD107a from activated NK cells as measured by a novel intracellular cytokine assay. HIV-specific ADCC directed toward Envelope proteins were present in the majority of 80 untreated HIV-infected individuals measured by killing function. Similarly, most subjects had HIV-specific Abs that mediated degranulation or cytokine expression by NK cells. Interestingly, there was a poor correlation between ADCC-mediated killing of fluorescently labeled whole Envelope protein-pulsed cell lines and Ab-mediated expression of IFN- γ by NK cells. However, in contrast to healthy donor NK cells, autologous patient NK cells more effectively degranulated granzyme B in response to ADCC activation. Activation of NK cells in response to stimulation by HIV-specific Abs occurs at least as rapidly as activation of Gag-specific CTLs. Our studies highlight the complexity of Ab-mediated NK cell activation in HIV infection, and suggest new avenues toward studying the utility of ADCC in controlling HIV infection. *The Journal of Immunology*, 2009, 182: 1202–1210.

HIV is an urgent global health problem and a safe and effective vaccine is desperately required. Critical to vaccine development goals is an improved understanding of immune responses that can control or prevent HIV infection. T cell based vaccine strategies have recently failed to prevent or ameliorate HIV infection in a large efficacy trial based on a recombinant adenovirus (1, 2). Efforts to generate vaccines that induce broad neutralizing Abs (Nab)³ have not yet been successful, in part owing to occlusion of useful Nab epitopes (3).

Ab-dependent cellular cytotoxicity (ADCC) represents a potentially effective humoral immune response, where the Fc portion of particular HIV-specific Abs, most commonly specific for the surface Envelope (Env) protein, bind to Fc receptors of effector cells, triggering lysis of infected target cells and secretion of immunomodulatory cytokines. A recent study demonstrates the ability of passively transferred Nabs with Fc binding activity to control simian HIV infection of macaques (4). Several human cohort studies have demonstrated that ADCC Abs correlate with slower progression of HIV-1 infection (5–10). The effector functions of ADCC Abs are mediated primarily by cells of the innate immune system,

particularly NK cells. Theoretically, such a response could be mobilized rapidly in response to a new infection and potentially prevent the spreading cellular infection.

Although HIV-specific ADCC responses have been described for some time, they have been relatively understudied due to technical hurdles in quantifying these responses using the laborious and relatively insensitive radioactive chromium release assay (9). Improved ADCC assays have recently become available. Robert-Guroff and colleagues have pioneered a recent fluorescent killing assay termed “rapid fluorescent ADCC” (RFADCC) assay (11). This assay relies on the release of intracellular fluorescent dye and is simpler and more quantitative than chromium release assays. This assay was used to show that ADCC Abs correlate with protection of monkeys from SIV challenge (12). Forthal and colleagues have recently used convincing assays of in vitro viral replication inhibition to illustrate the effectiveness of HIV- and SIV-specific ADCC responses (4, 13–15).

Very recently, our group explored an intracellular cytokine staining (ICS)-based assay studying NK cell expression of effector molecules following activation by HIV Ags and Abs (16). Here, small quantities of whole blood are incubated with HIV peptide Ags, and gated NK cells are monitored for the expression of cytokines such as IFN- γ or degranulation markers such as the expression of CD107a or loss of intracellular granzyme B. This assay is simpler than killing assays and can measure a variety of functions of NK cells triggered by HIV-specific Abs. Because this ICS-based assay of HIV-specific NK cell activation can use overlapping peptides as the Ag source, responses can be mapped to individual linear peptides. In our previous work, we mapped a strong Vpu-specific response (16). The ICS assay can be used with fresh whole patient blood to analyze NK cell function. In contrast, most killing assays use healthy donor PBMCs to provide the effector NK cells. To our knowledge, analyses of ADCC activity using autologous NK cells (which would be more relevant in vivo) in comparison to healthy donor cells have not previously been performed.

To study aspects of HIV-specific ADCC function, we recruited a large cohort of antiretroviral-naïve HIV-infected individuals and

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³ Abbreviations used in this paper: Nab, neutralizing Ab; ADCC, Ab-dependent cellular cytotoxicity; Env, Envelope; ICS, intracellular cytokine staining; KIR, killer Ig-related receptor; RFADCC, rapid fluorescent ADCC; NCR, natural cytotoxicity receptor.

analyzed blood and plasma samples for ADCC activity as measured by the RFADCC assay and Ab-mediated NK cell activation with the ICS-based assay.

Materials and Methods

HIV-infected subjects

HIV-infected adults ($n = 80$) not on antiretroviral therapy were recruited to donate blood samples. Subjects were recruited through the Melbourne Sexual Health Centre and the Alfred Hospital, the largest referral centers for HIV-infected individuals in Melbourne, Australia. All subjects provided informed consent. The relevant human research ethics committee approved all studies. The subjects provided 9–18 ml of Na-heparin anticoagulated blood for fresh whole blood ADCC assays using the ICS method and storage of plasma for the RFADCC and ICS assays using donor cells. Healthy HIV-uninfected control subjects ($n = 10$) also provided blood samples.

HIV-1 Ags

Fifteen amino acid long Env peptides overlapping by 11 amino acids of a consensus B subtype strain were provided by the National Institutes of Health AIDS reagent repository. The 212 Env peptides were solubilized in DMSO and pooled to a single pool for evaluation of Env responses. To identify and map ADCC activity across Env and in particular across the V3 loop of Env, we studied subpools of 30 Env peptides and the Env peptides which span the V3 loop individually (peptides 77–80). Soluble, uncleaved Env analogues (gp140) were generated by ablating the cleavage site between gp120 and gp41 and inserting a stop codon immediately before the transmembrane domain by mutagenesis. Gp140 from subtype B HIV-1_{NL4-3} (CXCR4-tropic) and HIV-1_{AD8} (CCR5-tropic) strains were produced from stably transfected HeLa cells and purified by lentil lectin affinity chromatography as previously described (17). An Env protein without the V3 loop was generated to assess ADCC responses to regions of Env excluding the V3 loop. Overlapping PCR-generated fragments were used to replace the wild-type HIV-1_{AD8} V3 loop with the amino acids CTGAGHC, where the amino acids GAG replaces a 31-amino acid segment of the wild-type AD8 V3 loop to produce V3-deleted gp140.

RFADCC assay

The RFADCC assay was used as previously described (11). In brief, the CEM-NK T lymphoblast cell line was labeled with the intracellular dye CFSE and the membrane dye PKH26 and then pulsed with gp140 proteins (5 $\mu\text{g}/\text{ml}$). Healthy donor PBMCs and plasma from the HIV-infected subjects were added to the labeled CEM-NK cells for 6 h. The cell mix was fixed and the proportion of cells that maintained membrane expression of PKH26 but had lost intracellular CFSE (i.e., lysed cells) were analyzed by flow cytometry. Data is shown as percentage of lysed target cells at a plasma dilution of 1:1000.

