

**Effect of combination antiretroviral therapy on HIV-1-specific antibody-dependent cellular cytotoxicity responses in subtype B- and subtype C-infected cohorts**

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**Conflicts of interest and Source of funding:** No reported conflicts. This work was supported by the Australia-India Strategic Research Fund (award 08020) from the Australian government, Department of Industry, Innovation and Science, Australian NHMRC grant (awards 1052979, 1041832, 1042632), the Australian Centre for HIV and Hepatitis Virology Research, Victorian government operational infrastructure scheme and the Indo-Australian Strategic Biotechnology Fund from the Department of Biotechnology, Government of India.

**ABSTRACT**

**Background**

There is growing interest in immune therapies to clear the latent HIV-1 after combination antiretroviral therapy (cART). There is limited information on the effect of cART on antibody-dependent cellular cytotoxicity (ADCC) and no studies have directly compared ADCC in HIV-1 subtype B- and subtype C-infected subjects. The effect of improving immunocompetence on ADCC to influenza also remains unexplored.

## Methods

The effect of cART on HIV-1- and influenza-specific ADCC was analyzed in two cohorts (39 subtype B- and 47 subtype C-infected subjects) before and after two years of cART. ADCC analyses included an ELISA-based dimeric recombinant soluble (rs) Fc $\gamma$ RIIIa-binding assay, antibody-dependent natural killer (NK) cell activation assay and ADCC-mediated killing assays.

## Results

HIV-1 subtype B and C Env-specific antibody binding to dimeric rsFc $\gamma$ RIIIa were reduced in subtypes B- and C-infected cohorts after two years of cART (both  $p < 0.05$ ). Reduced ADCC-mediated killing of target cells expressing subtype B Env in the subtype B-infected cohort ( $p = 0.003$ ) was observed after 96 weeks of cART, but not of subtype C Env in the subtype C-infected cohort. A greater reduction in ADCC was detected in subjects with baseline CD4 counts  $> 300$  cells/ $\mu$ l ( $p < 0.05$ ). The resolving immunodeficiency after 96 weeks of cART resulted in improved HA-specific ADCC to six strains of influenza (all  $p < 0.01$ ).

## Conclusions

cART results in HIV-1 antigen loss and reductions in HIV-1 Env-specific antibodies with Fc functionality in both subtype B- and C-infected subjects, particularly in immunocompetent subjects. Simultaneously, cART improves ADCC to diverse strains of influenza, suggesting reduction in influenza disease after cART.

## Key words

HIV-1, subtype B, subtype C, cART, ADCC, Fc $\gamma$ R, Influenza, Australia, India

## INTRODUCTION

The HIV-1 epidemic is a global health issue with no effective protective vaccine available. Combination antiretroviral therapy (cART) has improved the health and prolonged the life of HIV-1-infected individuals [1, 2]. Although cART reduces plasma HIV-1 RNA to very low levels, cellular HIV-1 DNA remains detectable during cART and more sensitive assays have shown that low level HIV-1 RNA remains present in the plasma of most patients on therapy [3]. This residual viremia may be due to the release of HIV-1 from reactivated latently infected cells or from low level virus replication in other tissue reservoirs.

cART reduces HIV-1-specific immune responses such as CTLs [4, 5] and neutralizing antibody (NAb) responses in subjects receiving therapy [6-9], however there is a limited amount of information on reductions in ADCC. We previously showed that the loss of HIV-1 antigen with cART results in reduced antibody-dependent effector functions in HIV-1 subtype AE infected cohort from Thailand [10].

HIV-1 diversity poses a major problem to designing globally effective prevention as well as cure strategies. HIV-1 subtype C accounts for almost half of worldwide infections and is prominent in less developed countries such as India and South Africa [11]. There is limited understanding of the effect of cART on antibody-based immune responses to HIV-1 subtype C compared to HIV-1 subtypes present in the more developed countries such as subtype B.

Further, little is understood about how cART influences ADCC responses to other viral infections such as influenza. Influenza infections are more severe in HIV-1-infected individuals [12, 13] and there is growing interest in the role of ADCC antibodies, which can recognize more diverse strains than NAb, in controlling influenza [14]. Evidence in mouse and macaque models, as well as human challenge studies, suggests ADCC responses can reduce influenza disease severity. We recently showed that HIV-1 infected individuals already on cART have lower influenza-specific ADCC responses than HIV-1

uninfected subjects but can generate effective influenza-specific ADCC responses after seasonal influenza vaccination [11]. The effect of cART on ADCC responses to influenza has not previously been studied.

