

# Effect of Combination Antiretroviral Therapy on HIV-1-specific Antibody-Dependent Cellular Cytotoxicity Responses in Subtype B- and Subtype C-Infected Cohorts

Vijaya Madhavi, PhD,\* Archana Kulkarni, PhD,† Ashwini Shete, MD,† Wen S. Lee, BBS (Hons),\* Milla R. Mclean, BSc,\* Anne B. Kristensen, BSc,\* Manisha Ghate, MBBS, PhD,† Bruce D. Wines, PhD,‡§|| Phillip M. Hogarth, PhD,‡§|| Matthew S. Parsons, PhD,\* Anthony Kelleher, PhD, MBBS,¶# David A. Cooper, MD,¶ Janaki Amin, PhD,¶ Sean Emery, PhD,\* ENCORE1 Study Group, Madhuri Thakar, PhD,† and Stephen J. Kent, MD\*\*\*††

**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 2 cohorts (39 subtype B- and 47 subtype C-infected subjects) before and after 2 years of cART. ADCC analyses included an enzyme-linked immunosorbent assay-based dimeric

recombinant soluble (rs) FcγRIIIa-binding assay, antibody-dependent natural killer cell activation assay, and ADCC-mediated killing assays.

**Results:** HIV-1 subtype B and C Env-specific antibody binding to dimeric rsFcγRIIIa were reduced in subtypes B- and C-infected cohorts after 2 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/ $\mu\text{L}$  ( $P < 0.05$ ). The resolving immunodeficiency after 96 weeks of cART resulted in improved HA-specific ADCC to 6 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, FcγR, influenza, Australia, India

(*J Acquir Immune Defic Syndr* 2017;75:345–353)

## 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.<sup>1,2</sup> 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 in the plasma of most patients on therapy.<sup>3</sup> 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 Cytotoxic T lymphocytes (CTLs)<sup>4,5</sup> and neutralizing antibody (NAb) responses in subjects receiving therapy<sup>6–9</sup>; however, there is a limited amount of information on reductions in antibody-dependent cellular cytotoxicity (ADCC). We previously showed

Received for publication November 21, 2016; accepted March 2, 2017.

From the \*Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria, Australia; †Department of Immunology and Serology, National AIDS Research Institute, Pune, India; ‡Centre for Biomedical Research, Burnet Institute, Melbourne, Victoria, Australia; §Department of Immunology, Monash University Central Clinical School, Melbourne, Victoria, Australia; ||Department of Pathology, the University of Melbourne, Melbourne, Victoria, Australia; ¶Kirby Institute, University of New South Wales, Sydney, Australia; #St Vincent's Centre for Applied Medical Research, Darlinghurst, New South Wales, Australia; \*\*Melbourne Sexual Health Clinic and Infectious Diseases Department, Alfred Hospital, Central Clinical School, Monash University, Carlton, Victoria, Australia; and ††ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, the University of Melbourne, Melbourne, Australia.

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.

The authors have no conflicts of interest to disclose.

V.M. and A.K. are joint first authors.

M.T. and S.J.K. are joint last authors.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site ([www.jaids.com](http://www.jaids.com)).

Correspondence to: Stephen J. Kent, MD, 792 Elizabeth Street, Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria 3010, Australia (e-mail: [skent@unimelb.edu.au](mailto:skent@unimelb.edu.au)).

Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.

that loss of HIV-1 antigen with cART results in reduced antibody-dependent effector functions in an HIV-1 subtype AE-infected cohort from Thailand.<sup>10</sup>

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

Furthermore, 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,<sup>12,13</sup> and there is growing interest in the role of ADCC antibodies, which can recognize more diverse strains than NAb, in controlling influenza.<sup>14</sup> Evidence from mouse and macaque models, as well as human challenge studies, suggests that 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 do HIV-1-uninfected subjects but can generate effective influenza-specific ADCC responses after seasonal influenza vaccination.<sup>11</sup> 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 Reverse transcriptase (RT) and protease

genotyping] were recruited as part of the ENCORE1 study.<sup>15,16</sup> Serum samples were stored before initiation and at 96 weeks after 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 before initiation and at 104 weeks after 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 2 years of cART (<115 copies/mL for the subtype B-infected cohort and <400 copies/mL for the subtype C-infected cohort).