Intracellular cytokine staining assay for ADCC activity

The ICS-based assay was used to measure HIV Ab-mediated NK cell cytokine expression and degranulation as previously described (16). In brief, for fresh whole blood assays, 200 μl of blood was incubated with either the pool of overlapping 15-mer Env peptides or gp140 Env (1 $\mu\text{g}/\text{ml}$) proteins for 6 h in the presence of Brefeldin A and Monensin (Sigma) but without costimulatory molecules. At the end of the incubation, CD56+ or CD2+, CD3- NK lymphocytes were studied for the expression of intracellular IFN- γ , surface CD107a, and loss of intracellular granzyme B. Fluorescent Abs used in the ICS assays were CD3 (catalog number 347344, fluorescent label PerCP), CD2 (556611, FITC), CD56 (555516, PE), CD8 α (335787 PE-Cy7), CD107a (624078, allophycocyanin), IFN- γ (557995, Alexa700) (all from BD Biosciences) and granzyme B (3485-7, FITC; Mabtech). These assays were also conducted using patient plasma and healthy donor PBMCs. To study the kinetics of the responses, we stopped the assay after varying intervals between 0 and 7 h, as previously described for T cell assays (18). To analyze differences between autologous blood cells and donor blood cells, we incubated 50 μl of plasma from the HIV-infected subjects with 150 μl of fresh whole blood from healthy HIV-uninfected donors. This ratio of plasma to donor blood cells was optimal for activation of NK cells and equivalent to using larger volumes of plasma on donor PBMC (not shown).

Statistical analyses

To compare ADCC responses to different Ags and with different assays across the cohort we used double-sided non-parametric Mann-Whitney U tests and Fisher's exact test for binary data.

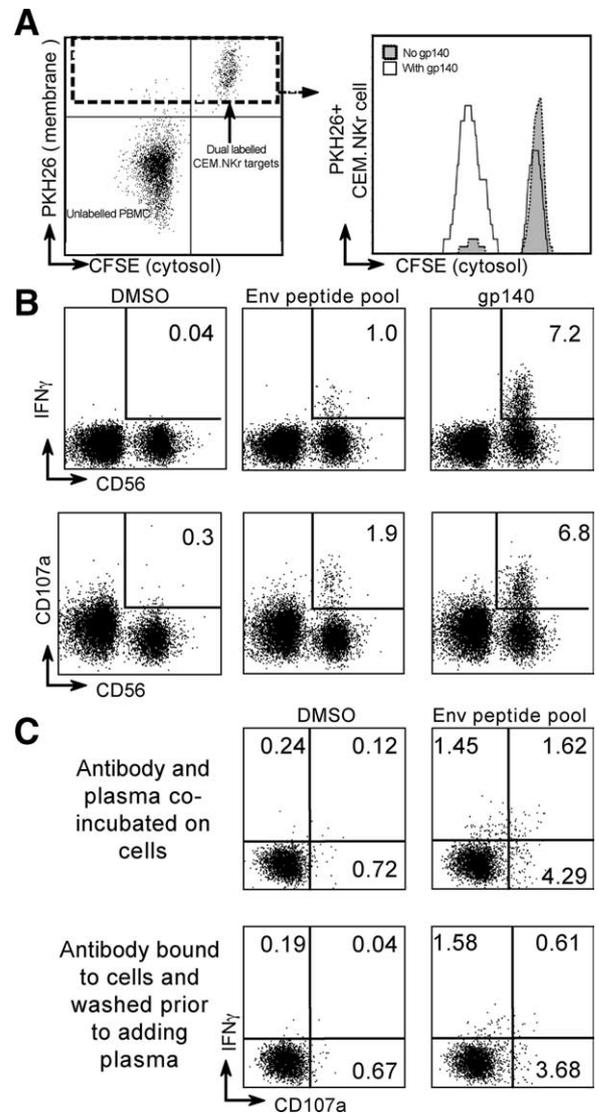


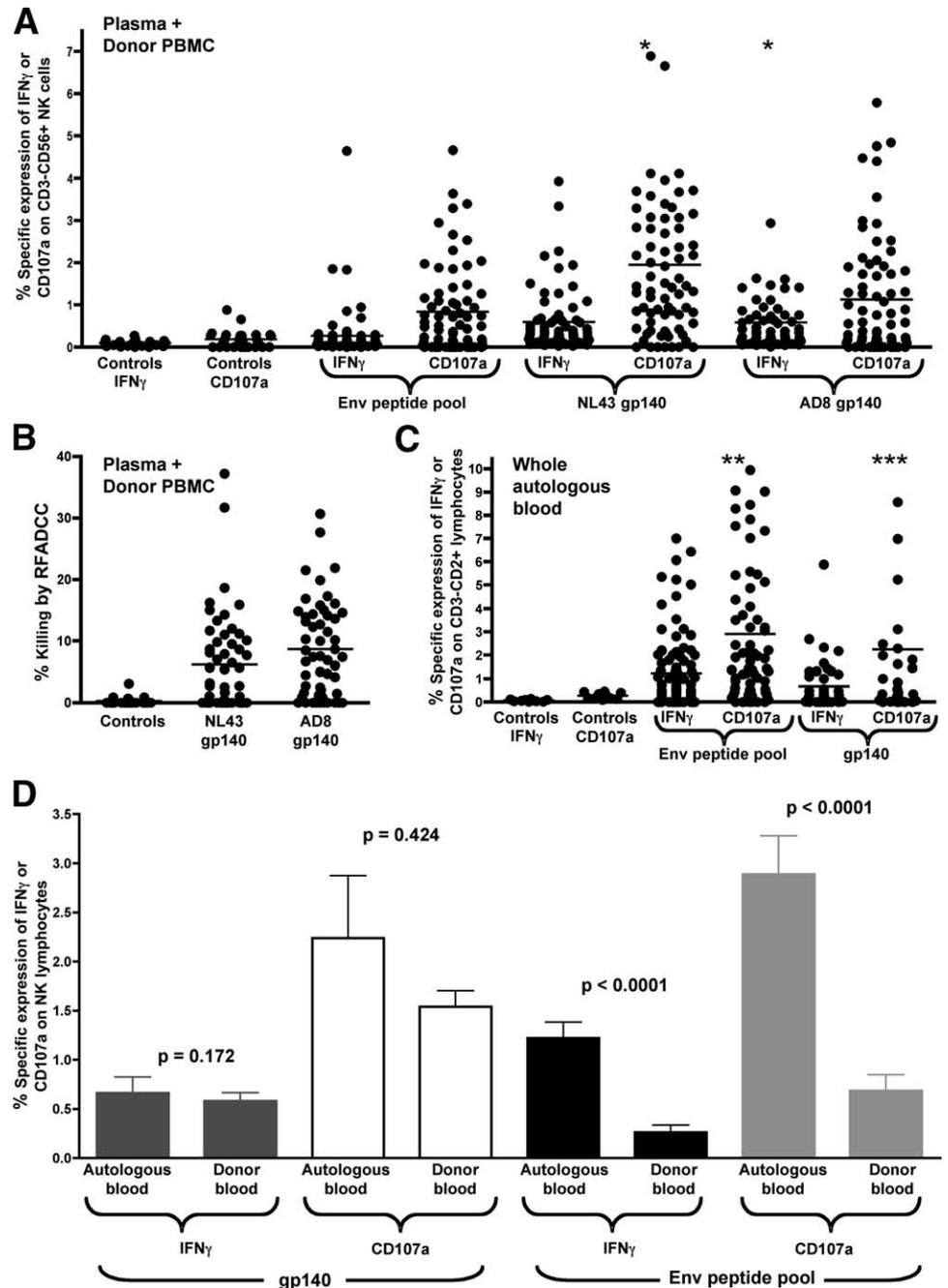
FIGURE 1. ADCC assays. *A*, The RFADCC assay. The CEM.NK cell line is labeled with both PKH26 and CFSE dyes and incubated together with gp140 HIV-1 Env Ag or no Ag together with plasma from HIV-infected subjects and unlabelled healthy donor PBMC. The PKH26+ cells are gated on and studied for the loss of CFSE (solid line), indicating cell lysis. *B*, The ICS assay of NK cell activation. Fresh autologous blood, or healthy donor blood together with plasma from the HIV-infected subjects, are incubated with either gp140 Env proteins or a pool of overlapping 15-mer Env peptides. Env-specific expression of intracellular IFN- γ or surface CD107a on the surface is monitored on CD56+CD3- NK cells. The percentage of NK cells expressing the relevant marker is shown in the upper right quadrant. *C*, Cell-bound Env peptides are sufficient to trigger Ab-mediated NK cell IFN- γ expression and degranulation. In the *upper panels*, plasma from an HIV-infected subject and Env peptides are added simultaneously to fresh donor blood depleted of plasma by aspiration and washing in PBS. In the *lower panels*, Env peptides are added first to the plasma-depleted blood for 1 h, and the Ag washed away before adding the HIV+ plasma. All samples are incubated for 5 h and gated CD3+56+ NK lymphocytes assessed for intracellular IFN- γ expression and surface CD107a expression. The percentage of NK cells expressing one or both of IFN- γ and CD107a are shown in the quadrants.

