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PATHOGENESIS AND IMMUNITY

Influence of the Envelope gp120 Phe 43 Cavity on HIV-1 Sensitivity to Antibody-Dependent Cell-Mediated Cytotoxicity Responses

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ABSTRACT HIV-1-infected cells presenting envelope glycoproteins (Env) in the CD4-bound conformation on their surface are preferentially targeted by antibody-dependent cellular-mediated cytotoxicity (ADCC). HIV-1 has evolved sophisticated mechanisms to avoid the exposure of Env ADCC epitopes by downregulating CD4 and by limiting the overall amount of Env on the cell surface. In HIV-1, substitution of large residues such as histidine or tryptophan for serine 375 (S375H/W) in the gp120 Phe 43 cavity, where Phe 43 of CD4 contacts gp120, results in the spontaneous sampling of an Env conformation closer to the CD4-bound state. While residue S375 is well conserved in the majority of group M HIV-1 isolates, CRF01_AE strains have a naturally occurring histidine at this position (H375). Interestingly, CRF01_AE is the predominant circulating strain in Thailand, where the RV144 trial took place. In this trial, which resulted in a modest degree of protection, ADCC responses were identified as being part of the correlate of protection. Here we investigate the influence of the Phe 43 cavity on ADCC responses. Filling this cavity with a histidine or tryptophan residue in Env with a natural serine residue at this position (S375H/W) increased the susceptibility of HIV-1-infected cells to ADCC. Conversely, the replacement of His 375 by a serine residue (H375S) within HIV-1 CRF01_AE decreased the efficiency of the ADCC response. Our results raise the intriguing possibility that the presence of His 375 in the circulating strain where the RV144 trial was held contributed to the observed vaccine efficacy.

Received 19 December 2016 **Accepted** 12 January 2017

Accepted manuscript posted online 18 January 2017

Citation Prévost J, Zoubchenok D, Richard J, Veillette M, Pacheco B, Coutu M, Brassard N, Parsons MS, Ruxrungtham K, Bunupuradah T, Tovanabutra S, Hwang K-K, Moody MA, Haynes BF, Bonsignori M, Sodroski J, Kaufmann DE, Shaw GM, Chenine AL, Finzi A. 2017. Influence of the envelope gp120 Phe 43 cavity on HIV-1 sensitivity to antibody-dependent cell-mediated cytotoxicity responses. J Virol 91:e02452-16. https://doi.org/10.1128/JVI.02452-16.

Editor Guido Silvestri, Emory University

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IMPORTANCE HIV-1-infected cells presenting Env in the CD4-bound conformation on their surface are preferentially targeted by ADCC mediated by HIV-positive (HIV^+) sera. Here we show that the gp120 Phe 43 cavity modulates the propensity of Env to sample this conformation and therefore affects the susceptibility of infected cells to ADCC. CRF01_AE HIV-1 strains have an unusual Phe 43 cavity-filling His 375 residue, which increases the propensity of Env to sample the CD4-bound conformation, thereby increasing susceptibility to ADCC.

KEYWORDS HIV-1, Env, gp120, Phe 43 cavity, ADCC, RV144

Entry is the first step of the human immunodeficiency virus type 1 (HIV-1) replication cycle and requires the mature viral envelope glycoproteins (Env), which results from the proteolytic cleavage of the gp160 precursor Env into the exterior gp120 and transmembrane gp41 subunits. These subunits are linked by noncovalent bonds, allowing conformational changes of the Env trimer during the entry process (1–3). The gp120 exterior subunit mediates the initial interaction with the CD4 receptor. gp120 residue 375 is located in what is known as the Phe 43 cavity, where Phe 43 of CD4 makes numerous contacts with conserved gp120 residues critical for CD4 binding (4). Some gp120 residues that line this cavity contribute to an aromatic array that helps stabilize the CD4-bound conformation (1, 4, 5). Upon CD4 binding, major conformational changes expose the binding site for coreceptor (i.e., CCR5 and CXCR4) interaction (6). Upon coreceptor engagement, additional conformational changes in gp41 bring together the viral envelope and the target cell membranes (7, 8). It has been shown that some of these conformational rearrangements could be impacted by large alterations in the Phe 43 cavity. The replacement of the well-conserved group M serine at position 375 by a large hydrophobic residue such as tryptophan fills the Phe 43 cavity; this substitution alters the Env conformation by predisposing gp120 to spontaneously assume a state closer to the CD4-bound conformation (9). In agreement with the important role played by Env conformation in the CD4 interaction (1, 9–11), a recent study using the simian-human immunodeficiency virus (SHIV) model in rhesus macaques showed that the replacement of residue 375 by larger hydrophobic or basic amino acids enhanced the affinity of Env for macaque CD4 and allowed better infection of macaque T lymphocytes in culture and *in vivo* (12), highlighting the importance of this residue for viral replication.

