HIV-1 Env- and Vpu-Specific Antibody-Dependent Cellular Cytotoxicity Responses Associated with Elite Control of HIV

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ABSTRACT Studying HIV-infected individuals who control HIV replication (elite controllers [ECs]) enables exploration of effective anti-HIV immunity. HIV Env-specific and non-Env-specific antibody-dependent cellular cytotoxicity (ADCC) may contribute to protection from progressive HIV infection, but the evidence is limited. We recruited 22 ECs and matched them with 44 viremic subjects. HIV Env- and Vpu-specific ADCC responses in sera were studied using a novel enzyme-linked immunosorbent assay (ELISA)-based dimeric recombinant soluble FcγRIIIa (rsFcγRIIIa)-binding assay, surface plasmon resonance, antibody-dependent natural killer (NK) cell activation assays, and ADCC-mediated killing assays. ECs had higher levels of HIV Env-specific antibodies capable of binding FcγRIIa, activating NK cells, and mediating granzyme B activity (all \( P < 0.01 \)) than viremic subjects. ECs also had higher levels of antibodies against a C-terminal 13-mer Vpu peptide capable of mediating FcγRIIa binding and NK cell activation than viremic subjects (both \( P < 0.05 \)). Our data associate Env-specific and Vpu epitope-specific ADCC in effective immune responses against HIV among ECs. Our findings have implications for understanding the role of ADCC in HIV control.

IMPORTANCE Understanding immune responses associated with elite control of HIV may aid the development of immunotherapeutic and vaccine strategies for controlling HIV infection. Env is a major HIV protein target of functional antibody responses that are heightened in ECs. Interestingly, EC antibodies also target Vpu, an accessory protein crucial to HIV, which degrades CD4 and antagonizes tetherin. Antibodies specific to Vpu are a common feature of the immune response of ECs that may prove to be of functional importance to the design of improved ADCC-based immunotherapy and preventative HIV vaccines.

KEYWORDS ADCC, elite controller, FcγRI, Vpu

The immune mechanisms of human immunodeficiency virus (HIV) control are not clearly understood. Investigation of HIV-infected individuals who naturally control HIV infection, known as elite controllers (ECs), may provide clues to the immune mechanisms facilitating HIV control. ECs represent <0.5% of HIV-infected individuals...
and have repeatedly undetectable HIV plasma viremia in the absence of antiretroviral therapy (ART) (1).

Numerous factors are likely involved in the control of HIV infection, including viral and host genetics, which cannot be easily manipulated in a broad-based vaccine or cure strategy. Particular HLA I alleles, such as HLA-B27, HLA-B57, and HLA-B51, are approximately twice as common in ECs as in people with progressive HIV infection (2–4). HIV epitopes restricted by these alleles (especially targeting Gag) mediate potent HIV suppression (1, 5). HIV-specific CD4 T-cell responses have higher functional avidities in ECs (6), but somewhat counterintuitively, neutralizing antibodies (NAbs) are found less frequently in ECs than in viremic subjects (7, 8). We previously reported that HIV-infected slow progressors have higher and broader antibody-dependent cellular cytotoxicity (ADCC) responses than people with progressive HIV (9, 10). Lambotte et al. have shown high HIV Env-specific ADCC responses in a heterogenous group of HIV controllers (11). However, more data on the role of ADCC in elite control of HIV would facilitate the designing of strategies to control and prevent HIV.

Most antibody-related HIV vaccine research has focused on targeting the envelope (Env) protein of HIV. However, Env has proven to be a difficult target due to its wide diversity across the globe, its high mutation rate, and often poor access of NAbs to critical conserved epitopes (12, 13). Since the partial efficacy seen in the RV144 trial and the results of the subsequent post hoc analyses, HIV-specific ADCC responses have gained importance as potential targets for prevention and control of infection (14). These responses have several possible advantages over NAbs, in that they can target cells within which HIV is replicating and they can also target non-Env proteins such as Pol and Vpu (10, 15).

In addition to Env, immune responses against the HIV regulatory transmembrane protein Vpu are of interest as potentially effective responses. We have previously identified HIV-specific NK cell activation by antibodies targeting the C-terminal portion of the Vpu protein and also showed that Vpu-specific ADCC responses were more common in subjects with slowly progressive HIV infection (16). Chen et al. showed that polymorphisms in the terminal sequence of Vpu are associated with EC status compared with HIV viremic subjects, although the drivers of these polymorphisms were not identified, other than that they was independent of KIR2DL2 status (17).

To investigate a potential role for ADCC in HIV control, we enrolled 22 ECs and compared these to a matched cohort of 44 viremic subjects and studied Env- and Vpu-specific ADCC responses.