Results

ADCC assays

Novel methods to measure HIV-specific Ab responses allowed us to probe the functions of HIV Ag-stimulated NK cells. We evaluated a RFADCC assay in comparison to a novel intracellular

FIGURE 2. Ab-mediated killing and NK cell degranulation responses in HIV-infected cohort. A cohort of 80 HIV-infected subjects were recruited and monitored for ADCC responses by either incubating plasma from the HIV infected cohort with healthy donor blood and either overlapping Env peptides or gp140 proteins from HIV-1_{NK3} or HIV-1_{AD8} strains and analyzing NK cell IFN- γ and CD107a expression (A), or incubating patient plasma with donor PBMC, PHK26, and CFSE-labeled target cells and analyzing for killing (B). C, Controls are uninfected healthy plasmas incubated similarly or studying fresh whole autologous blood from HIV-infected subjects and measuring IFN- γ and CD107a expression as in A. Each dot represents data from one of the subjects. Controls in all assays represent ten healthy HIV-uninfected donors. Responses in the controls were similar for all proteins and peptides and are pooled in the graphs. The asterix above panels A and C represent single subjects with responses marginally greater than 7 and 10%, respectively. D, A comparison of autologous blood and donor PBMC assays (from data in A and C). Env gp140 protein or Env peptide specific expression of IFN- γ or CD107a was compared in autologous blood and donor PBMC across the cohort. The mean SE of each group is shown. Values of p are a non-parametric two-sided Mann-Whitney U test.



cytokine based assay for Ab-mediated NK cell activation we recently published (16).

Both assays could reliably detect HIV Ab activities. In the case of the RFADCC assay, when HIV+ serum is cultured with healthy donor PBMC and a trimeric Env-protein derived from the HIV-1_{NL4.3} strain pulsed onto the fluorescent CEM.NK γ target cell line, there was a clear population of HIV-1 Env-pulsed CEM.NK γ cells that still retained the membrane dye PKH26 but had lost intracellular CFSE, indicating target cell lysis (Fig. 1A). We also showed that NK cells were specifically mediating this effect from within the PBMC population by using magnetic beads to deplete CD56+ NK cells and CD3+ T cells (not shown). No target cell lysis was measurable when the CEM.NK γ target cell line was pulsed with a pool of overlapping Env peptides alone (not shown).

The ICS-based assay also reliably detected activation of NK cells in the presence of Env HIV-1 Ags and plasma from HIV-

infected subjects (Fig. 1B). We have previously shown that this assay is dependent on the presence of the IgG fraction of Abs within the serum of infected individuals (16). This assay can be used either on whole blood from HIV-infected individuals or using uninfected donor PBMCs as the effector NK cell source. The assay measures both IFN- γ expression as well as expression of the degranulation marker CD107a simultaneously. In contrast to the RFADCC assay, the ICS-based assay is also effective at measuring NK cell activation using overlapping linear Env peptides (Fig. 1B).

It was formally possible in the ICS-based assay that the HIV-specific NK cell expression of IFN- γ and CD107a was being triggered by soluble Ag-Ab complexes, rather than Ab recognition of cell-presented Ag. We therefore explored whether incubating plasma-free blood cells with HIV Ags and washing away unbound Ag before the addition of plasma abrogated NK cell activation. Extensive washing to remove soluble Ag resulted in only a minor loss

of the activation of NK cells in the ICS assay (Fig. 1C). Similar findings were observed using different donor cells and to both HIV peptide and gp140 Ags (not shown). The minor (~20%) loss of activity could be related to immune complexes mediating this fraction of activity or alternatively some presented Ag lost during the washing process. This suggests that cell-based presentation of HIV Ags, as is typical of killing-based ADCC assays, is also required to stimulate the majority of Ab-mediated NK cell degranulation.

ADCC responses in HIV-infected cohort

We recruited 80 HIV-infected antiretroviral naive subjects between February 2004 and July 2008. Blood samples were obtained to measure ADCC activity in fresh blood samples and plasma was stored to batch test ADCC activity using healthy donor PBMC. The HIV-infected subjects had been infected for a mean of 5.0 years (range 0.8–25.7), and had a mean viral load of 47,000 HIV-1 RNA copies/ml (400–298,000) and CD4 T cell count of 557/ μ l (147–1147).