Besides its potential involvement in viral replication, the CD4-bound conformation of HIV-1 Env represents a major target of antibodies (Abs) present in sera of HIV-1-infected individuals that mediate antibody-dependent cellular cytotoxicity (ADCC) (13). It has been shown that HIV-1 minimizes the exposure of this ADCC-susceptible Env conformation using a highly sophisticated strategy to keep Env on the surface of infected cells in the unbound "closed" conformation. HIV-1 accomplishes this through its accessory proteins Nef and Vpu, which decrease the overall amounts of Env (via Vpu-mediated BST-2 downregulation) and CD4 at the cell surface (13–16). In addition, decreased amounts of Env at the cell surface due to efficient internalization also help the virus to avoid ADCC responses (17). In agreement with the need for HIV-1 to avoid exposing Env in the CD4-bound conformation, it was recently shown that forcing Env to adopt this conformation with small CD4-mimetic compounds (CD4mcs) sensitizes HIV-1-infected cells to ADCC responses (18–20). Accordingly, we recently reported that the transition of Env to the CD4-bound conformation is required for efficient interactions with ADCC-mediating antibodies (14). We reported that the replacement of serine 375 by a tryptophan residue (S375W) enhanced the exposure of epitopes recognized by anti-cluster A antibodies, known to mediate potent ADCC responses (13, 14, 18, 20–26). Thus, the transition of Env from the unbound to the CD4-bound conformation appears to be a prerequisite for the exposure of inner-domain ADCC epitopes. However, how the presence of naturally occurring Phe 43 cavity-filling residues affects ADCC responses remains unknown.

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While Ser 375 is well conserved in the majority of group M HIV-1 isolates, we noticed that CRF01_AE Env possess a Phe 43 cavity-filling residue at position 375 (His 375) (27). CRF01_AE is the predominant circulating strain in Thailand, where the RV144 clinical vaccine trial took place. In this trial, which resulted in a modest degree of clinical protection, ADCC responses were identified as a correlate of protection (28), raising the intriguing possibility that naturally occurring His 375 in CRF01_AE Env might have contributed to this outcome. Here we explored the influence of the integrity of the Phe 43 cavity on ADCC responses.

RESULTS

Comparison of residue 375 flanking the Phe 43 cavity among HIV-1 strains. The vast genetic variability of HIV-1 resulted in its classification into 4 major groups, groups M, N, O, and P. Group M, or "main" group, viruses are responsible for the majority of the global HIV-1 pandemic. Group M is comprised of nine major subtypes and circulating recombinant forms (CRFs) (29–33). To determine the variance in residue 375 among HIV-1 Env, we analyzed all available group M sequences in the NIH Los Alamos HIV database. Interestingly, while the vast majority (>84%) of group M strains have a serine at position 375 (S375) and another 9% have a closely related T375 substitution (Fig. 1A to D and F to H), nearly all CRF01_AE Env have a large basic aromatic histidine residue at this position. Indeed, alignments revealed >99% conservation for H375 in CRF01_AE strains (Fig. 1E), clearly differentiating them from the other circulating strains of group M.