RESULTS

ECs have high levels of total HIV Env-specific and Vpu-specific IgG antibodies compared to those of viremic subjects. ECs are a model to study HIV control (9, 18, 19), and we recruited 22 ECs to provide serum samples and matched these subjects to 44 viremic subjects. The clinical characteristics of the subjects are shown in Tables 1 and 2. Since functional antibody responses have been associated with the control of HIV in other settings, we studied antibody responses to both HIV Env and Vpu in ECs. Env is a common target for functional antibody responses (7), and we previously observed Vpu-specific functional antibody responses in a subset of HIV-infected subjects (10). In the current study, we first analyzed total antibody responses and found that sera from 22 ECs had higher levels of HIV Env-specific IgG antibodies than those from the 44 matched viremic subjects ($P < 0.0001$) (Fig. 1A, left panel). As a sensitivity analysis, we performed a conditional logistic regression test accounting for matching between cases and controls and observed a trend toward higher levels of Env-specific IgG in the ECs (adjusted $P$ value $[P'] = 0.069$). We also observed higher Vpu protein-specific IgG responses in the EC cohort ($P = 0.086$ and $P' = 0.027$) (Fig. 1A, middle panel). We had previously mapped an antibody response to the Vpu protein to a C-terminal 13-mer linear peptide (termed “Vpu19” since it is the 19th of 19 overlapping Vpu peptides previously studied) (20). We found that ECs had significantly higher levels of Vpu19-
specific antibodies than viremic subjects ($P < 0.0001$ and $P' = 0.002$) (Fig. 1A, right panel).

Certain HLA I alleles, particularly HLA-B57, -B27, and -B51 play an important role in controlling HIV through CD8$^+$ T cells and are enriched in EC cohorts (3). It has been postulated that ECs without protective HLA I alleles may be more enriched for non-CD8 T-cell responses capable of controlling HIV (21). Six of the 20 ECs tested had one of these three beneficial HLA I alleles (Table 1), and we therefore compared these 6 ECs to the other 14 ECs without beneficial HLA I alleles and to the viremic subjects for Env and Vpu antibody responses. We did not observe any overall difference in the binding antibodies against HIV Env and Vpu within the two EC subgroups, although there was a trend toward higher Vpu protein-specific antibody responses in the EC cohort without protective HLA I alleles (Fig. 1B, middle panel). Furthermore, the subset of ECs without protective HLA I alleles continued to have significantly higher levels of binding antibodies against Vpu19 than viremic subjects ($P < 0.05$) (Fig. 1B, right panel).

**ECs have high levels of FcγRIIIa-binding antibodies to HIV Env and Vpu compared to those of viremic subjects.** There is increasing interest in antibodies to HIV Env with Fc-mediated functions, although the protective importance of such

### TABLE 1 Cohort characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for:</th>
<th>Value for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>Elite controllers</td>
<td>22</td>
</tr>
<tr>
<td>HIV load, copies/ml</td>
<td>&gt;65% of VLs below detection limit$^b$</td>
<td>107,583 (12,449–740,839)</td>
</tr>
<tr>
<td>CD4 count, cells/µl</td>
<td>880 (337–2,475)</td>
<td>303 (113–490)</td>
</tr>
<tr>
<td>Duration of infection, yr</td>
<td>11.3 (2.0–24.0)</td>
<td>6.5 (2.1–21.5)</td>
</tr>
<tr>
<td>HIV-1 subtype(s)</td>
<td>B ($n = 13$), not done ($n = 9$)</td>
<td>B ($n = 39$), C ($n = 5$)</td>
</tr>
<tr>
<td>Gender</td>
<td>4 female, 18 male</td>
<td>8 female, 36 male</td>
</tr>
<tr>
<td>Age, yr</td>
<td>46 (34–61)</td>
<td>41 (23–62)</td>
</tr>
<tr>
<td>Favorable HLA</td>
<td>6 of 20 tested (2 B27, 3 B57, 2 B51)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

$^a$Unless indicated otherwise, values are means with ranges in parentheses.

$^b$See Table 2.

### TABLE 2 Longitudinal VL data for all ECs
functional antibodies to non-Env proteins is not clear. Measuring Fc-mediated antibody function is difficult to standardize because of genotypic differences and the variable activation statuses of effector cells. Further, many flow cytometry-based assays are low throughput and are not well suited for studying multiple antigens in larger cohorts.

To quantitate antibodies capable of binding FcγRI, we recently developed a high-throughput enzyme-linked immunosorbent assay (ELISA)-based assay to measure the ability of influenza virus-specific ADCC antibodies to bind dimeric recombinant soluble FcγRIIIa (rsFcγRIIIa) ectodomains (22–24). We adapted this assay to study the ability of HIV-specific antibodies to bind higher-affinity dimeric rsFcγRIIIa (Fig. 2A) and found that anti-HIV Env antibodies in the sera of ECs bound the dimeric rsFcγRIIIa more potently than antibodies in the sera of viremic subjects ($P < 0.0001$ and $P' = 0.002$) (Fig. 2B, left panel).

