



## Pol as a target for antibody dependent cellular cytotoxicity responses in HIV-1 infection

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### ABSTRACT

Antibody-dependent cellular cytotoxicity (ADCC) may assist in preventing HIV or delaying disease progression. Most prior studies have analysed Env-specific ADCC responses. We hypothesized that effective ADCC-based immunity may target conserved internal viral proteins such as Pol. We analysed the ability of overlapping Pol peptides to induce activation of NK cells via ADCC. We prospectively studied ADCC responses in 83 HIV+ subjects followed for 3 years. Pol peptides were commonly targeted by ADCC responses in these chronically infected subjects (in 32 of the 83 subjects). However, Pol-specific ADCC responses declined over time and did not correlate with delayed HIV progression, measured by either baseline CD4 T cells, CD4 T cell loss over time, baseline viral load or the need to start antiretroviral therapy. Although Pol is frequently targeted by ADCC in HIV+ subjects, the strength or specificity of Pol-specific ADCC responses needs to be modulated to be effective in delaying HIV progression.

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### Introduction

Human immunodeficiency virus type 1 (HIV-1) is an important global pandemic with close to 3 million new infections each year. An HIV vaccine is urgently needed. Protein subunit vaccines induce only narrowly directed neutralizing antibodies, and failed to protect in human and macaque trials (Pitisuttithum et al., 2006; Stott, 1991). Vaccination with recombinant adenoviruses expressing HIV proteins elicited HIV-specific CD8+ T lymphocytes (McElrath et al., 2008), but also do not protect against infection or reduce viral load set point in those individuals who became infected during follow-up (Buchbinder et al., 2008). These disappointing results after years of HIV vaccine research suggests newer concepts in immunity to HIV should be explored (Isitman et al., 2009). Importantly, a recent efficacy trial based on a recombinant Canarypox virus prime and envelope protein boost showed partial protection from infection, despite only inducing narrow Nab responses and minimal CTL responses. High levels of non-neutralizing antibodies were induced by this regime, suggesting such responses could play a role in protective immunity (Rerks-Ngarm et al., 2009).

ADCC is an immune response combining components of innate and humoral immunity. Cells that can be activated by ADCC to provide

effector functions via their Fc receptors include NK (Natural Killer) cells, neutrophils and macrophages. ADCC utilizes effector cells bearing Fc gamma (Fc $\gamma$ R) or CD16 receptors such as NK cells attracted by antibodies of the IgG isotype to the target antigens on the surface of virus-infected cells. NK cells comprise 15% of the peripheral blood lymphocytes. Lysis of virus-infected cells occurs once the ADCC antibodies bind to surface viral antigens and interact with the Fc $\gamma$ R of NK cells. The activation of NK cells elicits release of perforin, granzymes and cytokines including IFN $\gamma$ , IL-1, TNF $\alpha$  and GM-CSF. A series of *in vitro* studies have demonstrated the presence of ADCC antibodies against HIV in the plasma of the majority of subjects infected with HIV-1 (Forthal et al., 2001; Ljunggren et al., 1990). HIV-specific ADCC responses generally correlate with delayed HIV progression (Baum et al., 1996). Importantly, Hessel and colleagues showed significant decreases in the efficacy of mutated neutralizing antibodies that were no longer able to elicit ADCC functions (Hessell et al., 2007).

Despite the potential efficacy of ADCC antibodies, little is known about the specific HIV-1 epitopes that stimulate ADCC. To date, only Env (Alsmadi et al., 1997) and Nef (Yamada et al., 2004)-specific ADCC epitopes have been well characterized in HIV-1 infected subjects. Most ADCC responses described in the literature are to the HIV-1 envelope protein (Env). Env is highly variable across HIV strains and can readily mutate to escape Nab and CTL responses. Recent data from our group shows ADCC responses to Env epitopes also force immune escape (Chung et al., 2010). Ideal ADCC epitopes expressed by HIV vaccines would be to conserve internal proteins.