Given the paucity of information on the effect of cART on HIV-1 and influenza specific ADCC responses across different populations, we studied the effect of cART on ADCC responses in subtype B and C infected cohorts. We found reductions in HIV-1 Env-specific antibodies with Fc functionality in both subtype B and C infected subjects, particularly in subjects with high CD4 T cell counts. Conversely, ADCC responses to influenza were enhanced by cART.

## **MATERIALS AND METHODS**

### **Study subjects**

Thirty-nine cART-naive HIV-1 subtype B-infected subjects (based on RT and protease genotyping) were recruited as part of the ENCORE1 study [15, 16]. Serum samples were stored prior to and 96 weeks post initiation of cART (Table 1). The mean age of the subjects was 32 years (range 20-51).

Serum samples from 47 HIV-1 subtype C-infected subjects- 34 subjects recruited as part of the HPTN052 study and 13 from National AIDS Research Institute, Pune, India were obtained prior to and 104 weeks post initiation of cART (Table 1). Mean age of the study participants was 34 years (range 21-50). All HIV-1 infected subjects had sustained viral suppression after two years of cART (<115 copies/ml for subtype B-infected cohort and <400 copies/ml for subtype C-infected cohort).

All participants were consented to the study. Healthy HIV-negative volunteers provided effector PBMCs for the assays conducted. The study was performed under the auspices of participating institutional review boards at all recruiting sites.

## **HIV-1 and influenza antigens**

HIV-1<sub>AD8</sub> gp140 (subtype B) Env protein was kindly provided by Dr Rob Center, Burnet Institute, Melbourne; and was produced from stable Env-expressing HeLa cell lines, using previously published techniques [17]. HIV-1<sub>96ZM651</sub> gp120 (subtype C) Env protein was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. There is 70% amino acid sequence identity between HIV-1<sub>AD8</sub> subtypes B and HIV-1<sub>96ZM651</sub> subtype C Envs. Purified influenza Hemagglutinin (HA) proteins from six different strains of influenza A- H1N1 (New Caledonia/99), H2N2 (Japan/1957), H3N2 (Aichi/1968 and Brisbane/2007), H4N6 (Swine/Ontario/99) and H5N1 (Vietnam/2004) were procured from Sino Biologicals, Shanghai, China.

## **Dimeric recombinant soluble (rs) FcγRIIIa-binding assay**

HIV-1 antibody binding to FcγRs was measured *in vitro* using dimeric rsFcγRIIIa-binding assay recently described to be a sensitive measure of Influenza-specific ADCC antibodies [11, 18]. Briefly, 96-well Maxisorp® ELISA plates (Nunc, Rochester, NY) were coated with 50ng/well of HIV-1 Env protein in PBS overnight at 4°C. Wells were washed with PBS containing 0.05% Tween-20 (U-CyTech) and blocked with PBS containing 1mM EDTA and 1% human serum albumin for 1h at 37°C. Plates were washed and incubated with 1:100 diluted sera for 1h at 37°C, and then with 0.1µg/ml of biotinylated dimeric rsFcγRIIIa diluted in PBS containing 1mM EDTA and 1% bovine serum albumin (Sigma-Aldrich) for 1h at 37°C. Subsequently, 100ng/ml of Pierce™ High Sensitivity Streptavidin-HRP (Thermo Scientific, Pittsburgh, PA) diluted in PBS with 1mM EDTA and 1% bovine serum albumin was added for 1h at 37°C. Lastly, TMB substrate (Sigma-Aldrich) was added and colour development stopped with 1M Hydrochloric acid. Absorbance was read at 450nm. A positive signal was defined as one giving an OD higher than three times mean OD obtained using sera from three HIV-negative donors against each antigen tested.

Influenza-specific antibody binding to dimeric rsFcγRIIIa was measured using purified influenza Hemagglutinin (HA) proteins from six different strains of influenza A- H1N1

(New Caledonia/99), H2N2 (Japan/1957), H3N2 (Aichi/1968 and Brisbane/2007), H4N6 (Swine/Ontario/99) and H5N1 (Vietnam/2004). A serum dilution of 1:20 was tested in the assay and all data were normalised to 5µg/ml HIV IgG immunoglobulin (HIVIG; obtained from NIH AIDS Reagent Program) diluted in PBS.