Furthermore, there was a trend toward higher dimeric rsFcγRIIIa binding for Vpu protein ($P = 0.073$ and $P' = 0.010$) (Fig. 2B, middle panel) in EC sera than in sera from viremic subjects. We also found that antibodies to the Vpu19 peptide in ECs bound the
A. Coat plate with HIV antigen → Add sample → Add biotinylated dimeric rsFcyRIIia → Add streptavidin-HRP/TMB → Read A450nm

B. 

HIV Env

Normalised OD@450nm

ECs | Viremic subjects

HIV Vpu protein

Normalised OD@450nm

ECs | Viremic subjects

HIV Vpu 19 peptide

Normalised OD@450nm

ECs | Viremic subjects

C. 

HIV Env

Normalised OD@450nm

ECs with protective HLA I | ECs without protective HLA I | Viremic subjects

HIV Vpu protein

Normalised OD@450nm

ECs with protective HLA I | ECs without protective HLA I | Viremic subjects

HIV Vpu 19 peptide

Normalised OD@450nm

ECs with protective HLA I | ECs without protective HLA I | Viremic subjects

D. 

ECs (n=10)

Normalised OD@450nm

Plasma dilution (log10)

Viremic subjects (n=20)

Normalised OD@450nm

Plasma dilution (log10)

E. 

HIV Env

Normalised OD@450nm

Plasma dilution (log10)

receptor endpoint titre (log10)

ECs | Viremic subjects
dimeric rsFcγRlla more potently than those in viremic subjects’ sera ($P = 0.036$ and $P' = 0.014$) (Fig. 2B, right panel). When comparing protective- versus nonprotective-HLA-carrying ECs, we did not see any differences in the dimeric rsFcγRlla-binding antibodies against HIV Env (Fig. 2C, left panel), Vpu protein (Fig. 2C, middle panel), or Vpu19 peptide (Fig. 2C, right panel) within the two EC subgroups.

FcγR binding is influenced by several factors other than the amount of antibody, including IgG-Fc proximity, glycosylation, and antibody subtype. We surmised that the potency of dimeric rsFcγRlla binding may be distinct from antibody concentration and therefore performed endpoint titrations using half-log dilutions of sera against the HIV Env in a subset of 10 ECs and their 20 matched viremic subjects. For the samples at a 1:10 dilution, a normalized optical density (OD) threshold of 0.455 for the dimeric rsFcγRlla-binding activity almost evenly divided the viremic subjects into high- and low-FcγR-activity groups (see the receiver operator curve (ROC) analysis in Materials and Methods). As previously (Fig. 2B), most of the ECs had high FcR activity above this threshold (80%) (Fig. 2D). Interestingly, however, endpoint titration showed similar total levels of Env-specific dimeric rsFcγRlla-binding antibodies in ECs and viremic subjects (Fig. 2E). The higher levels of Env-specific dimeric rsFcγRlla-binding antibodies at low serum dilutions (1:10) despite similar overall titers may suggest greater breadth of epitope coverage in the ECs, consistent with our previous ADCC studies in slow progressors (9, 10). Alternatively, this improved EC plasma binding to the rsFcγRlla could suggest that EC plasma either binds gp140 more effectively or binds the rsFcγRlla more effectively.

**EC antibodies dissociate from gp140 more slowly than antibodies from viremic subjects.** We postulated that the mechanism of improved gp140-specific binding to the rsFcγRlla observed in the EC cohort could be through improved plasma antibody binding to gp140 and/or more effective binding to rsFcγRlla. To evaluate these possibilities, we developed a novel surface plasmon resonance (SPR) assay using polyclonal IgG purified from plasma to study both interactions. Purified IgG from ECs or viremic subjects was first passed across and allowed to bind to AD8 Env-gp140 immobilized onto the SPR chip. Following partial dissociation of bound IgG from the antibody-Env-gp140 complex, dimeric rsFcγRlla was injected to probe the FcγRlla-binding activity of the Env-specific IgG. As it is not practical to isolate Env-specific IgG from the patient samples, we purified and quantitated the total IgG from the samples using “melon” affinity chromatography. Figure 3A shows an example of data form dilutions of EC and viremic subjects’ IgG samples.