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Elucidating further ADCC epitopes has been slow, in part owing to the inefficient and complex nature of the historical assays for assessing ADCC responses. Typical killing-based ADCC assays measure responses to large proteins and are not suited to mapping ADCC responses. A novel ICS (intracellular cytokine staining) ADCC method has recently been developed in our lab that allows the fine mapping of linear ADCC epitopes (Stratov et al., 2008). This assay measures NK cell activation in response to ADCC antibodies targeting linear epitopes within overlapping peptide sets. Although this is not a “cytotoxicity” based assay, analysis of CD107a (a marker of cytotoxic granule release) provides a surrogate of cytotoxicity and correlates with a standard killing based ADCC assay (Chung et al., 2009). Compared to existing killing based ADCC assays, no artificial cell line is required, and either the patient’s NK cells or healthy donor NK cells used as the effector cells can be tested for ADCC activity along with the assessment of NK cytokine production, chemokine production and loss of perforin or granzymes (Chung et al., 2009). The ability to map linear ADCC epitopes from within large pools of overlapping peptides permits the identification of the particular ADCC antibody.

Two of the most conserved HIV proteins are the Gag and Pol proteins. Gag encodes capsid protein and Pol encodes for the replication enzymes including Protease (PR), Integrase (IN) and Reverse Transcriptase (RT). CTL responses that target Gag and Pol are often highly effective and escape mutations often result in large reductions in viral replicative capacity (large “fitness cost”) (Hue et al., 2009) (Clavel et al., 2000). Similarly, drug resistance mutations within Pol proteins frequently result in fitness costs (Rangel et al., 2009). ADCC epitopes within these Gag and Pol proteins could be valuable vaccine targets.

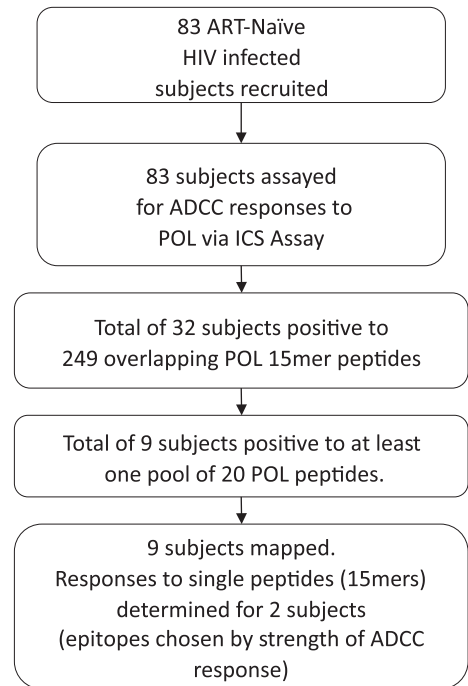
We prospectively studied ADCC responses to linear Gag and Pol peptides in a cohort of 83 HIV-infected subjects using our ICS-based ADCC assay. We found that Pol was commonly targeted by ADCC responses in these chronically infected subjects but Pol-specific ADCC responses did not correlate with delayed HIV progression.

## Results

### *Pol is a target for ADCC responses*

Envelope is the most well recognised ADCC target (Ahmad and Menezes, 1996; Alsmadi and Tilley, 1998; Baum et al., 1996), but ADCC responses to other more conserved HIV proteins such as Pol proteins could help mediate control of viremia. We studied ADCC responses to Pol peptide pools Pol 1 (Pol 1–124) and/or Pol 2 (125–249) using our novel ADCC-ICS assay (Fig. 1). Subject responses were mapped by gating on the lymphocyte population analysing the expression of IFN $\gamma$  and CD107a from CD56+ and CD2+ CD3– NK cells (Fig. 2A). Thirty-two (39%) subjects of the eighty-three assessed showed NK cells activated by one of the Pol overlapping peptide pools (Fig. 1). Similarly to our previous studies on ADCC responses to other HIV antigens (Chung et al., 2009), we found that Pol peptides were required to be present on presenting cells. The activation of NK cells was not diminished even if free peptides were washed out prior to adding serum. The proportion of IFN $\gamma$  expressing NK cells in response to Pol peptides was 0.24% when added directly to donor whole blood and serum, and 0.42% when peptides were first added to whole blood for 1 h then washed twice before adding serum.