All participants consented to the study. Healthy HIV-negative volunteers provided effector peripheral blood mononuclear cells (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.<sup>17</sup> 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 6 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 the dimeric rsFcγRIIIa-binding assay recently described

**TABLE 1.** Cohort Characteristics

	Week 0, Baseline	Week 96, Post-cART
HIV-1 subtype B-infected cohort		
n	39	39
CD4 count, cells/μL	Mean = 312 Range = 113–490	Mean = 519 Range = 210–944
HIV-1 RNA, copies/mL	Mean = 105,660 Range = 12,449–740,839	Mean = 42 Range = 40–114
HIV-1 RNA <40 copies/mL, no. of patients	0	37
HIV-1 subtype C-infected cohort		
n	47	47
CD4 count, cells/μL	Mean = 345 Range = 191–622	Mean = 618 Range = 190–1225
HIV-1 RNA, copies/mL	Mean = 99,170 Range = 2210–543,000	<400
HIV-1 RNA <400 copies/mL, no. of patients	0	47

to be a sensitive measure of influenza-specific ADCC antibodies.<sup>11,18</sup> Briefly, 96-well MaxiSorp enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Rochester, NY) were coated with 50 ng/well of HIV-1 Env protein in phosphate-buffered saline (PBS) overnight at 4°C. Wells were washed with PBS containing 0.05% Tween-20 (U-CyTech, Utrecht, the Netherlands) and blocked with PBS containing 1 mM EDTA and 1% human serum albumin for 1 hour at 37°C. Plates were washed and incubated with 1:100 diluted serum for 1 hour at 37°C, and then with 0.1 µg/mL of biotinylated dimeric rsFcγRIIIa diluted in PBS containing 1 mM EDTA and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MI) for 1 hour at 37°C. Subsequently, 100 ng/mL of Pierce High Sensitivity Streptavidin-HRP (Thermo Scientific, Pittsburgh, PA) diluted in PBS with 1 mM EDTA and 1% bovine serum albumin was added for 1 hour at 37°C. Last, 3,3'-5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added and color development stopped with 1 M hydrochloric acid. Absorbance was read at 450 nm. A positive signal was defined as one giving an Optical Density (OD) higher than 3 times the mean OD obtained using serum samples from 3 HIV-negative donors against each antigen tested.

Influenza-specific antibody binding to dimeric rsFcγRIIIa was measured using purified influenza hemagglutinin (HA) proteins from 6 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 normalized to 5 µg/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 (20 mM Tris pH 8.8, 100 mM 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, serum was added at 1:10,000 dilution. After 90-minute incubation, horseradish peroxidase-conjugated (HRP) rabbit anti-human IgG antibody (Sigma) was added and incubated for 1 hour. 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 3 times the mean OD obtained using serum samples from 3 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 20 µg/mL)—for their ability to bind a particular strain of Env protein.

### Rapid Fluorometric Antibody-Dependent Cellular Cytotoxicity Assay

The rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assay was used as previously described.<sup>19</sup> Briefly, 10<sup>6</sup> CEM.NKr-CCR5 cells in 100 µL of RF10 medium

(RPMI; Invitrogen Life Technologies, Scoresby, Victoria, Australia, 10% fetal calf serum, 1% penicillin-streptomycin, 2% glutamine) were coated with 3 µg of HIV-1 Env for 1 hour 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<sup>+</sup>CFSE<sup>+</sup> labeled CEM.NKr-CCR5 target cells were incubated with human serum (1:100 final dilution) for 30 minutes at 37°C followed by addition of 2 × 10<sup>5</sup> PBMCs (total volume of 100 µL) to achieve an E:T cell ratio of 10:1. Cells were incubated for 4 hour at 37°C and then stained with Per-CP-conjugated anti-CD3 (clone SK7; BioLegend, San Diego, CA) and APC-H7-conjugated anti-CD14 (clone MφP9; BD Biosciences, San Jose, CA) antibodies. Flow cytometry data were collected using an 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.<sup>19</sup> The assay readout is % PKH26<sup>+</sup>CD3<sup>-</sup>CD14<sup>+</sup> monocyte that is the measure of CFSE<sup>-</sup>CD3<sup>-</sup>CD14<sup>+</sup> 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 the mean of the response to HIV-1 Env using serum samples from 3 HIV-negative donors.