The complex polyclonal nature of the samples and the multivalent interactions of Env trimer with bivalent antibodies result in kinetics that cannot be strictly interpreted with simple defined binding models. Nonetheless, SPR analysis is highly informative, in particular, the dissociation rate ($k_d$), which is a concentration-independent measurement that can be examined quantitatively in polyclonal IgG preparations (25). We observed that the rate of IgG binding to Env was slower for the ECs than for the viremic subjects (Fig. 3B). However, since binding rates are related to analyze concentration, this could occur with lower concentrations of Env-specific IgG in the EC samples than in viremic samples. Bars represent medians (P values from Mann-Whitney U test). (C) Comparison of dimeric rsFcγRlla V158-binding antibody responses in ECs carrying protective versus nonprotective HLA I to those in viremic subjects. Bars represent medians (P values, Kruskal-Wallis test). For both panels B and C, the dashed lines represent three times the mean OD obtained using five different HIV-negative serum samples against each antigen tested. (D) Endpoint titration curves of dimeric rsFcγRlla V158 binding to half-log dilutions of sera against HIV Env for 10 ECs (left panel) and case-matched 20 viremic subjects (right panel); the dotted lines at OD = 0.455 represent a cutoff for dimeric rsFcγRlla binding activity calculated using ROC analyses of the data shown in panel B. (E) Dimeric rsFcγRlla V158-binding antibody endpoint titer comparison of ECs with viremic subjects. Bars represent medians (P values, Mann-Whitney U test).
However, of interest, the dissociation of the EC Env-specific IgG was slower than that of Env-specific IgG from the viremic subjects (median $k_d [1/s] = 1.01 \times 10^{-5}$ for ECs and $0.00131$ for viremic subjects). This may have functional importance for the EC Env-specific IgG, possibly reflecting more stable prolonged opsonization of Env protein, thereby enhancing the opportunities for IgG to engage and activate Fc-$\gamma$R-bearing cells.

When $k_a/k_d$ values were plotted as a measure of the apparent binding activities, there was no apparent difference between the two cohorts. In addition, when binding of the dimeric rsFc-$\gamma$Rlla to Env-specific IgG complexes was examined, there was no apparent difference in the kinetics of binding to IgG from the ECs or the viremic subjects (Fig. 3C).

**Higher levels of antibody-mediated NK cell activation in ECs than in viremic subjects.** In order to corroborate our finding using a dimeric rsFc-$\gamma$Rlla binding assay with a functional ADCC assay, we first performed antibody-mediated NK cell activation assays. This assay studies the ability of Env- or Vpu-specific antibodies to induce cytokine (gamma interferon [IFN-$\gamma$]) expression and/or degranulation (CD107a expression) in gated NK cells (9, 26). We found that Env-specific antibodies in the sera of ECs induced a higher proportion of NK cells to express IFN-$\gamma$ and/or CD107a than those in viremic subjects.
the sera of viremic subjects ($P = 0.006$ and $P' = 0.009$) (Fig. 4A, left panel). We also studied the capacity of Vpu19 peptide-specific antibodies to induce NK cell activation in both groups and that found sera from the ECs had higher levels than those from viremic subjects ($P = 0.005$ and $P' = 0.014$) (Fig. 4C, left panel). When comparing the effect of protective- versus nonprotective-HLA-carrying ECs, there were no significant differences between these EC subgroups against either HIV Env (Fig. 4B) or the Vpu19 peptide (Fig. 4D).

To dissect the ability of the antibodies to differentially stimulate killing functions through degranulation as represented by CD107a expression and cytokine secretion via IFN-γ expression, we analyzed NK cell expression of IFN-γ and CD107a against Env and Vpu19 separately in ECs compared to viremic subjects. We found significantly high IFN-γ as well as CD107a expression by NK cells against Env stimulation in ECs compared to viremic subjects (Fig. 4A, middle and right panels). We also found higher CD107a expression by NK cells against Vpu19 stimulation in ECs compared to viremic subjects (Fig. 4C, right panel); however, the Vpu19 antibody-stimulated IFN-γ expression did not differ between the two groups (Fig. 4C, middle panel).

**In vitro ADCC-mediated killing is higher in ECs than in viremic subjects.** Antibody clustering of FcγRs and subsequent NK cell activation are necessary steps...
toward granzyme B delivery to HIV Env-expressing target cells. To more directly study antibody-mediated granzyme B delivery to target cells, we employed the ADCC-GranToxiLux (ADCC-GTL) assay, which measures the entry of granzyme B into target cells as a surrogate of ADCC-mediated killing (27). In the ADCC-GTL assay, we found that Env-specific antibodies in the sera of ECs induced a higher granzyme B activity than those in sera of viremic subjects ($P_{0.0074}$ and $P_{0.014}$) (Fig. 5A). When comparing the effect of protective- versus nonprotective-HLA-carrying ECs, there were no significant differences between these EC subgroups (Fig. 5B).