We also measured responses to Gag and Env peptides and compared how frequent and robust ADCC responses to Pol were in comparison to Env and Gag. Comparing Pol to Env and Gag we found 11 subjects had CD3– CD2+ cells expressing IFN $\gamma$  to Pol 1 (Mean  $\pm$  SE = 0.30  $\pm$  0.05), and 14 subjects showed responses to Pol 2 (Mean  $\pm$  SE = 0.30  $\pm$  0.08), 34 responded to Env (Mean  $\pm$  SE = 1.165  $\pm$  0.19) and only 3 to Gag (Mean  $\pm$  SE = 0.06  $\pm$  0.05) (Fig. 3A). As compared to CD3– CD2+ IFN $\gamma$ + expressing cells, CD3– CD2+ CD107a+ cells responded as



**Fig. 1.** Flowchart of Pol-specific ADCC studies. 83 Subjects were initially tested for Pol-specific ADCC responses, 32 showed a positive Pol response. Further mapping of these 32 subjects was then undertaken.

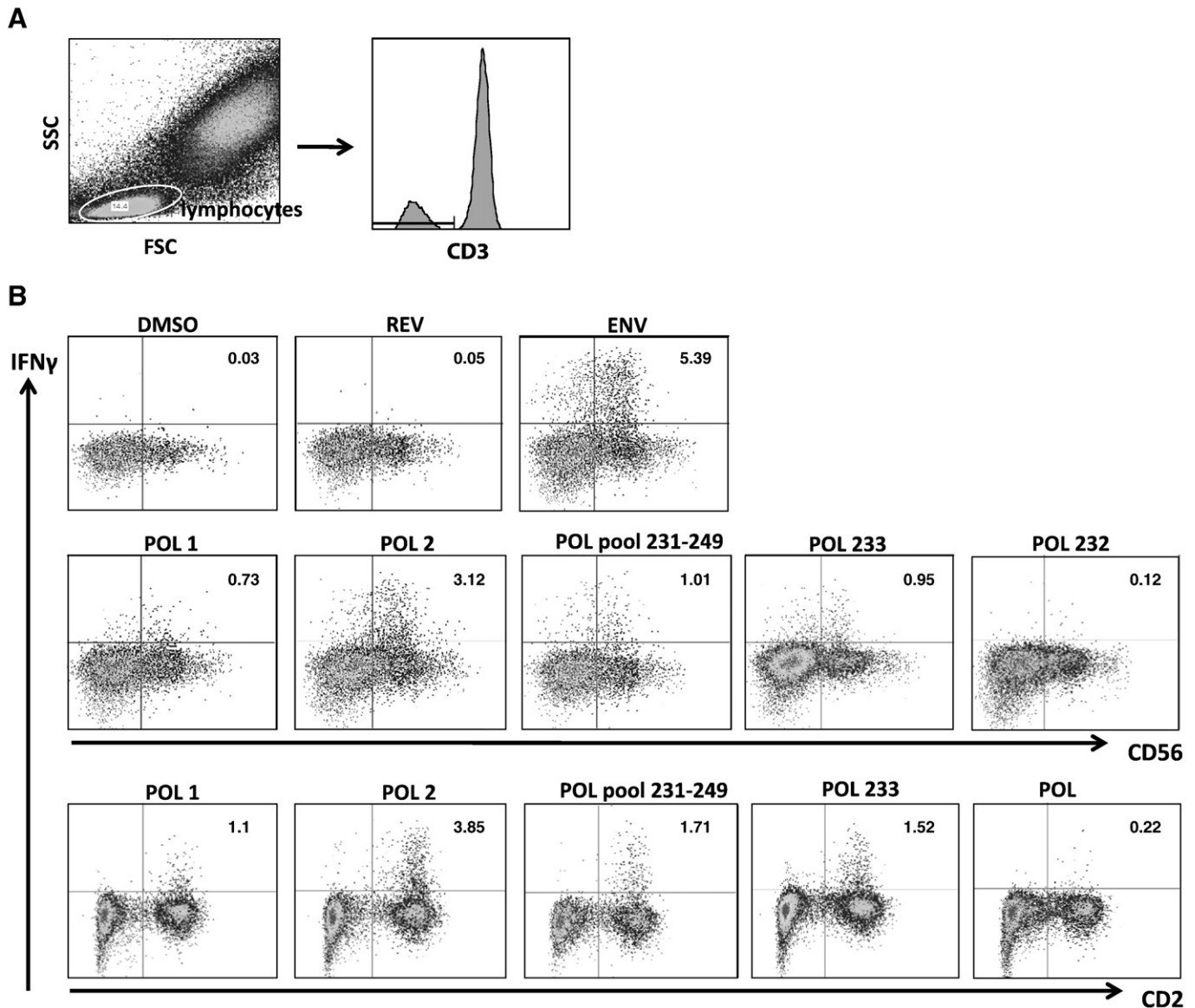
follows: 23 responses to Pol 1 (Mean  $\pm$  SE = 0.63  $\pm$  0.11) and 17 responses to Pol 2 (Mean  $\pm$  SE = 0.29  $\pm$  0.09), 48 responded to Env (Mean  $\pm$  SE = 2.32  $\pm$  0.40) and 11 to Gag (Mean  $\pm$  SE = 0.23  $\pm$  0.08) (Fig. 3B).