### Influenza HA Antibody-Mediated Natural Killer Cell Activation Assay

Natural killer (NK) cell activation to influenza HA protein was measured by the ability of antibodies bound to a plate coated with influenza protein to induce NK cell expression of CD107a as previously described.<sup>11,20–22</sup> Briefly, a 96-well ELISA plate (Nunc) was coated with 600 ng of purified influenza A/H3N2/Brisbane/2007 HA protein (Sino Biological, Shanghai, China) overnight at 4°C in PBS. The wells were washed 4 times with PBS and then blocked with PBS containing 5% bovine serum albumin (Sigma-Aldrich) for 2 hours at 37°C. Once blocked, plates were washed with PBS and incubated with heat-inactivated serum (1:10 dilution) for 2 hours at 37°C. Plates were washed with PBS, 1 × 10<sup>5</sup> GFP-CD16 (176V) NK-92 cells were added to each well, and incubated at 37°C with 5% CO<sub>2</sub> for 5 hours. Anti-human CD107a allophycocyanin (clone H4A3; BD Biosciences) and 1 mM EDTA were added to the cells for 30 minutes 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 were analyzed using Flow Jo version 10.0.6 software.

### Statistical Analysis

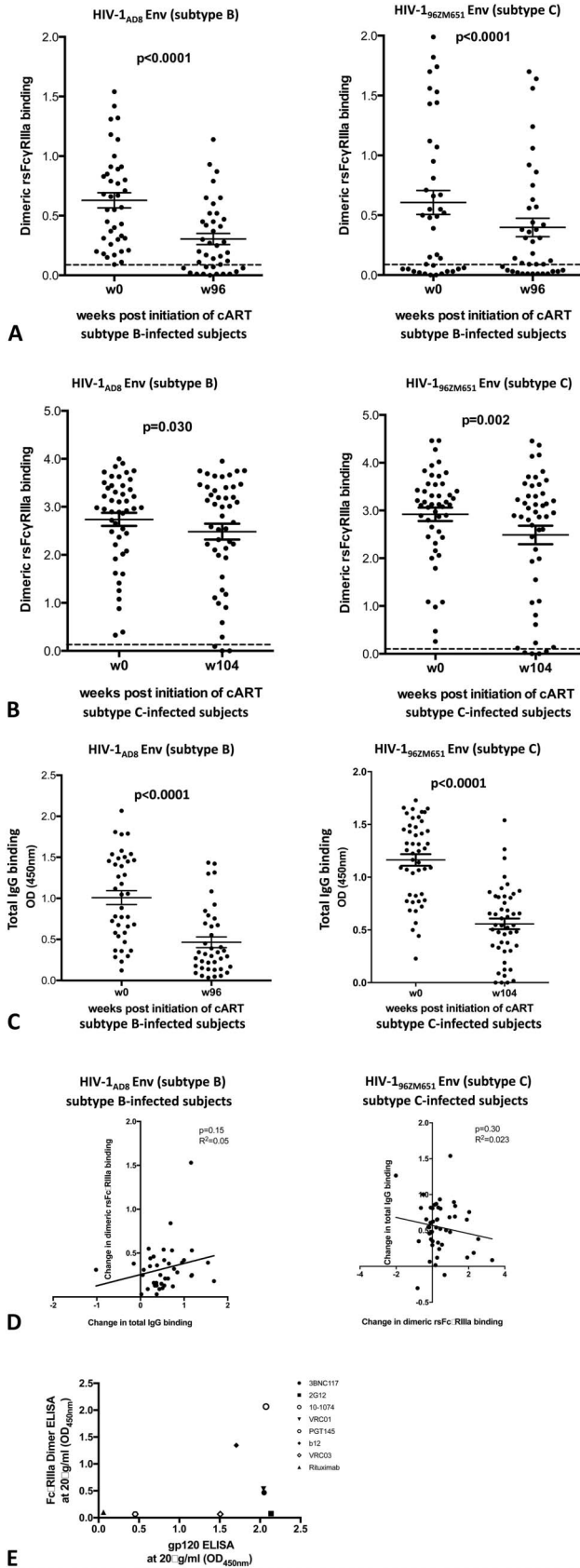
Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) and SPSS version 18 software (IBM, Armonk, NY). Data were analyzed by the Wilcoxon matched-pairs signed-rank test. *P* < 0.05 was considered to indicate a significant difference.

RESULTS

Reduced Dimeric rsFcγRIIIa-Binding Antibodies to HIV-1 Subtype B and C Envs in Study Subjects After 2 Years of cART