The rapid fluorometric ADCC (RFADCC) assay is another antibody-mediated functional assay that measures the loss of integrity of Env-coated target cells by measuring the uptake of target cell membrane by monocytes (28). Using the RFADCC assay, we observed only a weak trend toward increased uptake of Env-coated target cell membrane by gated monocytes (PKH26+ CD3− CD14+) in the sera of ECs compared to those of viremic subjects ($P = 0.139$ and $P_{0.296}$) (Fig. 6A). When comparing protective- versus nonprotective-HLA-carrying ECs, we did not see any difference in the killing within the two EC subgroups (Fig. 6B).

**DISCUSSION**

The investigation of ECs, who naturally control HIV infection, may provide valuable insights into immune correlates of protection from disease progression. Several viral,
genetic, and immunological factors are involved in HIV control (3, 17, 29–32). The role of HIV-specific ADCC is gaining prominence following post hoc analyses of the partially successful RV144 trial (33–35). There is increasing evidence from studies in both humans and macaques that correlate robust ADCC responses with slow disease progression (9, 10, 36, 37). ADCC responses have also been shown to develop rapidly following infection with HIV-1, which suggests that these responses could contribute to HIV control at the early stages of infection (38, 39). We previously showed a role for

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**FIG 5** ADCC-mediated killing is higher in ECs than in viremic subjects in the ADCC-GTL assay. (A) ADCC-mediated killing (percent granzyme B activity) of HIV Env-coated targets in the presence of 1:1,000 diluted sera from ECs (n = 22) compared to that in viremic subjects (n = 44). Bars represent medians (P values, Mann-Whitney U test). (B) Comparison of percent granzyme B activity in ECs carrying protective versus nonprotective HLA I to that in viremic subjects. Bars represent medians (P values, Kruskal-Wallis test). For both panels, the dotted lines represent three times the mean of background responses given by five HIV-negative serum samples against HIV Env.

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**FIG 6** ADCC-mediated killing in ECs compared to that in viremic subjects in the RFADCC assay. (A) ADCC-mediated killing (percent PKH26⁺ CD3⁰ CD14⁺ monocytes) of HIV Env-coated targets in the presence of 1:100 diluted sera from ECs (n = 22) compared to those from viremic subjects (n = 44). Bars represent medians (P values, Mann-Whitney U test). (B) Comparison of the percent PKH26⁺ CD3⁰ CD14⁺ monocyte responses in ECs carrying protective versus nonprotective HLA I to those in viremic subjects. Bars represent medians (P values, Kruskal-Wallis test). For both panels, the dashed lines represent three times the mean of background responses given by five HIV-negative serum samples against HIV Env.
ADCC in HIV control in viremic controllers (9, 10, 40). However, data on the role for ADCC in ECs is limited. Lambotte et al. found that ECs had high levels of HIV Env-specific ADCC, whereas levels of NAb were lower than those in viremic subjects (7). More recently, Ackerman et al. found polyfunctional antibody effector activity in ECs but not enhanced antibody responses compared to those in viremic subjects (41). Our data add weight to the concept that ADCC antibodies can assist durable suppression of HIV replication.

HIV Env is a key target for an effective antibody-based HIV vaccine, but its high genetic variability poses a large hurdle to vaccine design. Immune escape from broadly neutralizing antibodies that are directed against the surface-exposed parts of free virions is the norm (42). We previously showed Env escape from ADCC, and it is possible that immune escape reduced our detection of Env ADCC in viremic subjects (16). ECs may have less immune escape from ADCC responses, since there is so little virus replication and likely minimal evolution. Env-specific cytotoxic T lymphocytes (CTLs) are relatively less effective than Gag-specific CTL responses in controlling HIV (43, 44). We studied HIV Env-specific ADCC antibody responses in ECs compared to viremic subjects and found that ECs have more potent Env-specific ADCC responses than viremic subjects. This is despite the fact that ECs have less antigenic stimulation through undetectable plasma viral loads (VLs). A potential limitation of our functional assays is that we studied Env protein-pulsed target cells, and further work studying ADCC on HIV-infected cells, as a more relevant target, is warranted.

The dimeric rsFcγRIIIa ELISA-based assay to measure the binding of HIV-specific antibodies to FcγRIIIa is a relatively novel approach to studying functional antibodies for HIV, having previously been used to study influenza virus-specific antibodies (22–24). Commonly used ADCC assays can be difficult to reproduce across laboratories due to variability of effector and target cells, and they are difficult to perform on a large scale. The ELISA-based assay using soluble dimeric rsFcγRIIIa to detect the presence of HIV-specific ADCC antibodies in patient sera is robust and high throughput, and broadly consistent differences were observed between ECs and viremic subjects with the NK cell activation and ADCC killing assays. The dimeric rsFcγRIIIa detects pairs of closely spaced IgGs, and such antibodies may comprise some of the opsonizing antibodies that effectively cross-link FcγRIIIa and induce NK cell activation. The assay is rapid and easy to perform and does not require use of effector cells. This assay may prove useful for high-throughput analyses of future vaccine trials.