Significant ADCC responses to overlapping peptides of Pol 1 or Pol 2 were detected in a number of subjects (Fig. 3). The use of overlapping peptide pools as antigens allows the mapping of responses to smaller sets of peptides. We mapped responses to smaller pools of Pol consisting of 19–21 Pol peptides per pool. Nine subjects from the 32 Pol responders were mapped to at least one pool of Pol peptides. We were further able to fine map responses from 2 subjects to single Pol peptides (Fig. 2B). Subject 29 showed ADCC responses to Pol pool 231–249, which was then mapped to Pol peptides 233 (Fig. 2B).

### *Correlation of Pol specific ADCC responses with HIV progression*

We detected Pol-specific ADCC responses in just under half of the ART naïve, HIV positive subjects. We next asked whether Pol-specific ADCC responses correlated with markers of HIV disease progression. Pol-specific ADCC responses, as measured by NK cells expressing IFN $\gamma$  and CD107a in response to Pol peptides, were correlated to viral loads (Fig. 4A) and CD4 T cell counts were assessed (Fig. 4B). No correlation was evident between the subject’s baseline viral load or CD4 T cell count and ADCC response to Pol using the ADCC ICS assay, either by IFN $\gamma$  or CD107a expression analysis. We followed the subjects for a mean of 3 years (2006–2010) after enrolment, during which time they had regular CD4 T cell counts. The unpaired *t* test was used to assess the relationship between the loss of CD4 T cell (CD4 count slope) over time and Pol-specific ADCC responses (Fig. 4C). The decline in CD4 T cell count over time did not correlate with the presence of ADCC responses to Pol.

During follow up, the HIV infection of 45 subjects progressed such that ART was initiated. The effects of ART on subjects with a response to Pol were also calculated (Fig. 4D). Of the subjects, who progressed to require ART, 45% were Pol responders and 31% of ART naïve



**Fig. 2.** Intracellular cytokine staining ADCC assay and mapping process. A. Gating strategy: lymphocytes from all cells identified and isolated CD3<sup>+</sup> cells to allow assessment of CD2<sup>+</sup> and CD56<sup>+</sup> NK cells expressing IFN $\gamma$ . B. Subject response to Pol, Pol pool 1–124 and 125–249, mapped to Pol pool 231–249 and fine mapped to Pol peptide Pol 233. Responses for DMSO, Rev, Pol pool 126–146, and Pol 232 are included as irrelevant peptides or pools indicating negative ADCC responses.

subjects were also responding to Pol ( $P = 0.52$ ). Overall, we found that Pol-specific ADCC responses did not illustrate a significant correlation with progressive HIV disease.

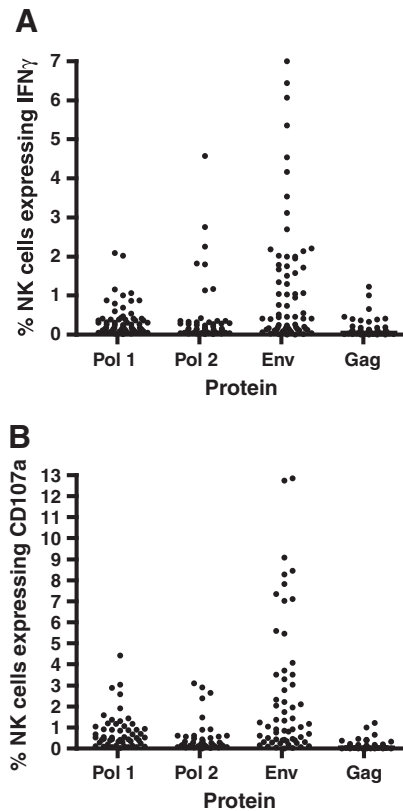
#### *ADCC Pol-specific responses decline over time*