### **Anti-HIV Env IgG ELISA**

HIV Env antigen in coating buffer (20mM Tris pH 8.8, 100mM NaCl) was coated onto 96-well flat bottom plates at 100 ng/well overnight. After blocking with 5% bovine serum albumin (Sigma-Aldrich) containing 0.1% Tween for 2 hours, sera was added at 1:10,000 dilution. After 90 min incubation, horseradish-peroxidase-conjugated (HRP) rabbit anti-human IgG antibody (Sigma) was added and incubated for 1h. Color reactions were developed using 3,3' -5,5' -Tetramethylbenzidine (TMB) and absorbance measured at 450 nm. A positive signal was defined as one giving an OD higher than three times mean OD obtained using sera from three HIV-negative donors against each antigen tested. We also tested a series of monoclonal anti-HIV broadly neutralizing antibodies-3BNC117, b12, 10-1074, 2G12, VRC01, VRC03, PGT145 (NIH AIDS reagent repository) and rituximab control mAb (all at 20µg/ml) for their ability to bind a particular strain of Env protein.

### **Rapid fluorometric ADCC (RFADCC) assay**

The RFADCC assay was used as previously described [19]. Briefly, 10<sup>6</sup> CEM.NKr-CCR5 cells in 100 µl of RF10 medium (RPMI, Invitrogen Life Technologies, 10% fetal calf serum, 1% penicillin-streptomycin, 2% glutamine) were coated with 3 µg of HIV-1 Env for 1h at room temperature. Uncoated CEM.NKr-CCR5 cells were used as control and were treated identically. Coated CEM.NKr-CCR5 cells were initially labeled with PKH26 (Sigma) and CFSE (Sigma). 2×10<sup>4</sup> PKH26+CFSE+ labeled CEM.NKr-CCR5 target cells were incubated with human sera (1:100 final dilution) for 30 min at 37°C followed by addition of 2×10<sup>5</sup> PBMCs (total volume of 100 µl) to achieve E:T cell ratio of 10:1. Cells were incubated for 4h at 37°C and then stained with Per-CP-conjugated anti-CD3 (clone SK7; Biolegend) and APC-H7-conjugated anti-CD14 (clone MφP9; BD Biosciences) antibodies. Flow cytometry data was collected using a FACS Fortessa Flow

cytometer (BD Biosciences). The data analysis was performed using Flow Jo version 10.0.6 software and gating strategy as previously described [19]. The assay readout is % PKH26+CD3-CD14+ monocyte that is the measure of CFSE-CD3-CD14+ monocytes that acquire the target cell PKH26 dye. For all HIV-1 Env-specific responses the no antigen control responses (average of 5.7%) were subtracted. A positive response was defined as higher than 3 times mean of the response to HIV-1 Env using sera from 3 HIV-negative donors.

### **Influenza HA antibody-mediated NK cell activation assay**

NK cell activation to influenza HA protein was measure by the ability of antibodies bound to a plate coated with influenza protein to induce NK cell expression of CD107a as previously described [11, 20-22]. Briefly, 96-well ELISA plate (Nunc, Rochester, NY) was coated with 600ng of purified influenza A/H3N2/Brisbane/2007 HA protein (Sino Biological, Shanghai) overnight at 4°C in PBS. The wells were washed four times with PBS and then blocked with PBS containing 5% Bovine serum albumin (Sigma Aldrich, St. Louis, MI) for 2 h at 37°C. Once blocked, plates were washed with PBS and incubated with heat-inactivated sera (1:10 dilution) for 2 h at 37 °C. Plates were washed with PBS,  $1 \times 10^5$  GFP-CD16 (176V) NK-92 cells were added to each well and incubated at 37°C with 5% CO<sub>2</sub> for 5 h. Anti-human CD107a allophycocyanin (clone H4A3; BD Biosciences) and 1 mM EDTA were added to the cells for 30 min at room temperature in the dark. GFP-CD16 (176 V) NK-92 cells were then washed twice with PBS, fixed with 1% formaldehyde and 50,000–80,000 events were generally acquired on the FACS LSR Fortessa Flow cytometer and data was analyzed using Flow Jo version 10.0.6 software.