Using the dimeric rsFcγRIIIa ELISA-based assay, we found that EC sera had greater capacity for both IgG binding to Env protein and binding of the IgG-opsonized Env to the dimeric rsFcγRIIIa, together suggesting a broader coverage of Env-specific epitopes in the B-cell response of the ECs. The lack of differences between endpoint titers of these antibodies in ECs compared to viremic subjects likely indicates that, although broader in epitope coverage, the EC Env-specific antibodies are not necessarily characterized by superior titer. This novel assay highlights that the quality of the antibody response with Fc-mediated function may be as important as or more important than the titer of the response. Future studies identifying conserved Env-specific ADCC epitopes commonly targeted by ECs could assist in designing improved HIV vaccines.

In addition, our SPR studies allowed us to examine the binding activity of Env-specific IgG from ECs and viremic subjects. The SPR data were fitted to the simplest 1:1 Langmuir binding model, but due to the polyclonal nature of patient IgG and the complexities of bivalent antibody binding to trimeric gp140 Env, this approach was only descriptive and not intended to be interpreted mechanistically. Nonetheless, the apparent dissociation rate, which is concentration independent, can be compared between samples. The significantly lower dissociation rates of Env-specific IgG of ECs suggest that these EC IgGs may have a prolonged ability to opsonize Env antigens, allowing for longer formation of immune complexes and increased opportunities for FcγR engagement activation of FcγR-bearing innate immune cells. Interestingly, we did not observe any intrinsic differences in the FcγR-binding activity of Env-specific IgG immune complexes by this SPR assay in the cohorts studied. However, highly functional
forms of IgG have been identified in spontaneous HIV controllers, for example, enhanced levels of afucosylated IgG (45) and isotypes such as IgG3, which have a more flexible hinge region (41), that can strongly enhance binding to FcγRIIIa and improve ADCC, which may not be apparent in these small quantities of bulk IgG.

We found Vpu-specific ADCC responses in ECs, consistent with our previous work on slow progressors (10). HIV Vpu is a crucial viral transmembrane protein that serves two important functions to promote HIV replication inside the host: (i) CD4 degradation to evade immune recognition and (ii) virus release by antagonizing the host restriction factor tetherin (46, 47). Tetherin is important for preventing HIV release and has more recently been shown to restrict cell-to-cell transmission of HIV (48, 49). Recent studies have identified a major role for Vpu in limiting Env expression on the surface of HIV-infected cells. The ability of Vpu to inhibit tetherin expression on the surface of infected cells reduces the binding and recognition of ADCC antibodies and thereby may protect infected cells from ADCC (50–52, 62). If ADCC antibody recognition of Vpu can disable Vpu-mediated tetherin inhibition, it would enhance the functionality of Env-specific ADCC antibodies and could significantly contribute to HIV control. In our IgG-ELISA and dimeric rsFcγRIIIa ELISA-based assay, while screening the cohort using the whole purified Vpu protein we detected some ECs with ADCC antibody responses to whole Vpu protein but not to Vpu19 peptide. This suggests that some antibodies might be targeting an extracellular domain of Vpu that could interfere with interactions with tetherin, thereby counteracting the antagonistic effect of Vpu. The importance of immune targeting of Vpu has further been noted by Alter et al., who described mutations within Vpu that are linked to recognition of Vpu-expressing cells for direct killing by specific NK cell subsets (53). The role of CTLs targeting Vpu has also been described previously (54). Taken together, the data suggest that Vpu is a potentially useful target for anti-HIV immune responses. Ongoing work to generate and test the ADCC function of Vpu-specific monoclonal antibodies is under way. We acknowledge that our work on both Env- and Vpu-specific ADCC does not prove a causal link between ADCC and EC status. Interventional passive-transfer studies in macaques are suggestive of a role for ADCC function in addition to neutralization capacity in prevention and control of simian-human immunodeficiency virus (SHIV) infection (55, 56), and further passive-transfer studies in macaques and humans should provide more definitive evidence.

In summary, elite control of HIV is a multifactorial phenomenon, and our findings suggest that ECs have strong ADCC responses to HIV Env and Vpu compared to those of viremic subjects. Prolonged binding of EC antibodies to Env may allow for the more effective engagement of FcγRs. Novel targets for ADCC antibodies to conserved HIV antigens such as Vpu may be relevant to target HIV with preventative and therapeutic vaccines.