Interestingly a number of subjects with initial responses to Pol and Pol pools (Table 1) were unable to be fine mapped to individual Pol peptides at subsequent blood samples (Fig. 5). For example subject 2 had a Pol response of approximately 1% in 2006–2008, which was mapped to Pol pools 168–188 (1.33%) and 210–230 (0.66%). (Table 1.) However we were unable to later fine map these responses to the individual Pol peptides, since the ADCC response to Pol declined to <0.1% in 2009–2010. This decline in Pol response over the years was evident in most of the subjects (Fig. 5). Inability to fine map some subjects to specific Pol peptides may be explained by this decline in response.

#### **Discussion**

Until HIV-specific broadly neutralizing antibodies to envelope can be reliably induced, it will be important to identify functional immune responses to conserved HIV proteins, which can effectively be induced by vaccination. Although some Gag-specific CD8<sup>+</sup> CTLs are associated with reduced HIV viral loads, only a subset of individuals have the HLA class I alleles that can present the most effective CTL responses. Effector antibody responses such as ADCC that recognise conserved internal proteins, such as Gag and Pol, is a potential method of targeting more broadly acting useful immune responses.

This is the first study to investigate in detail ADCC immune responses to HIV-1 Pol and Gag proteins. We employed a recently described ICS assay that assessed NK cell activation in response to overlapping HIV peptides and ADCC antibodies in plasma (Chung et al., 2009; Stratov et al., 2008). This assay measures both NK cell IFN $\gamma$  expression and CD107a expression (a degranulation marker), as



**Fig. 3.** Env, Gag and Pol specific ADCC responses. Initial ADCC responses to Pol were identified when ADCC responses were mapped for HIV proteins (Env, Gag and Pol). Significant ADCC responses for Env were ascertained as well as for Pol 1 and or Pol 2 by a number of subjects.

markers of cytotoxicity. This assay provides an alternate perspective on antibodies that trigger NK cell activation. We studied a cohort of 83 HIV+ subjects not on ART and found 32 subjects with ADCC responses to linear Pol peptides, although only 11 subjects targeted the Gag protein (including those that expressed either IFN $\gamma$  or CD107a or both). By mapping ADCC responses to smaller pools of Pol peptides we found that Pol proteins were broadly targeted by ADCC responses. The majority of the higher responses >0.5% was to pools 126–249, which correlates with the 5' end of the Pol protein. This region expresses RT and IN proteins. Targeting the RT and IN sections of Pol may prove useful targets for a vaccine that elicits ADCC antibodies to induce significant ADCC immune responses against HIV infection. However, we found no correlation between Pol-specific ADCC responses and several markers of HIV progression in the cohort, including loss of CD4 T cells over time and the requirement for anti-retroviral therapy. Interestingly, Pol-specific ADCC responses declined during the follow-up of this cohort.

ADCC responses to HIV are induced early during HIV infection and several studies have shown ADCC responses to Env proteins correlate with slower HIV progression (Ahmad et al., 2001; Alsmadi and Tilley, 1998; Baum et al., 1996; Chung et al., 2008; Tyler et al., 1990). Env readily mutates to escape other immune responses without significant loss in replicative capacity (i.e. minimal “fitness cost”) and is likely to mutate to avoid ADCC responses (Richman et al., 2003). Pol specific CTL epitopes can also mutate to escape immune pressure although there may be bigger fitness costs associated with escape from Pol specific ADCC responses in comparison to Env-specific responses. Similarly, Pol-specific mutations induced during the development of antiretroviral drug resistance also frequently result in significant fitness costs (Sun et al., 2009). Mapping of multiple Pol-specific responses to specific epitopes allows the assessment of

immune escape mutations within the Pol-specific ADCC epitope. Evidence of escape would suggest some immune pressure is being applied by Pol-specific ADCC responses and explain in part the lack of correlation with disease progression observed. It is important to note that our results are restricted to analysing linear rather than conformational Pol-specific ADCC responses. Conformational Pol-specific ADCC epitopes could be more effective at slowing HIV disease. Future studies could evaluate Pol-specific ADCC responses using purified whole Pol proteins.