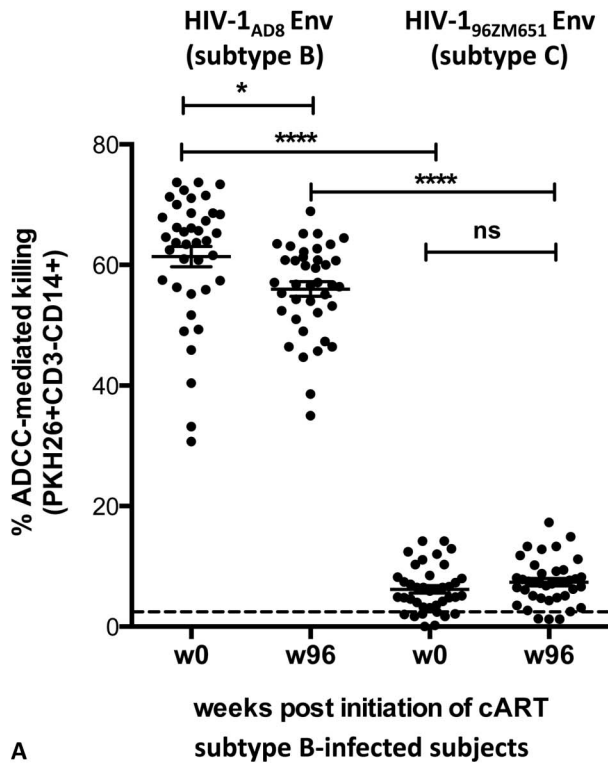
The process of ADCC is initiated primarily through the cross-linking of FcγRIIIa on innate immune cells such as NK cells by the Fc region of antibodies. To quantify antibodies capable of cross-linking FcγRs, we recently developed a high-throughput ELISA-based assay to measure the ability of influenza-specific ADCC antibodies to bind dimeric rsFcγRIIIa ectodomains.<sup>18</sup> This assay accurately models Fc-mediated antibody functions<sup>11,22</sup> 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 serum samples 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 B subtype Env protein across the group was 53%, and 12 of the 39 subjects had results below the cutoff of the assay after 96 weeks of cART. A more modest 34% mean reduction was observed against the heterosubtypic C subtype 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 serum samples 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

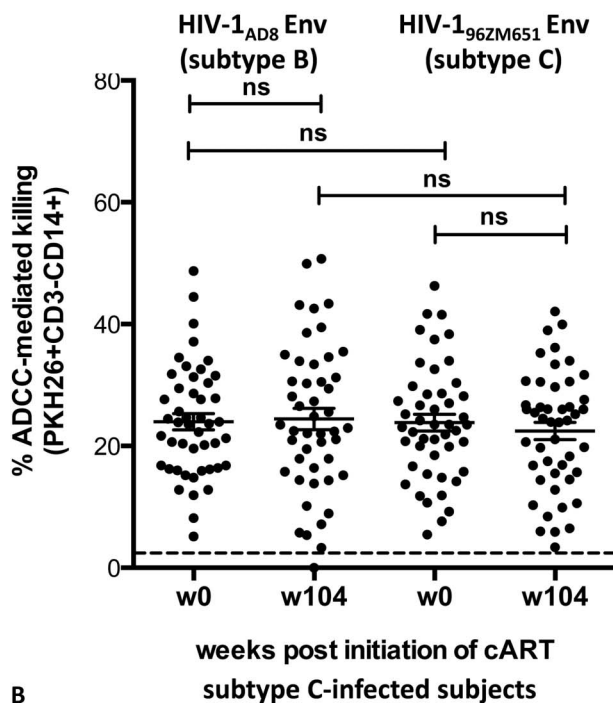


**FIGURE 1.** HIV-1 Env-specific dimeric rsFcγRIIIa-binding and total IgG antibodies after long-term cART. Dimeric rsFcγRIIIa-binding antibodies detected in (A) HIV-1 subtype B-infected cohort (n = 39) and (B) HIV-1 subtype C-infected cohort (n = 47) against HIV-1<sub>AD8</sub> subtype B Env (left graph) and HIV-1<sub>96ZM651</sub> subtype C Env (right graph) from baseline (w0) and 96 weeks (w96) after 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) after initiation of cART using 1:10,000 serum dilutions. Comparisons were made using the Wilcoxon matched-pairs signed-rank test; bars represent mean  $\pm$  SEM. Dotted line represents 3 times the mean OD obtained using 3 different HIV-negative serum 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.





A



B

**FIGURE 2.** HIV-1 Env-specific ADCC-mediated killing after 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) after initiation of cART using 1:100 serum dilutions. Comparisons were made using the Wilcoxon

cutoff (4 of 47 for the homosubtypic C subtype Env and 3 of 47 for the heterosubtypic B subtype Env protein).

To determine whether 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 rsFcyRIIIa binding. We observed an overall reduction in total anti-Env IgG antibodies after 96 weeks of cART in both cohorts as expected (Fig. 1C). However, the change in total anti-Env antibodies did not correlate with change in Env-specific dimeric rsFcyRIIIa binding (Fig. 1D). This suggests that additional factors may be influencing the change in dimeric rsFcyRIIIa binding other than total Env antibody levels.