MATERIALS AND METHODS

Study cohort. The clinical characteristics of the 22 elite controllers (ECs) and 44 viremic subjects are shown in Table 1. ECs were defined as having over 65% of the plasma HIV RNA measurements taken as below the detection limit in the absence of ART. An average of 88.6% of all viral loads (VLs) across ECs were below the limit of detection as determined by commercial HIV-1 RNA assays used in practice at the time (VLs ranged from <400 copies/ml in past years to <20 copies/ml more recently). All subjects were followed for at least 3 months. Longitudinal data for all the ECs are collated in Table 2. ECs (predominantly having HIV-1 subtype B) were recruited in Australia, both in Melbourne through the Melbourne Sexual Health Centre and Alfred Health (10 subjects) and in Sydney through Sydney LTNP cohort (12 subjects) (10). ECs were 1:2 case matched to the viremic subjects based on age (window of ±5 years), gender, subtype of HIV, and duration of infection. This case-control matching design enables comparison of outcomes among ECs and viremic subjects while reducing bias due to confounding variables. The viremic subjects with VLs of >10,000 copies/ml were recruited from subjects enrolled in the ENCORE1 clinical trial of reduced-dose efavirenz (57, 58). We studied 44 viremic subjects from baseline visit prior to initiation of ART (mean plasma HIV RNA of 5.1 log10 copies/ml). Healthy HIV-negative volunteers provided the donor whole blood and peripheral blood mononuclear cells (PBMCs). Research was conducted under the auspices of the Alfred Human Health Research Ethics Committee for the Melbourne EC cohort, the St Vincent’s Sydney Human Research Ethics Committee for the Sydney EC cohort, and participating institutional review boards at all recruiting sites for the ENCORE1 trial.
HIV antigens. HIV Env (subtype B, strain AD8 gp140) protein and a control simian immunodeficiency virus (SIVmac239, gp90) Env protein were produced from stable Env-expressing HeLa cell lines, using previously published techniques (59). HIV Vpu protein was kindly provided by Stanley Opella, University of California, San Diego (60). HIV Vpu19 peptide was obtained from the NIH AIDS Reagent Program (catalog number 6444).

Anti-HIV IgG ELISA. ELISA was used to measure the HIV Env- and Vpu-specific serum IgG titers. Briefly, HIV antigen in coating buffer (20 mM Tris [pH 8.8], 100 mM NaCl) was applied to 96-well flat bottom plates at 100 ng/well and left overnight. After blocking with 5% bovine serum albumin (BSA) (Sigma-Aldrich) containing 0.1% Tween for 2 h, sera were added at a 1:10 dilution. After 1 h of incubation, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody (Sigma) was added and incubated for 1 h. Color reactions were developed using 3,3',5,5'-tetramethylbenzidine (TMB) and absorbance measured at 450 nm. Data were normalized to 5 μg/ml HIV IgG (HIVIG) (obtained from the NIH AIDS Reagent Program) diluted in phosphate-buffered saline (PBS). A positive signal was defined as one giving an OD higher than three times the mean OD obtained using sera from five HIV-negative donors against each antigen tested.

Dimeric rsFcγRIIa-binding assay. HIV antibody binding to Fcγ receptors was measured in vitro using a dimeric recombinant soluble FcγRIIa (rsFcγRIIa)-binding assay recently described as a sensitive measure of influenza virus-specific ADCC antibodies (22–24). This novel assay uses dimeric rsFcγRIIa proteins to evaluate the cross-linking of pairs of antibody Fc by FcγRs, thereby modeling their potential to activate effector cells. Ninety-six-well MaxiSorp ELISA plates (Nunc, Rochester, NY) were coated with 50 ng/well of HIV antigens (NIH AIDS Reagent Repository) in PBS overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 (U-CyTech) and blocked with PBS containing 1 mM EDTA and 1% human serum albumin for 1 h at 37°C. Plates were washed and incubated with 1:10 diluted sera for 1 h at 37°C and then with 0.1 μg/ml of biotinylated dimeric rsFcγRIIa diluted in PBS containing 1 mM EDTA and 1% BSA (Sigma-Aldrich) for 1 h 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% BSA was added and left for 1 h at 37°C. Lastly, TMB substrate (Sigma-Aldrich) was added and color development stopped with 1 M hydrochloric acid. Absorbance was read at 450 nm. Data were normalized to 5 μg/ml HIVIG diluted in PBS. A positive signal was defined as one giving an OD higher than three times the mean OD obtained using sera from five HIV-negative donors against each antigen tested.