### **Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) and SPSS version 18 software (IBM, Armonk, NY). Data were analyzed by Wilcoxin matched-pairs signed rank test. A p value of <0.05 was considered to indicate a significant difference.

## RESULTS

### **Reduced dimeric rsFcγRIIIa-binding antibodies to HIV-1 subtype B and C Envs in study subjects after two years of cART**

The process of ADCC is initiated primarily through the cross-linking of FcγRIIIa on innate immune cells such as NK cells by the Fc region of antibodies. To quantitate antibodies capable of cross-linking FcγRs, we recently developed a high throughput ELISA-based assay to measure the ability of Influenza-specific ADCC antibodies to bind dimeric rsFcγRIIIa ectodomains [18]. This assay accurately models Fc-mediated antibody functions [11, 22] and was used to evaluate the ability of HIV-1-specific antibodies to bind dimeric rsFcγRIIIa. We found that 96 weeks of cART resulted in a reduction in dimeric rsFcγRIIIa-binding antibodies in the sera of 39 HIV-1 subtype B-infected subjects against both HIV-1<sub>AD8</sub> subtype B and HIV-1<sub>96zm651</sub> subtype C Env proteins (both  $p < 0.0001$ , Fig. 1A). The mean % reduction in the OD value of dimeric rsFcγRIIIa-binding to the homosubtypic subtype B Env protein across the group was 53% and 12 of the 39 subjects had results below the cut-off of the assay after 96 weeks of cART. A more modest 34% mean reduction was observed against the heterosubtypic subtype C Env protein. A proportion of subtype B infected subjects (14 of 39) were negative for cross-reactive dimeric rsFcγRIIIa-binding antibodies at baseline.

A modest reduction in dimeric rsFcγRIIIa-binding antibodies was also observed in sera of 47 subtype C-infected subjects against both subtype B and C Env proteins (both  $p < 0.05$ , Fig. 1B) after 104 weeks of cART. The mean % reduction in dimeric rsFcγRIIIa-binding antibodies was more modest in the subtype C-infected cohort against both subtype C and B Envs tested (15% and 10% respectively) with a smaller proportion falling below the cut-off (4 of 47 for the homosubtypic C subtype Env and 3 of 47 for the heterosubtypic B subtype Env protein).

To determine if there was a concomitant reduction in total Env-specific antibodies, we

measured the anti-HIV Env IgG levels and correlated them with the changes in Env-specific dimeric rsFcγRIIIa-binding. We observed an overall reduction in total anti-Env IgG antibodies after 96 weeks of cART in both cohorts as expected (Figure 1C). However, the change in total anti-Env antibodies did not correlate with change in Env-specific dimeric rsFcγRIIIa-binding (Figure 1D). This suggests additional factors may be influencing the change in dimeric rsFcγRIIIa-binding other than total Env antibody levels.

To analyse whether this disconnect between Env binding and dimeric rsFcγRIIIa-binding was specific to polyclonal sera or was recapitulated with monoclonal antibodies, we studied a series of anti-HIV broadly neutralizing antibodies, comparing their ability to bind a particular Env protein by ELISA with their ability to bind the dimeric rsFcγRIIIa. We found that while some monoclonal antibodies bound well to both Env protein and the dimeric rsFcγRIIIa well (e.g. b12, 10-1074), other monoclonals (e.g. 2G12, VRC03) had poor binding of the dimeric rsFcγRIIIa (Figure 1E). This is consistent with our observations on changes in Env binding and dimeric rsFcγRIIIa binding in subjects starting cART.

### **HIV-1 Env-specific ADCC-mediated killing following long-term cART in subtype B and C-infected cohorts**

To assess the capacity of patient's antibodies to eliminate Env-expressing target cells, we employed the RFADCC assay that measures the loss of integrity of Env-coated target cells by measuring the uptake of fluorescent target cell membrane by healthy donor PBMCs [19]. Using the RFADCC assay, we observed that 96 weeks of cART resulted in decreased uptake of HIV-1<sub>AD8</sub> subtype B Env-coated target cell membrane by gated monocytes (%PKH26+CD3-CD14+) in the sera of subtype B-infected cohort (p=0.003, Fig. 2A). ADCC responses against the heterosubtypic subtype C Env-coated targets were substantially lower than responses to the subtype B Env-coated targets in the subtype B-infected cohort at baseline and were unchanged after cART.