Effect of Phe 43 cavity-filling changes on recognition and ADCC-mediated killing of HIV-1-infected cells. It has been shown that the replacement of serine 375 by a histidine or tryptophan residue fills the Phe 43 cavity and predisposes Env to assuming a slightly more CD4-bound-like conformation (9). Moreover, it has been shown that sampling of the CD4-bound conformation results in an enhanced exposure of epitopes recognized by anti-cluster A antibodies (14, 34). To evaluate whether this enhanced recognition translates into enhanced susceptibility to ADCC, primary $CD4^+$ T cells from healthy HIV-1-negative donors were infected with HIV-1 NL4-3-GFP infectious molecular clones (IMCs) expressing wild-type (WT) strain ADA Env or mutant Env with a histidine (S375H) or a tryptophan (S375W) substitution at position 375. In parallel, primary $CD4^+$ T cells were infected with SHIVs expressing clade C and D HIV-1 Env with the same substitutions in residue 375 (WT and S375H and S375W mutants). Since it is now well established that CD4 present on the cell surface affects Env conformation by forcing it to assume the CD4-bound conformation (13, 14, 21, 22, 35), we first evaluated the ability of all the IMCs to downregulate CD4. Figure 2 shows that all IMCs tested, independently of the residue present at position 375, efficiently downregulated CD4 from the cell surface (Fig. 2A to C). Therefore, any detected effects on Env conformation cannot be attributed to differential CD4 downregulation but rather to the nature of the residue present within the Phe 43 cavity. Since Env levels present on the cell surface have been shown to affect ADCC responses (17), we verified the overall amount of Env present on the surface of infected cells. Env levels present on the cell surface were unaffected by the nature of the residue present at position 375. Indeed, we detected similar levels of Env expression using the gp120 outer-domain-specific 2G12 antibody for clade B and D Env (Fig. 2D and F). Due to the poor recognition of clade C Env by the 2G12 antibody, we utilized the broadly neutralizing anti-gp41 10e8 antibody to measure clade C Env expression (Fig. 2E). No effect of the changes at residue 375 on Env expression on the surface of cells infected with a SHIV expressing a clade C Env was observed (Fig. 2E). In agreement with an effect on Env conformation, we observed that in all the cases, filling of the Phe 43 cavity by the replacement of residue 375 with a histidine or a tryptophan residue significantly increased the binding of Env by CD4-induced (CD4i) antibodies that recognize the coreceptor binding site (CoRBS) (17b) or the cluster A epitope (A32) (Fig. 2G to L). Interestingly, we observed that Env with a "filled" Phe 43 cavity (S375H and S375W mutants) were also recognized significantly better by HIV-positive (HIV^+) sera than those with an "empty" Phe 43 cavity (S375)

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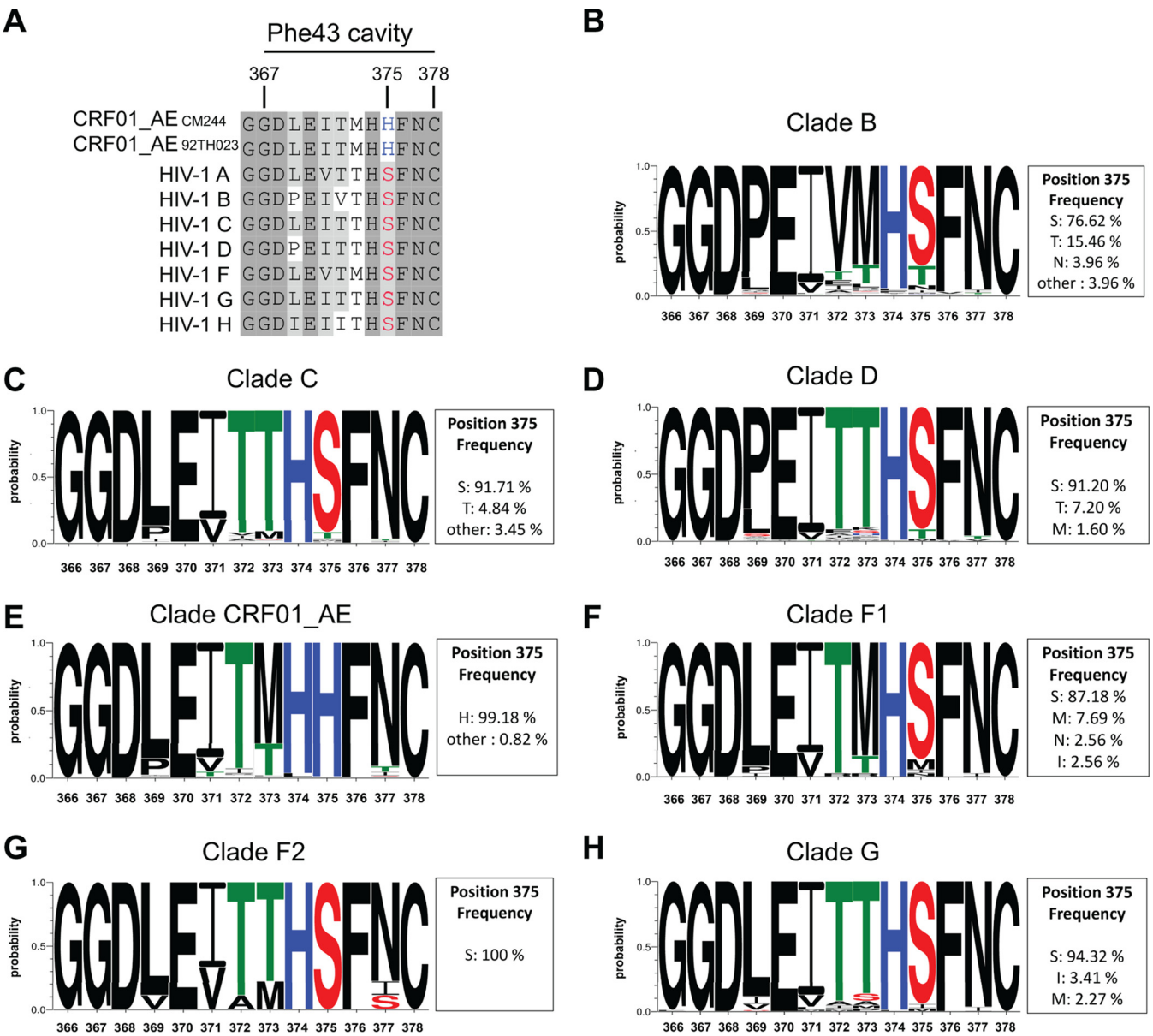