To analyze whether this disconnect between Env binding and dimeric rsFcyRIIIa binding was specific to polyclonal serum 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 rsFcyRIIIa. We found that although some monoclonal antibodies bound well to both Env protein and the dimeric rsFcyRIIIa (eg, b12, 10-1074), other monoclonals (eg, 2G12, VRC03) had poor binding of the dimeric rsFcyRIIIa (Fig. 1E). This is consistent with our observations on changes in Env binding and dimeric rsFcyRIIIa binding in subjects starting cART.

### HIV-1 Env-Specific ADCC-Mediated Killing After 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 used the RFADCC assay that measures loss of integrity of Env-coated target cells by measuring the uptake of fluorescent target cell membrane by healthy donor PBMCs.<sup>19</sup> 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<sup>+</sup>CD3<sup>-</sup>CD14<sup>+</sup>) in the serum 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 remained unchanged after cART.

RFADCC responses in the serum of subtype C-infected cohort were detectable against both subtype C and B Env protein-coated targets but did not change after 2 years of cART (Fig. 2B). There was substantial cross-reactivity toward subtype B Env ADCC responses in sera samples from the subtype C-infected cohort such that the ADCC responses were not different from subtype C or subtype B

matched-pairs signed-rank test; bars represent mean  $\pm$  SEM. The dotted line represents 3 times the mean OD obtained using 3 different HIV-negative serum samples against each antigen tested.

Env-coated targets in this cohort both at baseline and at 104 weeks after cART.

### ADCC Changes After cART Influenced by CD4 T Cell Count

The absence of a reduction in RFADCC responses after 2 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 rsFcyRIIIa-binding and RFADCC assays, based on the mean CD4 T cell count at baseline of the cohorts, which was around 300 cells/ $\mu$ L.

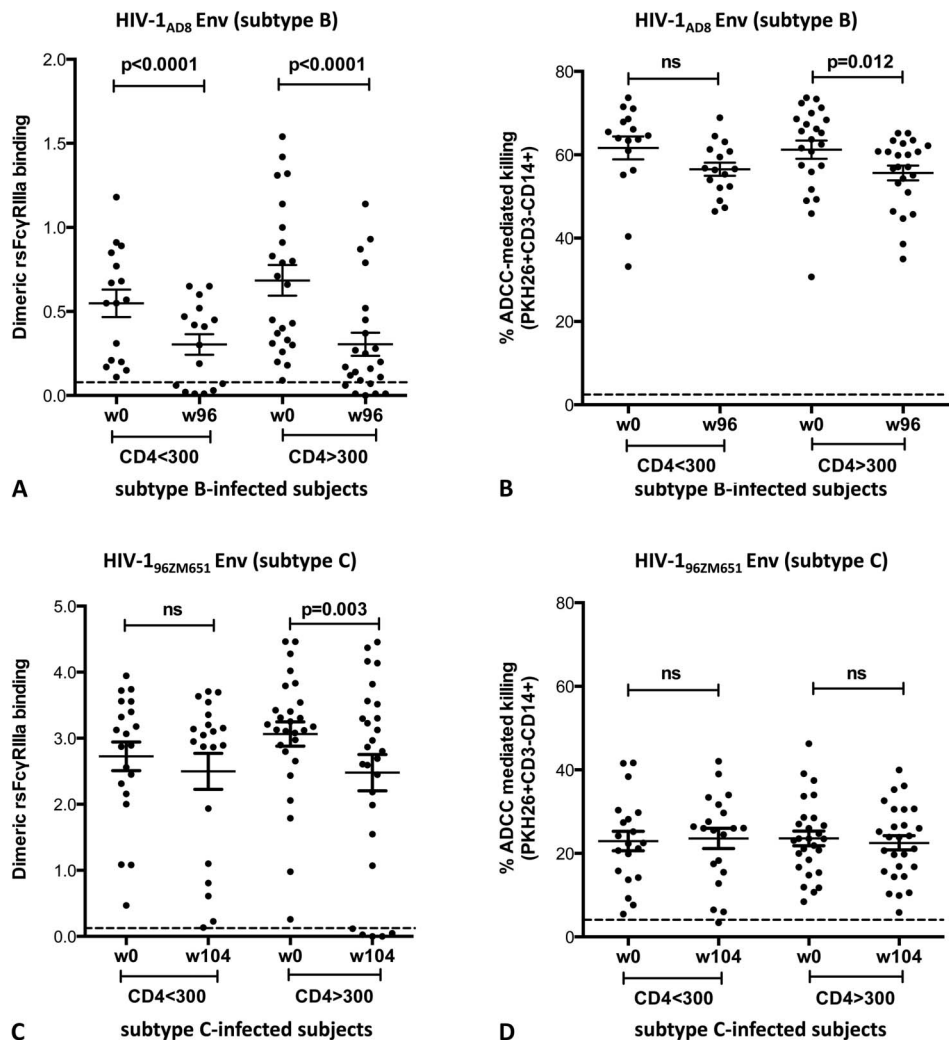
In the serum from the subtype B-infected cohort, we found greater reductions in ADCC in subjects with baseline CD4 counts  $>300$  cells/ $\mu$ L. In the dimeric rsFcyRIIIa-binding assay, subjects with CD4 T cell counts of both  $>300$  and  $<300$  cells/ $\mu$ L had reduced responses after cART, although the magnitude of the reduction was greater in the  $>300$  cells/ $\mu$ L 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/ $\mu$ L (Fig. 3B).