RFADCC responses in the sera of subtype C-infected cohort were detectable against both subtype C and B Env protein-coated targets but did not change after two years of cART (Fig. 2B). There was substantial cross-reactivity towards subtype B Env ADCC responses in sera from the subtype C-infected cohort such that the ADCC responses were not different against subtype C or subtype B Env-coated targets in this cohort both at baseline as well as at 104 weeks post-cART.

### **ADCC changes after cART influenced by CD4 T cell count**

The absence of a reduction in RFADCC responses after two years of cART in the subtype C-infected cohort was curious given the results observed in the subtype B-infected cohort. We hypothesized that levels of immunodeficiency may influence the magnitude of the changes in ADCC responses after cART. We therefore stratified ADCC responses, as measured by both the dimeric rsFcγRIIIa-binding and RFADCC assays, based around the mean CD4 T cell count at baseline of the cohorts, which was around 300 cells/μl.

In sera from the subtype B-infected cohort we found greater reductions in ADCC in subjects with baseline CD4 counts >300 cells/μl. In the dimeric rsFcγRIIIa-binding assay, subjects with CD4 T cell counts of both >300 cells/μl and <300 cells/μl had reduced responses after cART, although the magnitude of the reduction was greater in the >300 cells/μl subset, reflecting higher responses at baseline (Fig 3A). In the RFADCC assay, a significant reduction in responses after cART was observed in subjects with CD4 T cell counts of >300 cells/μl (Fig 3B).

In sera from the subtype C-infected cohort we found a significant reduction in dimeric rsFcγRIIIa-binding antibodies only in subjects with CD4 T cell counts of >300 cells/μl at baseline (Fig 3C). There was however no significant differences in responses after cART as measured by the RFADCC assay in this cohort when stratified by CD4 T cell count (Fig 3D).

## **Increased Flu-HA-specific ADCC in HIV-1 subtype B-infected subjects following long-term cART**

The reduced HIV-1-specific ADCC responses after cART were more marked in subjects with preserved immunocompetence (higher CD4 T cells counts) suggesting that immune deficiency plays a role in the generation or expansion of ADCC responses. To analyze whether responses to other important pathogens were influenced by cART we studied ADCC responses to influenza, an important cause of morbidity and mortality in HIV-1-infected subjects [12, 13]. We previously showed that ADCC responses to influenza are diminished in HIV-1-infected subjects compared to uninfected subjects, although influenza vaccination could at least partially restore this deficit [11].

In the dimeric rsFcγRIIIa-binding assay, we found that cART resulted in significant increases in influenza HA-specific dimeric rsFcγRIIIa-binding antibodies in the sera of 39 HIV-1 subtype B-infected subjects. This was the case for HA proteins from six separate strains of influenza (all  $p < 0.005$ , Fig. 4A) after 96 weeks of cART. The increase in influenza-specific ADCC responses was most marked to strains of influenza that have been circulating in recent years such as H3N2 and H1N1, but significant increases were also observed to HA proteins from strains that would not have been encountered by most subjects such as H2N2, H5N1 and H4N6. This is consistent with priming of cross-reactive influenza-specific ADCC responses by infections or vaccinations with related strains. Indeed, subjects that had higher ADCC responses to H3N2 and H1N1 HA proteins also tended to have higher responses to other HA proteins both before and after cART, as shown by the lines for each subject in supplementary Fig. S1 <http://links.lww.com/QAI/A999>.

Efficient cross-linking of FcγRIIIa on the surface of NK cells should lead to activation of the cells, degranulation, and killing of infected targets. To further corroborate the dimeric rsFcγRIIIa-binding results, we performed an antibody-mediated NK cell activation assay to study the ability of influenza HA-specific antibodies to induce degranulation (CD107a expression) of NK-92 cell line. We observed that two years of cART led to a significant

increase in antibody-mediated NK cell activation against influenza HA from the H3N2/Brisbane/2007 strain in sera of subtype B-infected cohort ( $p < 0.0001$ , Fig. 4B).