FIG 1 Sequence alignment of gp120 residues flanking the Phe 43 cavity of different HIV-1 isolates. (A) Primary sequence alignment of gp120 residues flanking the Phe 43 cavity based on a single representative sequence from each clade, including HIV-1 clade A (GenBank accession number ABB29387.1), HIV-1 clade B (accession number K03455), HIV-1 clade C (accession number AAB36507.1), HIV-1 clade D (accession number P04581.1), HIV-1 clade F (accession number ACR27173.1), HIV-1 clade G (accession number ACO91925.1), and HIV-1 clade H (accession number AAF18394.1), and two CRF01_AE strains, CM244 (accession number AY713425) and 92TH023. Residue numbering is based on that of the HXBc2 strain of HIV-1. Identical residues are shaded in dark gray, and conserved residues are shaded in light gray. S375 is shown in red, and H375 is shown in blue. (B to H) Logo depictions of the frequency of each amino acid from the Phe 43 cavity at positions 366 to 378 in isolates from clade B, clade C, clade D, CRF01_AE, clade F1, clade F2, and clade G. The height of the letter indicates its frequency within the clade. The box beside each logo indicates the frequency of all the amino acids at position 375. The 2016 Los Alamos database-curated Env alignment was used as the basis for this figure, which contains 4,397 amino acid HIV-1 group M sequences (1,897 of subtype B, 1,363 of subtype C, 125 of subtype D, 39 of subtype F1, 9 of subtype F2, 88 of subtype G, and 486 of CRF01_AE).

(Fig. 2M to O), thus supporting the notion that the CD4-bound conformation affects the recognition of Env by ADCC-mediating CD4i antibodies and antibodies within HIV$^+$ sera (13).

To evaluate whether the enhanced recognition of HIV-1-infected cells by A32 and antibodies within HIV$^+$ sera translated into increased susceptibility to ADCC responses, we used a previously described fluorescence-activated cell sorter (FACS)-based ADCC assay (18, 21). Briefly, primary CD4$^+$ T cells infected for 48 h were incubated with autologous peripheral blood mononuclear cells (PBMCs) (effector-to-target cell ratio of