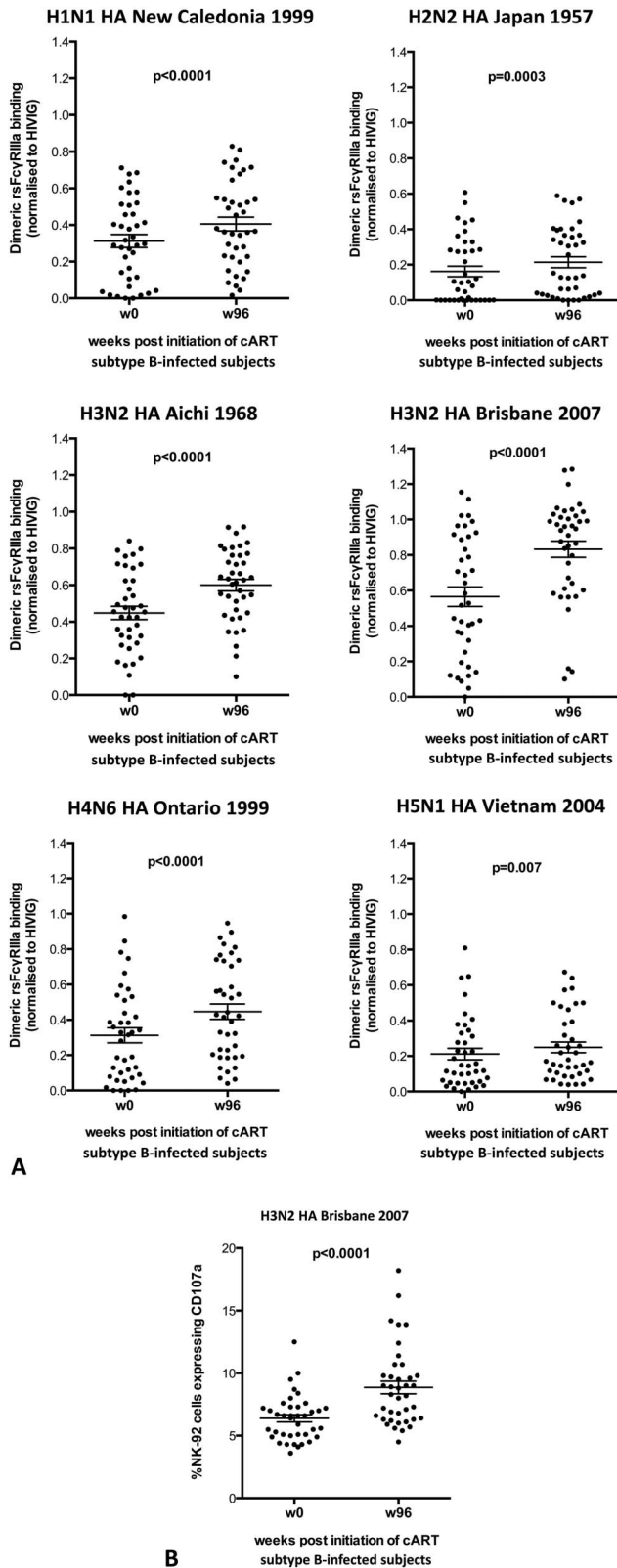
In the serum from the subtype C-infected cohort, we found a significant reduction in dimeric rsFcyRIIIa-binding antibodies only in subjects with CD4 T cell counts of  $>300$  cells/ $\mu$ L at baseline (Fig. 3C). There were however no significant differences in responses after cART as measured by the 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 After 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), which suggests 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



**FIGURE 3.** ADCC changes after cART influenced by the CD4 count. Analyses of dimeric rsFcyRIIIa-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 after cART in the HIV-1 subtype B-infected cohort (A, B) and the HIV-1 subtype C-infected cohort (C, D). Comparisons were made using the Wilcoxon matched-pairs signed-rank test; bars represent mean  $\pm$  SEM. The dotted line represents 3 times the mean OD obtained using 3 different HIV-negative serum samples against each antigen tested.