ADCC responses generally target viral proteins expressed on the surface of infected cells (Ahmad and Menezes, 1996). Since Pol is composed of several enzymes which act intracellularly, with very little of the protein expressed within virions, it is not clear how Pol proteins would be recognised by ADCC antibodies. Alternatively, the ADCC recognition of Pol proteins may primarily be induced by recognition of viral debris from lysed infected cells – in this scenario Pol would not be expected to delay disease progression as we observed. Precisely which cells would present Pol peptides is unclear. Phagocytic cells such as neutrophils and macrophages may scavenge Pol antigens and present them to ADCC antibodies. Future studies of purified Pol-specific ADCC antibodies could assess whether HIV replication is inhibited *in vitro* using the antibody-dependent cellular viral inhibition assay described by Forthal et al., (2006).

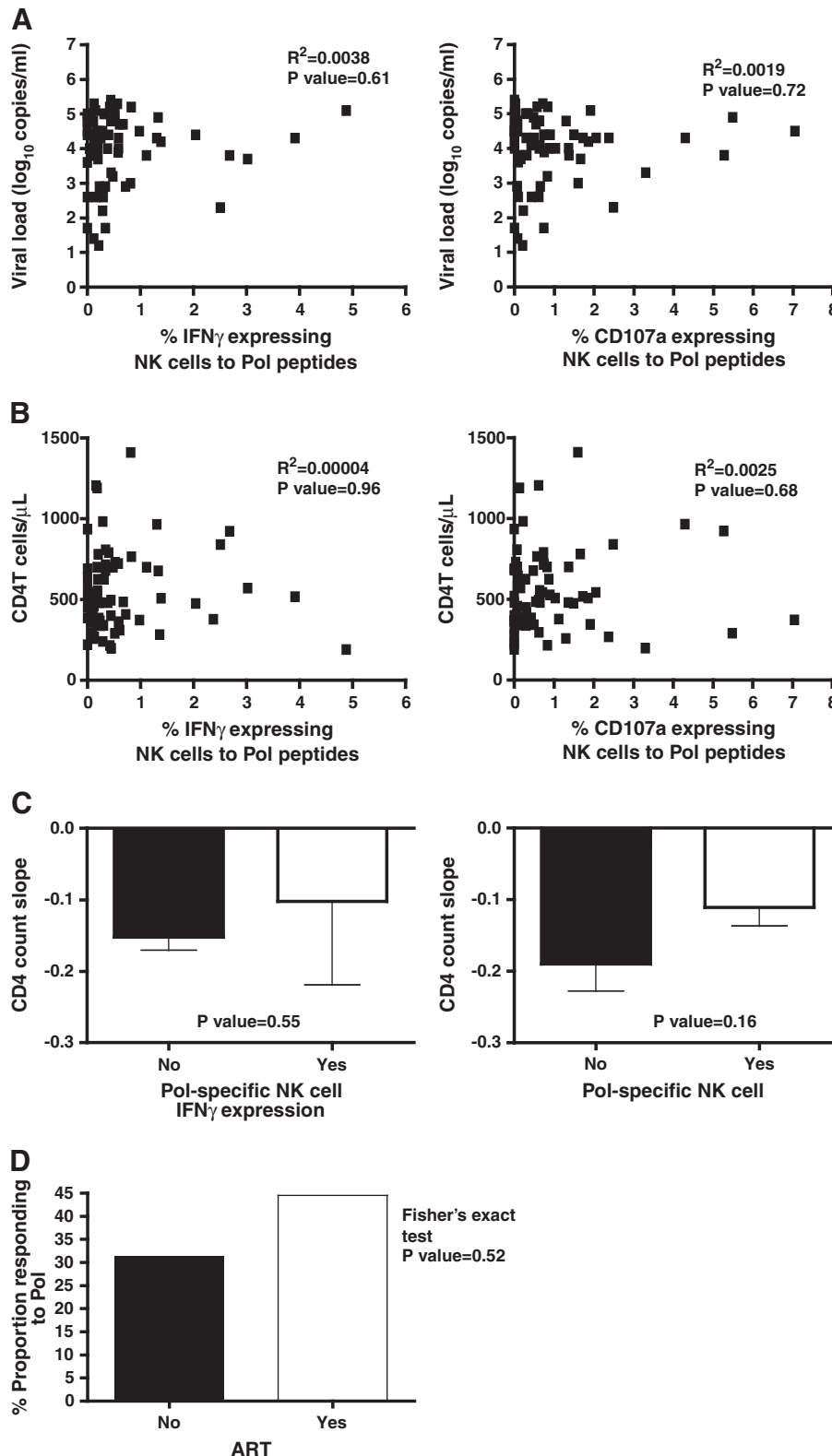
Interestingly, we observed a general decline in linear Pol-specific ADCC responses during follow up of the cohort. This restricted our ability to map responses to minimal epitopes. Others have also observed decline in HIV-specific ADCC responses over time (Baum et al., 1996). Decreases in numbers and function of circulating NK cells is likely to contribute in part to this decrease in ADCC activity during HIV infection (Fauci et al., 2005; Kottlil et al., 2003; Mavilio et al., 2005, 2006). A decrease in the function of ADCC effector cells over time would restrict the ability of ADCC responses to slow HIV disease progression as we observed. In addition, a decline in all HIV-specific antibodies is commonly observed in late stage HIV disease (Popovic et al., 1984). However, we might expect that ADCC responses induced by vaccination (prior to HIV infection) would be fully functional and could be capable of contributing to prevention of HIV infection. A modest reduction in numbers of new infections was recently observed in the Thai RV 144 HIV vaccine efficacy trial (Rerks-Ngarm et al., 2009). The vaccine regimen infrequently induced CTL responses and induces only narrowly directed Nab responses but does induce robust ADCC responses (Karnasuta et al., 2005). Several groups have speculated that ADCC responses may be in part responsible for the protection observed by SIV vaccine regimens in macaques (Flores et al., 2006; Gomez-Roman et al., 2005).