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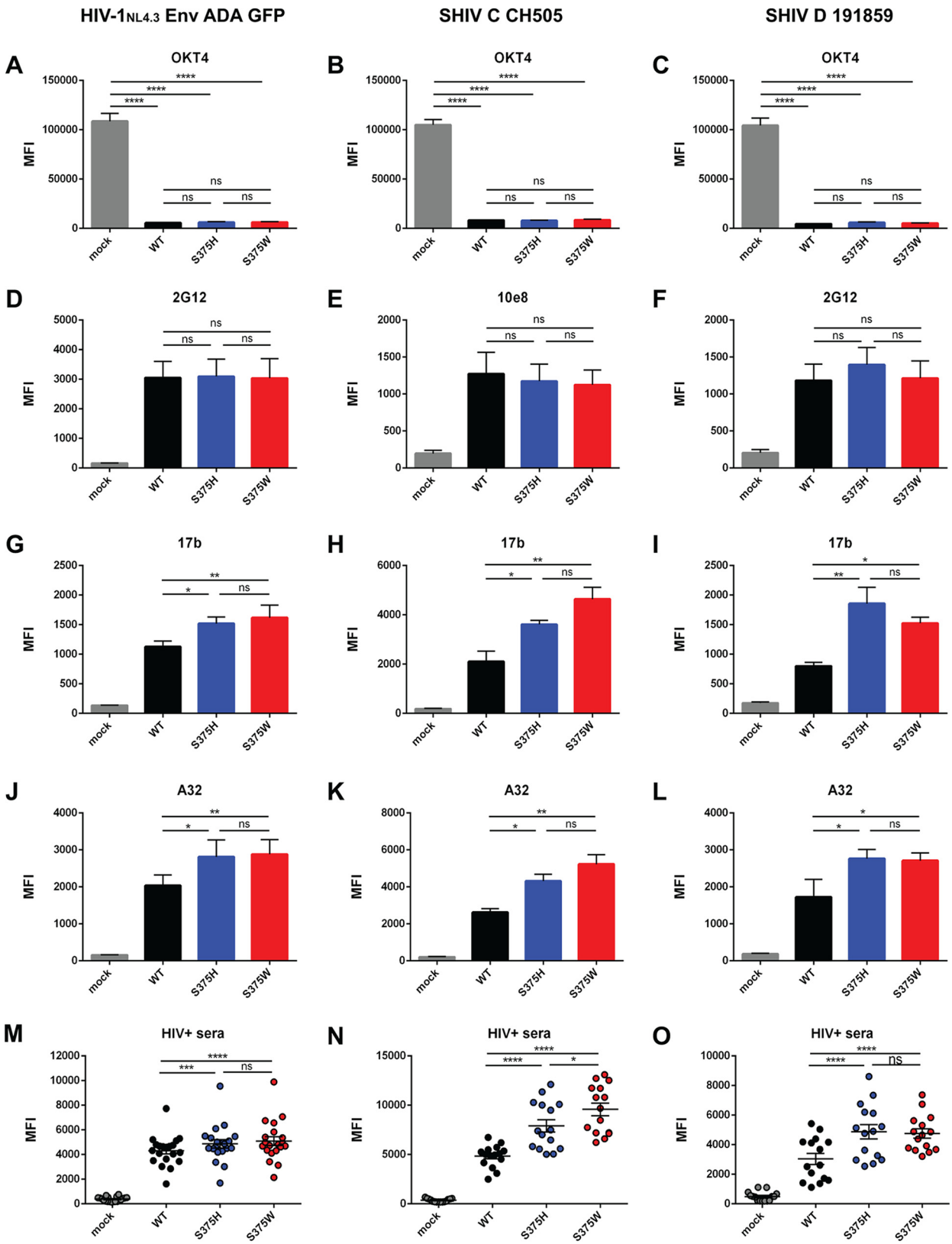

FIG 2 The Phe 43 cavity modulates recognition of clade B, C, and D HIV-1-infected cells by CD4i antibodies and antibodies within HIV$^+$ sera. Shown are data for cell surface staining of primary CD4$^+$ T cells that were either mock infected or infected with HIV-1 NL4.3-GFP expressing the primary R5 ADA Env (clade B) or SHIV expressing either CH505 (clade C) or 191859 (clade D) Env with anti-CD4 antibody OKT4 (A to C); the Env-specific antibody 2G12 (D and F) or 10e8 (E), which are not influenced by changes in residue 375; CD4i antibodies 17b (G to I) and A32 (J to L); and antibodies within HIV$^+$ sera (M to O). Shown are the MFIs obtained from at least 3 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using paired one-way analysis of variance with a Holm-Sidak posttest (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, nonsignificant).