Env-specific ADCC activity in stored plasma from all cohort subjects was analyzed both using the RFADCC killing activity (to two separate gp140 Env proteins, NL4.3 and AD8 HIV-1 strains) and by ICS again to both gp140 Env proteins as well as to overlapping Env peptides using healthy donor PBMC (Fig. 2, A and B). For the ICS-based assay we studied both cytokine (IFN- γ) expression and degranulation (CD107a expression) to all Env Ags. Plasma from HIV-negative control subjects had negligible NK cell activation in both assays, although background levels of activity are marginally higher for CD107a expression compared with IFN- γ expression.

The majority of plasma from HIV-infected subjects have HIV-antibody activity that is detectable by both assays. In response to the HIV-1_{NL4.3} Env gp140 protein, 74% of subjects had >0.5% of NK cells expressing either IFN- γ and/or CD107a in the ICS assay and 60% of subjects had >5% killing of CEM.NKr cell targets in the RFADCC assay. Although Env is highly variable between strains, there was no overall difference in the recognition of either the CXCR4-tropic HIV-1_{NL4.3} Env gp140 protein or the CCR5-tropic HIV-1_{AD8} Env gp140 protein by either assay.

The ICS assay was also used to study responses to Env peptides and the gp140 proteins when added to fresh whole autologous blood (Fig. 2C). This assay analyzes both the effect of the Ab and the effect of autologous NK cells from the HIV-infected subjects as the effectors. High levels of responses are also observed in this untreated HIV-infected cohort when this simplified whole blood ICS assay is used. We were particularly interested in this comparison, because it was possible either that the autologous NK cells would be dysfunctional and result in reduced activity or that the autologous NK cells would be more efficiently primed to respond to the Abs. We found that responses to gp140 protein were similar in the fresh autologous whole blood ICS assay compared with the study of patient plasma incubated with donor PBMC, with a mean (\pm SE) specific IFN- γ expression of $0.66 \pm 0.16\%$ vs $0.58 \pm 0.08\%$ and CD107a expression of 2.2 ± 0.6 vs 1.6 ± 0.6 in the autologous blood assay compared with donor PBMC respectively (both $p > 0.1$, double-sided Mann-Whitney U test, Fig. 2D). Surprisingly however, responses to Env 15-mer peptides were significantly higher in the autologous blood assay compared with responses seen using stored plasma. Mean levels of expression of IFN- γ expression were 1.2 ± 0.2 vs 0.26 ± 0.07 and CD107a expression of 2.9 ± 0.4 vs 0.68 ± 0.17 in the autologous blood assay compared with donor PBMC, respectively ($p < 0.0001$).

The cohort of antiretroviral therapy-naive HIV-infected subjects had been infected for varying lengths of time and exhibited a large range of viral loads and CD4 T cell counts. We assessed whether

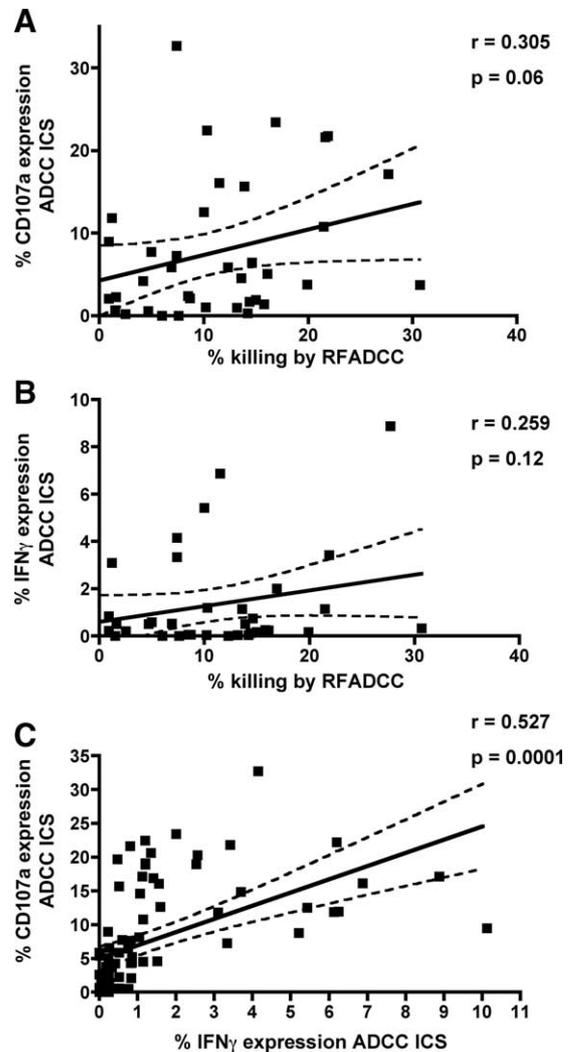


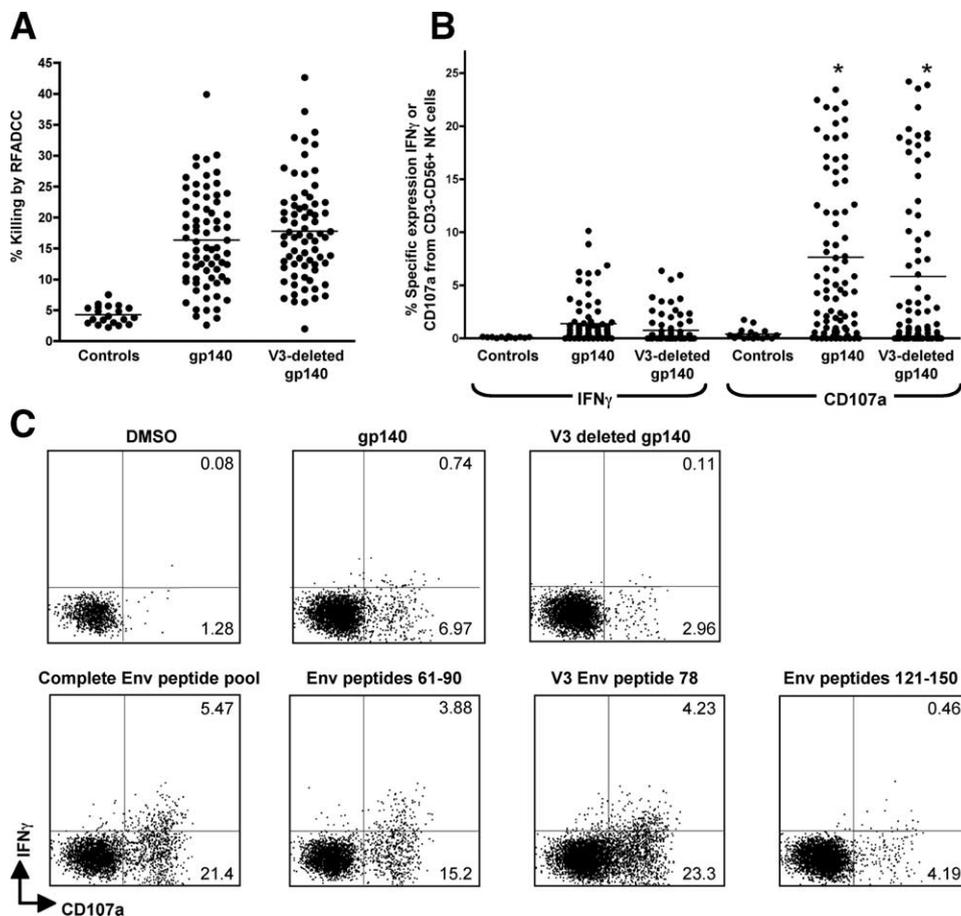
FIGURE 3. Correlation between ICS assay for NK cell activation and the killing-based RFADCC assay. Plasma from the HIV-infected cohort was analyzed in response to gp140 protein stimulation and healthy donor PBMC. Correlations were performed between CD107a expression by the ADCC ICS assay and killing by the RFADCC assay (A), IFN- γ expression and killing by the RFADCC assay (B), and comparing CD107a and IFN- γ expression on HIV Ab-activated NK cells by the ICS assay (C). Dashed lines represent 95% confidence intervals around the correlation.