In summary, we found Pol is a significant target for ADCC responses in HIV-1 infected subjects but that Pol-specific ADCC responses declined over time and did not correlate with slower progression of HIV infection. To further probe these findings, future studies are needed to resolve both the mechanisms of induction and presentation of Pol-specific ADCC responses and whether such responses force immune escape over time.

## Materials and methods

### Study subjects

To study Pol-specific ADCC responses in HIV-infected adults we recruited 83 HIV positive individuals not on antiretroviral therapy prospectively to donate blood samples in 2006 to 2007 with a follow-up period of 2 years (range 1–3 years). Subjects were recruited through the Melbourne Sexual Health Centre and the Alfred Hospital, Melbourne, Australia. All subjects provided informed consent. Blood samples were analysed at the start of follow-up and 2 years after recruitment. The subjects provided 9–18 ml of



**Fig. 4.** Correlation of Pol-specific ADCC responses with HIV progression. Correlation of Pol responses, NK cells expressing IFN $\gamma$  and CD107a to A. viral loads and B. CD4 T cell at recruitment to the study were undertaken. No correlation was evident with all parameters analysed against Pol-specific ADCC responses. C. The unpaired *t* test was used to assess the relationship between the loss of CD4 T cell (CD4 count slope) over time and whether or not subjects had a detectable Pol-specific ADCC response. The decline in CD4 T cell count over time did not correlate with the presence of an ADCC response to Pol. D. The effects of ART on subjects with a response to Pol were also calculated. Of the subjects, who progressed to require ART, 45% were Pol responders and 31% of ART naïve subjects were also responding to Pol ( $P=0.52$ ).

Na-heparin anticoagulated blood for analysis using the ADCC-ICS assay. During the follow up period 45 of the 83 subjects started antiretroviral therapy for HIV progression at the discretion of the

treating physician and were censored for follow up at that time. The relevant human research ethics committee approved all studies.

**Table 1**  
Mapping of Pol-specific ADCC responses to pools of 20 overlapping peptides.

Pol peptide pools												
Subject	1–20	21–41	42–62	63–83	84–104	105–125	126–146	147–167	168–188	189–209	210–230	231–249
2									1.33*		0.66*	
11											0.68#	0.63+
29	0.7+	0.8^				1.31+						1.71+
46	0.37+	0.35+										
53		2.45^	0.92^	0.65^	1.202^	0.862^	0.621^	1.102^	0.872^	1.042^	0.752^	0.658^
62	0.7^											
65											0.445^	
67		0.59^	0.43*				0.57#					1.32^
80		0.383^					0.368^			0.362^		0.51^

Maximum response for each pool shown based on gating \*CD56+ CD107a+; + CD56+ IFNγ+; #CD2+ IFNγ+; ^CD2+ CD107a+.