**FIGURE 4.** A, Influenza HA-specific dimeric rsFcγRIIIa-binding antibodies after long-term cART. Dimeric rsFcγRIIIa-binding antibodies detected in the HIV-1 subtype B-infected cohort (n = 39) against 6 different strains of influenza HA proteins from

morbidity and mortality in HIV-1-infected subjects.<sup>12,13</sup> We previously showed that ADCC responses to influenza are diminished in HIV-1-infected subjects compared with uninfected subjects, although influenza vaccination could at least partially restore this deficit.<sup>11</sup>

In the dimeric rsFcγRIIIa-binding assay, we found that cART resulted in significant increases in influenza HA-specific dimeric rsFcγRIIIa-binding antibodies in the serum of 39 HIV-1 subtype B-infected subjects. This was the case for HA proteins from 6 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 in 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 who 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 Supplemental Digital Content, Figure 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 2 years of cART led to a significant increase in antibody-mediated NK cell activation against influenza HA from the H3N2/Brisbane/2007 strain in the serum 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 FcγRIIIa-binding and ADCC-mediating antibodies across subjects with HIV-1 subtype B-infection (predominantly from developed countries) and reduced levels of FcγRIIIa binding in HIV-1 subtype C-infected subjects from India. Overall, our results are concordant with previous

baseline (w0) and 96 weeks (w96) after initiation of cART using 1:20 serum dilutions. Comparisons were made using the Wilcoxon matched-pairs signed-rank test; bars represent mean  $\pm$  SEM. B, Influenza HA-specific antibody-mediated NK cell activation after 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) after initiation of cART using 1:10 serum dilutions. Comparisons were made using the Wilcoxon matched-pairs signed-rank test; bars represent mean  $\pm$  SEM.

ADCC studies in a Thai cohort, predominantly with subtype AE infection,<sup>10</sup> and with studies that analyze other HIV-1 specific immune responses after cART.

Interestingly, we found that 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 in this study 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 2 years of cART. Third, we speculate that 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 interrelationships 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 optimizing 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 that the results of HIV-1 vaccine trial correlate analyses.<sup>23,24</sup> 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 that subjects who control HIV-1 infection have both stronger and broader HIV-1-specific ADCC responses compared with subjects with progressive HIV-1 infection.<sup>25</sup> 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 that 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 2 different study groups result from inherent 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, because these data potentially inform 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 noncirculating influenza strains. The improvement in influenza-specific ADCC responses is concordant with the partial recovery in B cell immunocompetence after cART.<sup>26</sup> 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 toward the more variable HA head and therefore more strain specific. The role of strain-transcending antibodies with FcγR 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.<sup>27</sup> 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.<sup>28</sup>

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 thank all the study subjects who donated blood samples for this study and 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.*

## REFERENCES

1. Hogg RS, O'Shaughnessy MV, Gataric N, et al. Decline in deaths from AIDS due to new antiretrovirals. *Lancet*. 1997;349:1294.
2. Hogg R, Lima V, Sterne JA, et al. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet*. 2008;372:293–299.
3. Palmer S, Wiegand AP, Maldarelli F, et al. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human



- immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 2003; 41:4531–4536.
4. Ogg GS, Jin X, Bonhoeffer S, et al. Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. *J Virol*. 1999;73:797–800.
  5. Casazza JP, Betts MR, Picker LJ, et al. Decay kinetics of human immunodeficiency virus-specific CD8<sup>+</sup> T cells in peripheral blood after initiation of highly active antiretroviral therapy. *J Virol*. 2001;75:6508–6516.
  6. Notermans DW, de Jong JJ, Goudsmit J, et al. Potent antiretroviral therapy initiates normalization of hypergammaglobulinemia and a decline in HIV type 1-specific antibody responses. *AIDS Res Hum Retroviruses*. 2001;17:1003–1008.
  7. Morris L, Binley JM, Clas BA, et al. HIV-1 antigen-specific and -nonspecific B cell responses are sensitive to combination antiretroviral therapy. *J Exp Med*. 1998;188:233–245.
  8. Morris MK, Katzenstein DA, Israelski D, et al. Characterization of the HIV-1 specific humoral immune response during highly active antiretroviral therapy (HAART). *J Acquir Immune Defic Syndr*. 2001;28:405–415.
  9. Binley JM, Trkola A, Ketas T, et al. The effect of highly active antiretroviral therapy on binding and neutralizing antibody responses to human immunodeficiency virus type 1 infection. *J Infect Dis*. 2000;182:945–949.
  10. Madhavi V, Ana-Sosa-Batiz FE, Jegaskanda S, et al. Antibody-dependent effector functions against HIV decline in subjects receiving antiretroviral therapy. *J Infect Dis*. 2015;211:529–538.
  11. Kristensen AB, Lay WN, Ana-Sosa-Batiz F, et al. Antibody responses with Fc-mediated functions after trivalent influenza vaccination of HIV-infected subjects. *J Virol*. 2016;90:5724–5734.
  12. Kroon FP, van Dissel JT, de Jong JC, et al. Antibody response to influenza, tetanus and pneumococcal vaccines in HIV-seropositive individuals in relation to the number of CD4<sup>+</sup> lymphocytes. *AIDS*. 1994;8:469–476.
  13. Kelly D, Burt K, Missaghi B, et al. Responses to pandemic AS03-adjuvanted A/California/07/09 H1N1 influenza vaccine in human immunodeficiency virus-infected individuals. *BMC Immunol*. 2012; 13:49.
  14. Jegaskanda S, Reading PC, Kent SJ. Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine. *J Immunol*. 2014;193:469–475.
  15. Carey D, Puls R, Amin J, et al. Efficacy and safety of efavirenz 400 mg daily versus 600 mg daily: 96-week data from the randomised, double-blind, placebo-controlled, non-inferiority ENCORE1 study. *Lancet Infect Dis*. 2015;15:793–802.
  16. Puls R, Amin J, Losso M, et al. Efficacy of 400 mg efavirenz versus standard 600 mg dose in HIV-infected, antiretroviral-naive adults (ENCORE1): a randomised, double-blind, placebo-controlled, non-inferiority trial. *Lancet*. 2014;383:1474–1482.
  17. Center RJ, Wheatley AK, Campbell SM, et al. Induction of HIV-1 subtype B and AE-specific neutralizing antibodies in mice and macaques with DNA prime and recombinant gp140 protein boost regimens. *Vaccine*. 2009;27:6605–6612.
  18. Wines BD, Vandervan HA, Esparon SE, et al. Dimeric Fcγ3R ectodomains as probes of the Fc receptor function of anti-influenza virus IgG. *J Immunol*. 2016;197:1507–1516.
  19. Kramski M, Schorch A, Johnston AP, et al. Role of monocytes in mediating HIV-specific antibody-dependent cellular cytotoxicity. *J Immunol Methods*. 2012;384:51–61.
  20. Jegaskanda S, Job ER, Kramski M, et al. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. *J Immunol*. 2013;190:1837–1848.
  21. Jegaskanda S, Laurie KL, Amarasena TH, et al. Age-associated cross-reactive antibody-dependent cellular cytotoxicity toward 2009 pandemic influenza A virus subtype H1N1. *J Infect Dis*. 2013;208:1051–1061.
  22. Vandervan HA, Ana-Sosa-Batiz F, Jegaskanda S, et al. What lies beneath: antibody dependent natural killer cell activation by antibodies to internal influenza virus proteins. *EBioMedicine*. 2016;8:277–290.
  23. Haynes BF, Gilbert PB, McElrath MJ, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med*. 2012;366:1275–1286.
  24. Chung AW, Ghebremichael M, Robinson H, et al. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. *Sci Transl Med*. 2014;6:228ra238.
  25. Madhavi V, Wren LH, Center RJ, et al. Breadth of HIV-1 Env-specific antibody-dependent cellular cytotoxicity: relevance to global HIV vaccine design. *AIDS*. 2014;28:1859–1870.
  26. Wheatley AK, Kristensen AB, Lay WN, et al. HIV-dependent depletion of influenza-specific memory B cells impacts B cell responsiveness to seasonal influenza immunisation. *Sci Rep*. 2016;6:26478.
  27. Schoofs T, Klein F, Braunschweig M, et al. HIV-1 therapy with monoclonal antibody 3BNC117 elicits host immune responses against HIV-1. *Science*. 2016;352:997–1001.
  28. Lu LL, Chung AW, Rosebrock TR, et al. A functional role for antibodies in tuberculosis. *Cell*. 2016;167:433–443.e414.