ADCC response measured by the RFADCC assay or HIV-Ab mediated NK cell activation correlated with levels of viral load or immunodeficiency in this cross-sectional analysis. Subjects with higher viral load (>20,000 copies/ml) had non-significantly lower mean HIV-specific NK cell activation compared with subjects with lower viral load (2.15 ± 0.32 vs $3.16 \pm 0.64\%$ of NK cells specifically expressing IFN- γ in response to HIV peptide pools, two-tailed t test $p = 0.12$). There was no difference in HIV-specific NK cell activation at recruitment stratified by peripheral CD4 T cell or NK cell levels (not shown). Similarly, there were no differences in numbers of other potential APCs or effector cells such as monocytes or neutrophils, or ratios of these cells to NK effector cells, in subjects with higher or lower levels of HIV Ab-mediated NK cell activation or ADCC by the RFADCC assay (not shown).

Poor correlation between ADCC killing assay and cytokine expression by NK cells

The differences in levels of expression of the cytokine IFN- γ and the degranulation marker CD107a to HIV-1 Env Ags in the ICS

FIGURE 4. ADCC responses to the V3 loop. Responses were studied either by the RFADCC assay on the HIV-infected cohort and healthy uninfected controls comparing whole gp140 protein to V3 deleted gp140 protein (A), or by the ICS assay using the same proteins (B). C, In an HIV subject where the response to the V3 deleted gp140 was reduced, we studied responses to a complete envelope peptide pool or smaller peptide pools. The majority of the response in this subject was directed toward the V3 loop (peptide 78).



assay suggested that there may be differences in NK cells functional responses to ADCC Abs. We therefore analyzed the correlation between the ADCC ICS assay and the RFADCC killing assay using the same plasma sample and the same HIV-1_{AD8} Env gp140 protein. The correlation between killing of the labeled target cell line in the RFADCC assay and degranulation of NK cells as assessed by CD107a expression was almost statistically significant ($p = 0.06$, Fig. 3A). However, there was a poorer correlation between gp140-specific IFN- γ expression from NK cells and killing in the RFADCC assay (Fig. 3B). This suggests that the various functions of NK cells (cytokine secretion, degranulation, and killing) do not necessarily occur simultaneously during HIV-Ag stimulation. Another possibility is that presentation of gp140 protein via the CEM.NKr cell line used in the RFADCC assay may be different to presentation by primary APCs in the ICS-based assay. Consistent with this possibility, there was a strong overall correlation between gp140-stimulated NK cell expression of CD107a and IFN- γ within the same ICS assay (Fig. 3C). Even within this tighter correlation there were clearly subjects who expressed high levels of CD107a with more modest levels of IFN- γ expression (for example, upper left dots in Fig. 3C), again suggesting differential function of NK cells responding to ADCC Abs.

Contribution of V3 loop of Env to ADCC activity

ADCC activity to whole Env protein or large Env peptide pools likely involves Abs with multiple specificities and ADCC Abs to some epitopes may stimulate NK cell function differently compared with ADCC Abs to alternate epitopes. We took two approaches to dissect the differential functions of specific Env ADCC Abs. First, because the V3-loop is a common target of Abs (19), we generated gp140 protein devoid of the V3 loop to determine

whether different levels of killing or cytokine expression in the presence or absence of the V3 loop segment of gp140 in both the ICS-based assay and the RFADCC assay. Second, because the Env peptide pool can be broken down to smaller pools of peptides or individual 15-mer peptides, we studied in detail one of our subjects where differential NK cell activation by the ICS-based assay between to wild-type gp140 and V3-deleted gp140 protein was detected.

Surprisingly, with the RFADCC assay, there was little overall mean difference in ADCC responses to whole HIV-1_{AD8} gp140 protein and the V3-deleted gp140 protein (16.3 ± 0.9 vs $17.8 \pm 0.9\%$ killing, respectively, Fig. 4A). There was a modest mean reduction in both IFN- γ expression and CD107a expression using the ICS assay to compare responses to wild-type and the V3-deleted gp140 protein (1.3 ± 0.24 vs 0.75 ± 0.16 , $p < 0.01$, 7.6 ± 0.9 vs 5.8 ± 0.9 , $p = 0.02$ respectively, Fig. 4B). This data is consistent with multiple specificities of ADCC responses to whole Env proteins and the total ADCC responses are minimally impaired by loss of reactivity to the V3-loop in most subjects.

To more definitively probe differential Env epitope-specific responses, we selected an HIV-infected subject who had a reduction in activity to V3-deleted Env gp140 protein to study in detail the linear Env epitopes to which Ab-mediated NK cell activation was occurring (Fig. 4C). Plasma from this subject had a significant reduction in CD107a expression to the V3-deleted gp140 (7% to 3%) and an almost complete loss of IFN- γ expression to the V3-deleted gp140 (0.7% to 0.1%). There was a large response to the pool of all 212 Env overlapping peptides. When this was mapped first to pools of 30 peptides, and then individual peptides, most of the reactivity in this subject was confined to Env peptide 78 (RKRIHIGPGRAFYYT), a linear peptide that spans the V3 loop.