**Peptide antigens**

ADCC responses were detected using 249 Consensus B subtype Pol peptides (15-mers, overlapping by 11 amino acids, solubilized in pure dimethyl sulfoxide (DMSO), divided into two pools (Pol 1–124, Pol 125–249)), spanning all HIV-1 Pol proteins using a consensus subtype B (kindly supplied by the NIH AIDS Reagent Repository). Pol-specific responses were further mapped by creating sub-pools of 20 or 21 individual peptides and further mapped to single Pol 15mer peptides. Fine mapping of Pol-specific ADCC responses was conducted approximately two years after enrolment of subjects in the study. Pol-specific responses were compared to responses to a pool of 212 consensus B subtype Env and 123 Gag 15mer overlapping peptide pools (NIH AIDS Reagent Repository).

**Analysis of Pol-specific ADCC responses**

The NK cell activation ADCC ICS assay was performed on all subjects using fresh sodium-heparinized whole blood to measure responses to Pol 1 and Pol 2 peptide pools. To map Pol-specific ADCC responses we studied stored plasma mixed with fresh healthy donor whole blood as previously described (Stratov et al., 2008). Samples were incubated at 37 °C for 5 h with peptides at a final concentration of 1 µg/ml of peptide in the presence of Brefeldin A (0.25 mg/ml) and Monensin (5 mg/ml) (Sigma). Negative (DMSO alone) and positive control (combined SEB (*Staphylococcus enterotoxin B*) Sigma) wells were included. Cells were surface stained with antibodies obtained from BD Biosciences CD2-FITC (fluorescein isothiocyanate), CD3-PE (phycoerythrin), CD56-PerCP (peridinin chlorophyll protein) and CD107a-APC (allophycocyanin). Lysis was performed on the stained

cells and permeabilized, and stained intracellularly with IFN- AF700 (AlexaFlour700). Data was collected using the FACS Canto analyser and data analysis was performed on FlowJo 9.0.2 software for CD56+ and CD2+ NK cells expressing IFNγ and CD107a within a CD3–lymphocyte gate. A Pol specific response was defined into two parts with a Pol response to Pol 1 (Pol 1-124) as >0.65% and to Pol 2 (Pol 125-249) as >0.32%. This criteria was based on greater than 2 standard deviations above the mean response to Pol 1-124 and Pol 125-249 in HIV-1 negative subjects (n = 12).

**Statistical analysis**

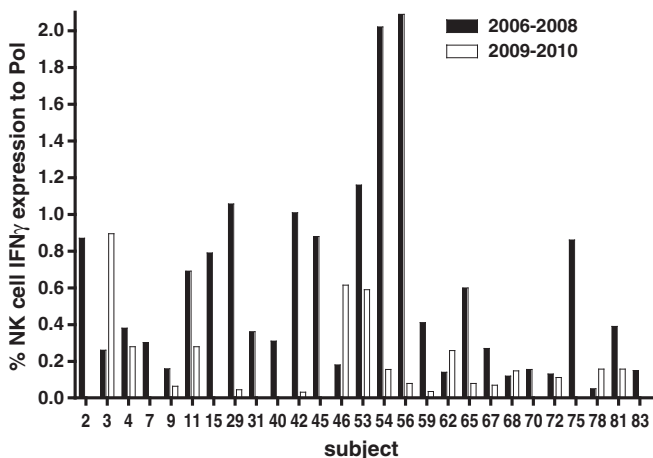
All statistical analyses were performed using Prism 4.0c (Graph-Pad). Analysis of correlation between CD4 counts and viral load with percent IFNγ and CD107a expression of NK cells to Pol peptides were evaluated using the linear regression test (Fig. 4A and B). Calculations of CD4 count slope (Fig. 4C) were determined in Excel (Microsoft) before assessing the significance of the results using the unpaired t test and the Fisher's exact test for Fig. 4D.

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**Fig. 5.** Changes in Pol-specific ADCC responses over time. A decline in Pol-specific ADCC responses over time occurred in the majority of subjects.

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