## DISCUSSION

There is interest in harnessing immune responses, including antibodies with Fc-mediated functions, to control HIV-1 and eliminate the latently infected cells that remain despite cART. We found however that cART reduces overall levels of Fc $\gamma$ RIIIa-binding and ADCC-mediating antibodies across subjects with HIV-1 subtype B-infection (predominantly from developed countries) and reduced levels of Fc $\gamma$ RIIIa-binding in HIV-1 subtype C-infected subjects from India. Overall, our results are concordant with previous ADCC studies in a Thai cohort, predominantly with subtype AE infection [10], and with studies that analyse other HIV-1 specific immune responses after cART.

Interestingly, we found the overall reduction in HIV-1-specific ADCC responses was more prominent in subjects with higher CD4 T cell counts at baseline and more marked in the subtype B-infected cohort than the Indian subtype C-infected cohort. We speculate that there may be opposing factors mediating changes in ADCC responses after cART. First, the reduction in antigen associated with cART will result in less stimulation of anti-HIV-1 antibodies, reducing HIV-1-specific ADCC responses. This effect is likely to be more prominent in subjects with reasonable immunocompetence at baseline, as we observed in subjects with higher CD4 T cell counts. We note that the ADCC response in the subtype B-infected cohort was higher at baseline, potentially resulting in a greater dynamic range for reduction in this cohort. Second, the improved immunocompetence associated with cART will improve B cell immunity and lead to an increased capacity to generate effective HIV-1-specific antibody responses. Recovery of immune responses to multiple pathogens is a well-recognized benefit of cART, as we observed herein with influenza-specific ADCC responses. This net recovery of B cell immune responses will be more marked in subjects with more severe baseline immunodeficiency. The overall effect of improved B cell capacity versus loss of antigenic stimulus in subjects with low

baseline CD4 T cell counts may be minimal change in HIV-1-specific ADCC responses, at least for the first two years of cART. Third, we speculate there will be genetic and environmental factors that also influence the magnitude of changes observed in HIV-1-specific immune responses after cART. Immune activation status, low level of HIV-1 replication and exposure to other pathogens may influence the expected reduction of HIV-1-specific immune responses associated with loss of HIV-1 antigen on cART. It will be of interest in future studies to focus on studying the inter-relationships between activation, very low levels of HIV-1 RNA expression and low level of HIV-1-specific immune response on cART. One limitation of our study is that both the levels of Env and their overall presentation on gp120-pulsed CEM cells do not necessarily model that on naturally infected cells *in vivo*. We are currently optimising our assays, including the rsFcγRIIIa-binding assay, to use on virus-infected cells which may reveal additional insights.

HIV-1-specific ADCC responses are of interest given the results of HIV-1 vaccine trial correlates analyses [23, 24]. A potentially useful feature of ADCC antibody responses is their ability to bind multiple HIV-1 strains, unlike the strain specificity of many NAb. Indeed, we previously found subjects who control HIV-1 infection have both stronger and broader HIV-1-specific ADCC responses compared to subjects with progressive HIV-1 infection [25]. In this study we found that subjects with both subtype B and C HIV-1 infection usually generate ADCC responses that can recognize both subtypes. Interestingly, however, we found the degree of cross-reactive ADCC responses generated was more robust in the subtype C infected subjects than in the subtype B-infected subjects. Whether the different anti-HIV-1 Env antibody responses elicited in these two different study groups results from inherit differences in humoral immunity or in the different HIV-1 subtype infections is unclear. In either case there is more common recognition of conserved subtype cross-reactive FcγR functional ADCC epitopes in subtype C-infected Indian subjects. This work should be confirmed with larger panel of Env strains in future studies, since this data potentially informs the choice of Env immunogens that are most likely to generate broadly protective ADCC responses.

There is increasing interest in ADCC responses to influenza, with *in vitro*, animal model

and human studies pointing to a role for ADCC in reducing influenza disease severity. We found that influenza HA-specific ADCC responses improved substantially after cART to HA proteins derived from both circulating and non-circulating influenza strains. The improvement in influenza-specific ADCC responses is concordant with the partial recovery in B cell immunocompetence after cART [26]. The improved ADCC response to a diverse array of influenza strains is consistent with recognition of conserved epitopes within the stem of influenza HA. Recognition of conserved epitopes by influenza-specific ADCC responses reflects potential advantages of ADCC responses over NAb responses that are more commonly directed towards the more variable HA head and therefore more strain specific. The role of strain transcending antibodies with Fc $\gamma$ R function in antigen presentation will also potentially result in the accelerated response to strain specific antibody response, including the generation of NAb, as has been seen in other systems including in HIV-1 infection [27]. The improved influenza-specific ADCC responses after cART may be one reason why cART reduces influenza-specific morbidity in this at risk group. We speculate that improved ADCC responses after cART to other pathogens, such as *M. tuberculosis*, may also contribute to reduced disease burdens after cART [28].