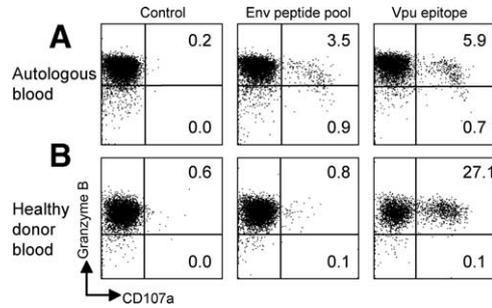


FIGURE 5. Degranulation of autologous vs healthy donor NK cells in response to HIV Abs. Fresh autologous blood (A) was compared with fresh healthy donor blood (B) when stimulated with Env peptides or a known Ab epitope to Vpu. CD3-CD56+ NK lymphocytes were gated on and studied for the expression of CD107a and loss of granzyme B.

We were also able to detect NK cell activation in response to the pool of 30 Env peptides containing peptides 121–150. The reactivity of this response was subsequently determined to be within 15mer peptide 138 (IVQQNNLLRAIEAQ), lying within the N-terminal portion of gp41 of Env (not shown). The NK cell reactivity was less to this epitope compared with the V3 epitope, particularly for IFN- γ expression (3.9% vs 0.5%), broadly consistent with the results using the V3-deleted gp140 protein. Thus, within particular subjects, responses to different epitopes trigger different levels of NK cell expression of CD107a and IFN- γ .

Ab-stimulated degranulation of healthy vs HIV+ donor NK cells

We noted above that the mean Env peptide pool-specific expression of CD107a was greater in NK cells from autologous HIV+ blood compared with healthy donor PBMC. However, expression of the degranulation marker CD107a does not necessarily indicate that cytolytic molecules such as granzyme B are actively being exported from the cell. Further, whether such a finding could be generalized to other non-Env epitopes was unclear. We therefore studied blood and plasma from a previously described HIV-infected subject that had Ab-mediated NK-cell responses to both Env and an epitope within the Vpu protein. We stimulated either the subject’s whole fresh blood or the subject’s plasma with healthy donor blood with these Ags and analyzed the concurrent expression of CD107a and loss of granzyme B from gated NK cells.

Autologous blood NK cells had high levels of Env and Vpu-specific expression of CD107a and the cells highly expressing CD107a were losing granzyme B (Fig. 5A). There were, however, some remarkable differences when the same fresh plasma was incubated with blood containing fresh NK cells from a healthy HIV-uninfected donor (Fig. 5B). There was substantial loss of Env peptide-specific reactivity, with minimal CD107a expression (3.5% to 0.8%) and virtually no granzyme B loss. In contrast, Vpu epitope-specific reactivity was enhanced, with CD107a expression in the healthy donor cells compared with autologous NK cells (27% vs 5.9%). Again, there was virtually no granzyme B loss from the healthy donor cells in comparison with autologous NK cells (0.7% vs 0.1%). Thus, there was substantial variability in both CD107a expression and granzyme B loss of donor, compared with autologous, NK cells following activation by different HIV epitopes.

To probe the differences between the HIV Ab-mediated activation of autologous and healthy donor cells we analyzed the surface expression of two killer Ig-related receptor (KIR) molecules (KIR3DL1 and KIR2DL1), a natural cytotoxicity receptor (NCR) molecule (NKp44), the activation marker CD69 and the NK cell surface molecule CD56. We had previously shown that CD56 is down-regulated on the HIV Ab-activated cells from HIV+ donors but not the healthy donor cells (16) and this was confirmed in this study (Table I). There were however no marked differences in KIR, NCR, or CD69 expression in the Ab-activated cells in comparison to unactivated cells, nor between autologous and healthy donor cells, nor across either Vpu- or Env-specific responses (Table I).

Kinetics of degranulation of Ab-mediated NK cell responses

Maximally effective anti-HIV immune responses will likely need to respond very quickly and robustly to free HIV or HIV-infected cells to limit a rapidly expanding infection (20). We recently showed that SIV-specific CD8 T cells armed by live attenuated SIV vaccination in macaques more rapidly expressed CD107a and lost granzyme B in comparison to CD8 T cells armed by standard DNA/viral vector vaccination (18). As cells of the innate immune system, there is the potential for NK cell-mediated effector functions to very rapidly generate cytolytic activity. We therefore studied the kinetics of expression of HIV-specific CD107a, IFN- γ , and granzyme B in both NK cells (to Env and Vpu) and CD8 T cells (to Gag, Pol, and Env) within the same HIV-infected individuals.

Table I. Surface molecule expression on HIV-Ab activated NK lymphocytes

Plasma Stimulation	% Expression														
	NKp44 (NCR)			CD158a (KIR2DL1)			NKB1 (KIR3DL1)			CD69			CD56		
	Donor 1 ^a	Donor 2	Autologous	Donor 1	Donor 2	Autologous	Donor 1	Donor 2	Autologous	Donor 1	Donor 2	Autologous	Donor 1	Donor 2	Autologous
HIV+ Subject 1															
None ^b	48.3	63.9	55.0	30.4	20.7	21.2	16.3	36.1	4.6	7.9	9.1	6.0	37.0	20.5	59.2
Vpu ^c	41.8	43.5	53.0	18.6	13.9	10.1	30.1	40.1	8.4	1.5	19.2	3.5	95.5	85.7	62.7
Env	48.6	59.7	55.5	9.1	12.4	7.9	19.2	31.2	9.4	0.0	3.0	1.6	86.9	82.0	58.6
gp140	42.9	46.1	45.1	15.1	10.3	10.7	25.9	45.0	3.9	1.2	13.6	2.5	97.2	85.1	64.9
HIV+ Subject 2															
None	48.8	50.7	56.1	7.5	18.7	14.9	35.1	42.0	32.2	9.5	9.4	6.8	37.7	18.5	16.9
Env	49.4	44.8	66.4	6.5	13.7	23.0	15.1	28.4	12.9	5.0	2.0	1.5	89.3	85.2	49.5
gp140	51.5	47.9	66.9	2.6	11.4	18.5	30.0	37.0	7.7	3.1	10.8	4.3	89.2	81.1	39.7

^a Donors 1 and 2 were fresh blood from healthy HIV-negative subjects and autologous was fresh blood from the HIV+ subject.
^b None refers to blood incubated with plasma but no HIV-antigen stimulation. The proportion of cells expressing the 5 difference surface molecules is on total CD3-negative lymphocytes.
^c Antigens used were a Vpu peptide epitope previously described (ref. 16), overlapping Env peptides or HIV-1_{AD8} gp140 protein. The proportion of cells expressing the 5 different markers refers to the IFN- γ -expressing fraction of cells.