ADCC-GTL assay. Antibody-dependent cellular cytotoxicity (ADCC) activity mediated by the patient sera was detected using the previously described GranToxiLux (GTL) cell-mediated cytotoxicity procedure (27). Briefly, 10⁶ CEM.NK-CCR5 cells were coated with 3 μg HIV Env and left for 1 h at room temperature. Coated CEM.NK-CCR5 target cells were then labeled with a fluorescent target-cell marker (TFL4; OncoImmunity, Inc., Gaithersburg, MD) and a viability marker (NFL1; OncoImmunity, Inc.) for 15 min at 37°C as specified by the manufacturer. The NK cell line GFP-CD16 (176V) NK-92 (61) was used as an effector cell line. CEM.NK cells were incubated with HIV Env and left for 1 h at room temperature. The mixture was analyzed for ADCC activity using a dimeric recombinant soluble FcγRIIa (rsFcγRIIa) (Thermo Scientific) binding to FcγRs (clone SK7; BioLegend) and phycoerythrin (PE)-Cy7-conjugated anti-CD56 (clone HCD56; BioLegend). Following incubation, cells were stained with peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 (clone SK7; BioLegend) and phycoerythrin (PE)-Cy7-conjugated anti-CD56 (clone HCD56; BioLegend) antibodies. Next, whole blood was treated with lysing solution (BD Biosciences) and stained with allophycocyanin (APC)-conjugated anti-IFN-γ antibody (clone B27; BioLegend). Flow cytometry data were collected using a fluorescence-activated cell sorter (FACS) LSR II flow cytometer (BD Biosciences) and were analyzed using FlowJo version 10.0.6 software. NK cells were identified as CD3⁺ CD56⁺, and responses were considered positive if the percentage (of NK cells expressing IFN-γ or CD107a) was more than three times the mean of the response to HIV Env and Vpu19 against five HIV-negative sera.
and effecter cells at a 5:1 effector-to-target (E:T) cell ratio. The GFP-CD16 (176V) NK-92 cell line was kindly provided by Kerry Campbell from the Institute for Cancer Research in Philadelphia, PA. Twenty-five microliters of each effector and target cell suspension and 75 μl of granzyme B substrate (OncoImmunin, Inc.) were dispensed into each well of a 96-well V-bottom plate. After incubation for 5 min at room temperature, a 1:1,000 dilution of sera was added and incubated for 15 min at room temperature. The plates were subsequently centrifuged for 1 min at 300 × g and incubated for 1 h at 37°C and 5% CO₂. After two washes with wash buffer, cells were resuspended in 50 μl of wash buffer and placed at 4°C, and data were acquired with the LSRII flow cytometer (BD Bioscience, San Jose, CA) within 2 h. Data analysis was performed using FlowJo version 10.0.6 software. For all HIV Env responses, the SIV Env control responses (average of 4.8%) were subtracted. A positive response was defined as a percent granzyme B activity higher than three times the mean of the response to HIV Env using sera from five HIV-negative donors.

**RFADCC assay.** The rapid fluorescent ADCC (RFADCC) assay was used as previously described (28). Briefly, 10⁵ CEM.Nkr-CRRS cells were coated with 3 μg of HIV Env and left for 1 h at room temperature. SIV Env-coated CEM.Nkr-CRRS cells were used as a control and were treated identically. Coated CEM.Nkr-CRRS cells were initially labeled with PKH26 (Sigma) and carboxyfluorescein succinimidyl ester (CFSE) (Sigma). PKH26+/CFSE⁻ labeled CEM.Nkr-CRRS target cells (2 × 10⁶) were incubated with human sera (1:100 final dilution) for 30 min at 37°C, followed by addition of 2 × 10⁵ PBMCs (total volume of 100 μl) to achieve an E:T ratio of 10:1. Cells were incubated for 4 h at 37°C, and then stained with PerCP-conjugated anti-CD3 (clone SK7; BioLegend) and APC-H7-conjugated anti-CD14 (clone M/H9262/4). Flow cytometry data were collected using a FACS LSR II flow cytometer (BD Biosciences). The data analysis was performed using FlowJo version 10.0.6 software and a gating strategy as previously described (28). The assay readout is percentage of PKH26⁺ CD3⁻CD14⁺ monocytes that acquire the target cell PKH26 dye. For all HIV Env-specific responses, the SIV Env control responses (average of 5.7%) were subtracted. A positive response was defined as higher than three times the mean of the response to HIV Env using sera from five HIV-negative donors.

**Statistical analysis.** Statistical analyses were performed with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) and SPSS version 18 software (IBM, Armonk, NY). For comparisons between ECs and viremic subjects, an unadjusted P value was derived using nonparametric Mann-Whitney U tests (referred to here as the P value). As a sensitivity analysis, adjusted P values (P') were derived using conditional logistic regression tests accounting for matching between cases and controls. The median was used to describe the data for Mann-Whitney U tests. For multiple comparisons in Fig. 1B, 2C, 3B, 4B, and 5B, Kruskal-Wallis tests were performed. A P value of <0.05 was considered to indicate a significant difference. Receiver operator curve (ROC) analysis was performed to establish the dimeric rsFcRfllα-ligation activity threshold (normalized OD = 0.455) that best distinguished between the ECs and viremic subjects (data analyzed from Fig. 2B, left panel, area under the curve = 81.9% and P < 0.0001).

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