In summary, we show that, overall, cART reduces HIV-1-specific ADCC responses in both subtype B and C infected subjects, although this effect is more marked in subjects with higher CD4 T cell counts. At the same time, cART improves influenza-specific ADCC responses, one factor that may assist in the reduction in influenza disease after cART.

## **ACKNOWLEDGMENTS**

Authors wish to thank all the study subjects who donated blood samples for this study. We thank Dr Rob Center, Burnet Institute, Australia for kindly providing the HIV-1<sub>AD8</sub> Env protein. The authors also acknowledge the NIH-sponsored HIV Prevention Trials Network for the use of some of the stored samples and corresponding data included in this study.

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## FIGURE LEGENDS

### **Figure 1. HIV-1 Env-specific dimeric rsFcγRIIIa-binding and total IgG antibodies following long-term cART**

Dimeric rsFcγRIIIa-binding antibodies detected in (A) HIV-1 subtype B-infected cohort (n=39) and (B) HIV-1 subtype C-infected cohort (n=47) against HIV-1<sub>AD8</sub> subtype B Env (left graph) and HIV-1<sub>96ZM651</sub> subtype C Env (right graph) from baseline (w0) and 96 weeks (w96) post initiation of cART using 1:100 serum dilutions. (C) Anti-HIV Env IgG antibodies detected in HIV-1 subtype B-infected cohort (n=39) against HIV-1<sub>AD8</sub> subtype B Env (left graph) and HIV-1 subtype C-infected cohort (n=47) against HIV-1<sub>96ZM651</sub> subtype C Env (right graph) from baseline (w0) and 96 weeks (w96) post initiation of cART using 1:10000 serum dilutions. Comparisons were made using Wilcoxon matched-pairs signed rank test; bars represent mean $\pm$ SEM. Dotted line represents three times mean OD obtained using three different HIV-negative sera samples against each antigen tested. (D) Correlations between change in dimeric rsFcγRIIIa-binding and total IgG antibodies in subtype B and C-infected cohorts by linear regression analyses. (E) Comparison of dimeric rsFcγRIIIa-binding and gp120-binding for a series of anti-HIV broadly neutralizing antibodies- 3BNC117, b12, 10-1074, 2G12, VRC01, VRC03, PGT145 and rituximab control.

### **Figure 2. HIV-1 Env-specific ADCC-mediated killing following long-term cART**

ADCC-mediated killing assay performed on serum from (A) 39 HIV-1 subtype B-infected subjects and (B) 47 HIV-1 subtype C-infected subjects using HIV-1<sub>AD8</sub> subtype B Env and HIV-1<sub>96ZM651</sub> subtype C Env from baseline (w0) and 96 weeks (w96) post initiation of cART using 1:100 serum dilutions. Comparisons were made using Wilcoxon matched-pairs signed rank test; bars represent mean $\pm$ SEM. Dotted line represents three times mean OD obtained using three different HIV-negative sera samples against each antigen tested.

### **Figure 3. ADCC changes after cART influenced by CD4 count**

Analyses of dimeric rsF $\gamma$ RIIIa-binding antibodies (A, C) and ADCC-mediated killing (B, D) based on CD4 counts <300 or >300 cells/ $\mu$ l at baseline and week 96 post-cART in HIV-1 subtype B infected cohort (A, B) and HIV-1 subtype C-infected cohort (C, D). Comparisons were made using Wilcoxon matched-pairs signed rank test; bars represent mean $\pm$ -SEM. Dotted line represents three times mean OD obtained using three different HIV-negative sera samples against each antigen tested.

### **Figure 4A. Influenza HA-specific dimeric rsF $\gamma$ RIIIa-binding antibodies following long-term cART**

Dimeric rsF $\gamma$ RIIIa-binding antibodies detected in HIV-1 subtype B-infected cohort (n=39) against six different strains of influenza HA proteins from baseline (w0) and 96 weeks (w96) post initiation of cART using 1:20 serum dilutions. Comparisons were made using Wilcoxon matched-pairs signed rank test; bars represent mean $\pm$ -SEM.