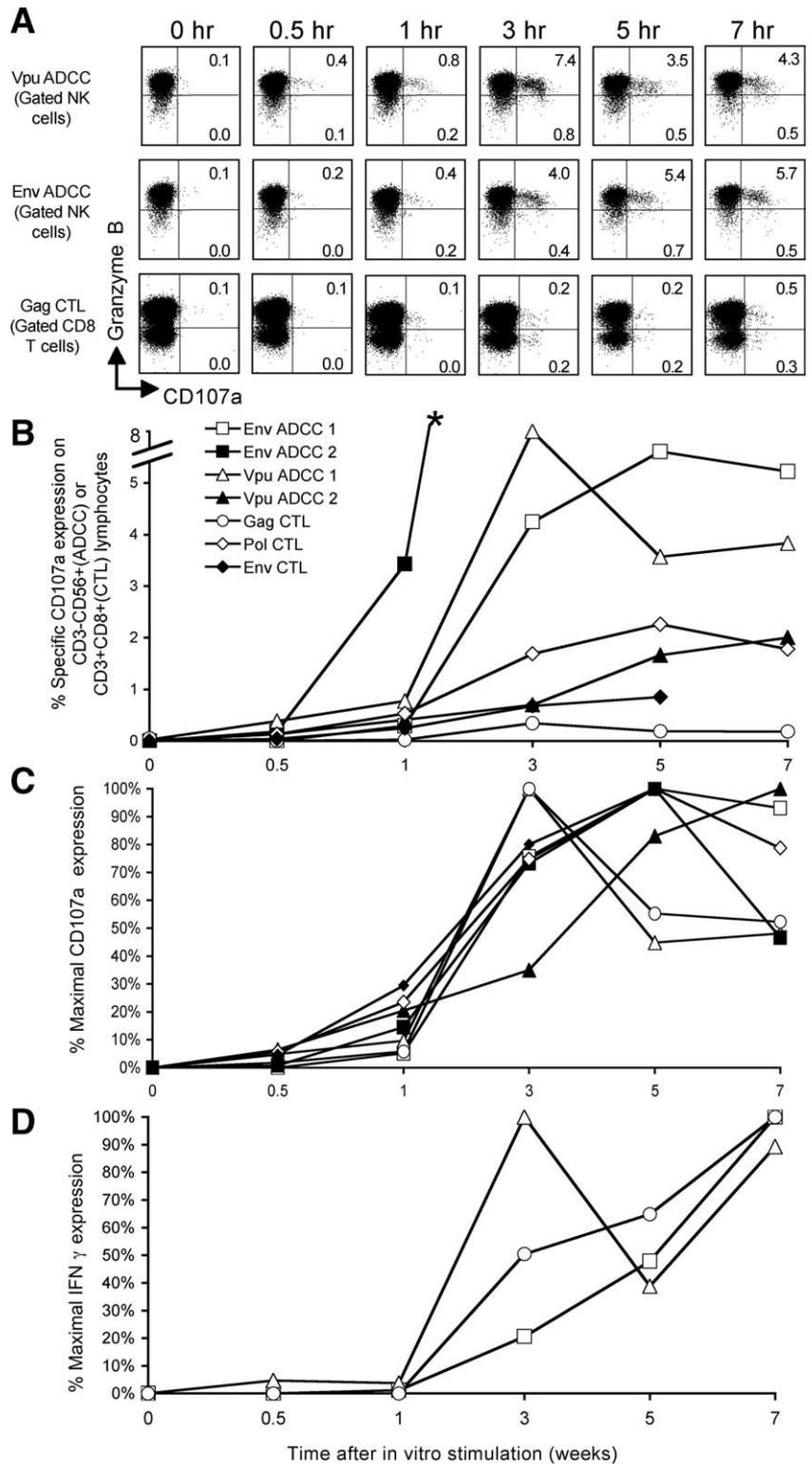


FIGURE 6. Kinetics of degranulation of NK cells and CTLs. Autologous blood from two HIV-infected subjects was stimulated with either the Vpu epitope, or the overlapping Env, Gag, or Pol peptide pools. After varying time intervals the specific expression of CD107a, IFN- γ , and granzyme B were studied. *A*, Panels of flow cytometry plots showing the kinetic expression of CD107a and loss of granzyme B are shown for subject 1. *B*, The kinetic expression of CD107a by Ab-mediated NK cell responses and the Gag CTL response is shown over time for both subjects. The Env-specific NK cell expression of CD107a in subject 2 peaked at 21% (asterisk). The data is graphed as a percentage of the maximal response in the 7 h assay (*C* for CD107a for both subjects and *D* for IFN- γ in subject one). Legends for *C* and *D* are as for *B*.

Fresh autologous blood from two HIV-infected subjects was stimulated for varying intervals between 0 min and 7 h with overlapping Env, Gag, and Pol peptides and a known Vpu ADCC-epitope peptide, with the assay stopped by placing the cells at 4°C. We chose two donors where we could follow multiple specificities simultaneously. With no stimulation, there was minimal CD107a or IFN- γ expression (0 h, Fig. 6A). Within 30 min, there was expression of CD107a on NK cells activated by ADCC to the Vpu epitope (0.5 h Vpu panel 0.36% at 0.5 h compared with

baseline response of 0.08%, Fig. 6A), but no up-regulation of CD107a on Gag-stimulated CD8 T cells. Within 1 h, there was also increased CD107a expression on NK cells stimulated by Env peptides and CD8 T cells stimulated by Gag peptides. The proportions of NK cells expressing of CD107a in response to Env or Vpu peptides were much higher than the CTLs stimulated with Gag, Pol, or Env peptides (Fig. 6, A and B). Interestingly, degranulation and granzyme B loss peaked at 3 h in the Vpu-stimulated NK cells in donor 1 (which had begun to

degranulate the fastest), but rose steadily out to 7 h in the NK cells stimulated by Env peptides and CD8 T cells stimulated by Gag peptides (Fig. 6, A–C). The results show that HIV-specific Ab stimulation of NK cells can occur as fast or even faster than activation of HIV-specific CTLs.

Discussion

There is growing interest in the potential utility of ADCC and related Fc-mediated functions of HIV-specific Abs to prevent or control HIV infection (4). We comprehensively analyzed Env-specific ADCC and NK cell activation activity in 80 untreated HIV-infected subjects. A more traditional killing-based ADCC assay was compared directly with a novel ICS-based assay that analyses multiple effector functions of activated NK cells and can map linear Ab responses to peptides. Although we found the majority of HIV+ subjects to have effector Ab responses to Env Ags using either assay, there was a remarkably poor correlation between the assays, particularly between cytokine expression and killing of target cell lines. Further, we identified differences in HIV-specific Ab stimulation of NK cell functions using healthy donor NK cells compared with autologous patient cells, between whole Env protein and Env peptides, across different epitopes, and in the kinetics of cytotoxicity. Our studies, particularly the use of a novel ICS-based assay, provided new insights into ADCC recognition of HIV infection.

Given recent emphasis on the differential functions (“polyfunctionality”) of T cells and their role in controlling HIV infection (21, 22), perhaps it should be no surprise that HIV Ab-stimulated NK cell functions vary between subjects across different epitopes. Indeed, NK cells have multiple additional effector function other than IFN- γ secretion, expression of degranulation markers and loss of granzyme B (and granzyme A–K, perforin, and others), and secretion of other cytokines such as TNF- α and secretion of β -chemokines (23). Evaluating the most effective anti-HIV functions of NK cells to stimulate via ADCC and related Abs is clearly an important goal of future HIV research. The relatively poor correlation between Env-specific ADCC directed lysis of immortalised target cell lines and expression of IFN- γ and CD107a by activated NK cells suggests that presentation of Env Ags by cell lines may be substantially different from that of primary PBMC. This is supported by our finding that PBMC, but not the CEM.NKr cell line, can present linear peptides to ADCC Abs to activate NK cells. Precisely which primary cells within blood present peptides to HIV Abs, and which cellular surface molecules are required (for example lectins or HLA molecules), and the role of soluble immune-complexes in stimulating NK cells, is not understood but a subject of important future research. The differential presentation of Env Ags by cell lines and primary cells to Ab-aimed effector cells suggests future opportunities to more clearly define precisely how ADCC recognize infected cells. Functions of ADCC effector cells other than NK cells (e.g., neutrophils, monocytes) may also contribute substantially to ADCC activity and will be important to analyze in future studies.

A key finding of this work is the marked differences observed in HIV Ab-stimulated NK cell functions when using autologous NK cells compared with healthy donor NK cells using the ICS-based assay, hitherto ignored in studies of HIV-specific Abs. Studying ADCC with fresh autologous NK cells by standard killing assays is logistically complicated by the need to set up fresh, high-quality, labeled target cell lines on the day of each assay. Our findings uncover a substantial level of complexity when studying ADCC responses. Arguably, autologous NK cells are the most important to study, because these cells will need to mediate effector activity in vivo. A surprising finding across our cohort was the enhanced

ability of autologous Ab-activated NK cells to recognize Env peptides, both in CD107a and IFN- γ expression, in comparison to healthy donor NK cells. Further, in more detailed individual studies, we found the ability to secrete granzyme B was markedly reduced in healthy donor cells in comparison to autologous NK cells (Fig. 5). This was despite well-described defects in various NK cell functions that occur with progressive HIV infection that could have reduced ADCC activity (24–26). Our data should be interpreted cautiously because only a fraction on the CD107a+ NK cells lose granzyme B following the in vitro stimulation (Fig. 5), making these studies technically difficult in subjects with smaller responses. Nonetheless, our findings suggest the possibility that autologous NK cells in HIV infected subjects may be pre-activated and capable of responding rapidly to their own ADCC Abs in vivo, a feature not shared by donor NK cells. It is possible that some autologous NK cells may already have bound the Fc portions of HIV-antibodies, pre-arming the cells for APC recognition. Against this possibility is the lack of NK cell stimulation by HIV Ags without the addition of further Abs in plasma ((16) and not shown).

The substantial genetic variability in NK cell receptors and ligands could also underlie some differences observed between donor and autologous NK cells, although our studies of some KIR and NCR receptors have not yet revealed significant differences between activated and unactivated autologous or donor NK cells or across different HIV Ags (Table I). Clear associations between HLA-B alleles and particular NK cell KIR receptors have emerged recently (27–29). Future studies should attempt to determine whether common genetic differences between NK cells from different subjects correlate with effective ADCC activity.

The V3 loop of the Env protein has long been recognized as a major target of Env-specific Abs (19). By using gp140 protein devoid of the V3-loop and mapping the specificity of Ab epitopes using overlapping peptides, we demonstrated, not surprisingly, that some patients preferentially target the V3 loop. However, across the entire 80-patient cohort there was no overall difference in Ab-mediated lysis or NK cell IFN- γ /CD107a expression between wild-type and V3-deleted Env protein. This implies that multiple epitopes are recognized by these Abs. Similar to recent observations on HIV-specific T cell immunity (30), it is highly likely that some Abs are more effective at controlling HIV infection in vivo than others. Identifying particular Ab epitopes that are associated with delayed disease progression or prevention of HIV infection will be critical in harnessing ADCC Abs to treat or prevent infection. In the absence of being able to induce broadly Nab, it will be crucial to identify useful binding HIV Abs that effectively trigger multiple effector systems from NK and other innate immune cells.

Our studies on the kinetics of degranulation and IFN- γ expression by Ab-activated NK cells show that, at least in the setting of HIV infection, NK cells can very rapidly respond to in vitro peptide Ag stimulation. NK cells responding to an ADCC epitope identified in Vpu were able to respond marginally quicker (30 min) than either NK cells responding to Env or CTLs responding to Gag within the same individual (Fig. 6A). Our studies predict that NK cells will likely respond at least as quickly as CTLs to HIV Ag and suggest the possibility that such Fc-mediated Ab responses could be effective in limiting a spreading infection. This concept is supported by the recent finding of Hessel et al. on the effectiveness of passive transfer of neutralizing Abs with ADCC activity (4). It should be noted however, that in vivo virus-infected cells will need to present Ag on the surface of the cells; recent studies highlight different rates of MHC I presentation of Env and Gag Ags (20). How, and how quickly, HIV-infected primary cells present Ags to Abs is poorly studied, particularly for Ags such as Vpu. Further, it

may be difficult to maintain activated NK cells in the setting of inducing ADCC responses by preventative HIV vaccine approaches.

This study does not yet, however, define the utility of ADCC responses in HIV-infected subjects. At the time we measured responses across the cohort we recruited, there were no significant cross-sectional correlations between baseline CD4 T cell counts or viral load and ADCC responses using the RFADCC assay or NK cell activation with the ICS assay. This contrasts with other cohorts of HIV-infected subjects using cell line killing-based or viral inhibition ADCC assays (5, 8). We are currently longitudinally following our patient cohort to define whether ADCC responses are associated with reduced CD4 T cell depletion. In particular, we are interested in whether ADCC responses to particular, more conserved, epitopes are associated with delayed progression. Indeed, CD8 T cell responses to HIV and SIV that target conserved epitopes within viral proteins such as Gag are more useful than CD8 T cell responses to other Ags (31, 32), and related findings may also be true for ADCC responses as further epitopes are mapped and studied. Including the most effective ADCC epitopes, rather than ineffective ADCC epitopes, is a clear priority for future HIV vaccine research.

In summary, our findings shed light on the complexity on NK cell mediated effector functions stimulated by HIV Abs. HIV-specific Abs commonly trigger a series of overlapping functions by autologous NK cells, only some of which are shared by healthy donor NK cells. Ab-activated NK cells are capable of rapidly responding to HIV Ag in vitro, suggesting such responses should be a useful activity to stimulate by vaccination.

Disclosures

The authors have no financial conflict of interest.

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