### **Figure 4B. Influenza HA-specific antibody-mediated NK cell activation following long-term cART**

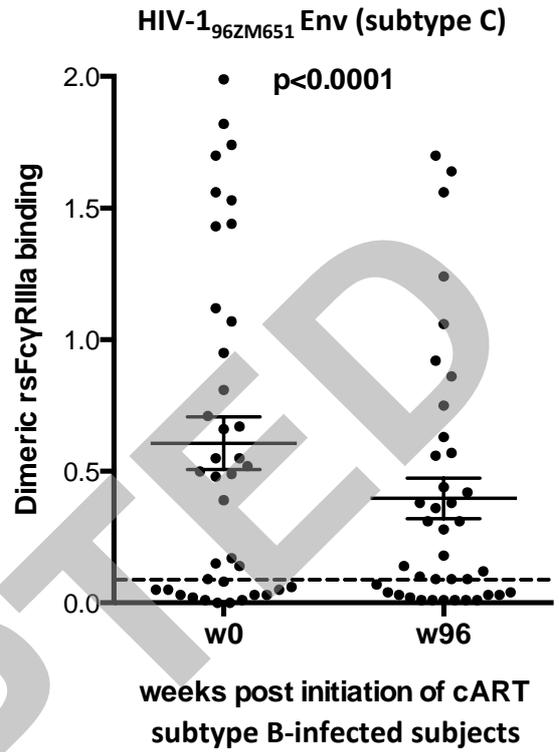
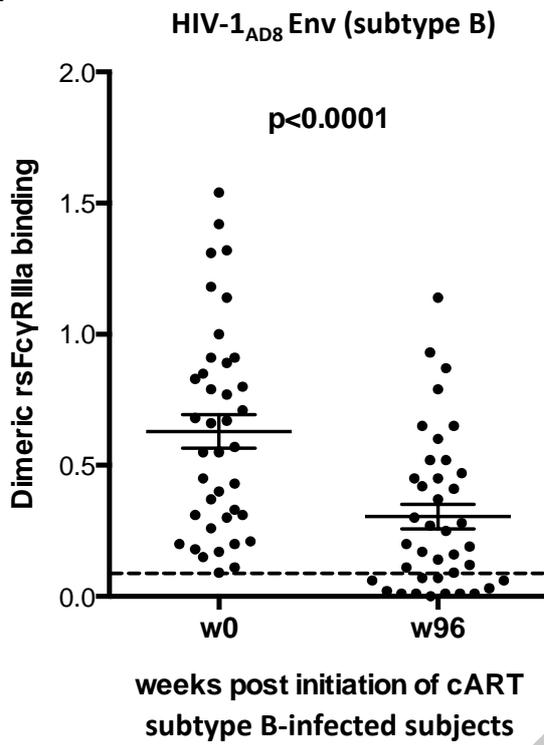
Serum samples from HIV-1 subtype B infected subjects (n=39) analyzed for %NK-92 cells expressing CD107a against influenza HA protein from H3N2 HA Brisbane/2007 strain from baseline (w0) and 96 weeks (w96) post initiation of cART using 1:10 serum dilutions. Comparisons were made using Wilcoxon matched-pairs signed rank test; bars represent mean $\pm$ -SEM.

**Table 1. Cohort characteristics**

		<b>Week 0 baseline</b>	<b>Week 96 post-cART</b>
<b>HIV-1 subtype B- infected cohort</b>	n	39	39
	CD4 count, cells/ $\mu$ l	Mean=312 Range=113-490	Mean=519 Range=210-944
	HIV-1 RNA, copies/ml	Mean= 105660 Range=12449-740839	Mean=42 Range=40-114
	HIV-1 RNA <40 copies/ml,  Patients no.	0	37
<b>HIV-1 subtype C- infected cohort</b>	n	47	47
	CD4 count, cells/ $\mu$ l	Mean= 345 Range=191-622	Mean= 618 Range=190-1225
	HIV-1 RNA, copies/ml	Mean= 99170 Range=2210-543000	<400
	HIV-1 RNA <400 copies/ml,  Patients no.	0	47

Fig 1.

A.



B.