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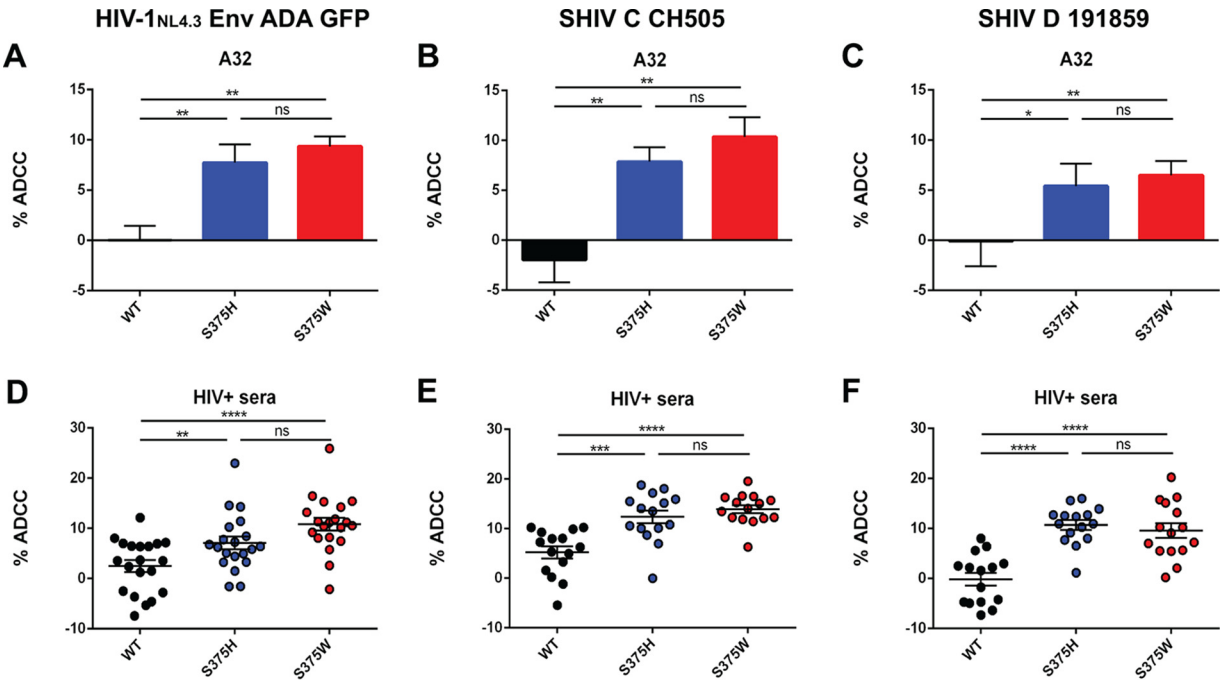

FIG 3 The Phe 43 cavity modulates ADCC responses mediated by the A32 antibody and antibodies within HIV$^+$ sera. Primary CD4$^+$ T cells infected with NL4.3-GFP expressing the primary R5 ADA Env (clade B) or SHIV expressing either CH505 (clade C) or 191859 (clade D) Env were used as target cells and autologous PBMCs were used as effector cells in our FACS-based ADCC assay, as described in Materials and Methods. Shown are the percentages of ADCC activity in the presence of anti-cluster A monoclonal antibody A32 (A to C) or in the presence of HIV$^+$ sera from HIV-1 clade B-infected individuals (D to F). These results are representative of those obtained in at least 3 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using paired one-way analysis of variance with a Holm-Sidak posttest (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, nonsignificant).

10:1) in the presence of A32 or HIV$^+$ sera (1:1,000). The percentage of cytotoxicity was calculated as described previously (18). In agreement with the poor recognition of infected cells expressing wild-type Env (S375), we observed low ADCC activity mediated by A32 (Fig. 3A to C) or sera from >15 HIV-1-infected individuals (Fig. 3D to F). However, replacing S375 with a histidine or tryptophan residue resulted in a significant increase in susceptibility to ADCC for the three IMCs tested. Of note, no staining of infected cells or ADCC responses were observed when sera from healthy HIV-1-negative donors were used (not shown).

The Phe 43 cavity modulates recognition of CRF01_AE HIV-1-infected cells by CD4i antibodies and HIV$^+$ sera. CRF01_AE HIV-1 isolates have a highly conserved histidine residue at Env position 375 (Fig. 1E), which was recently shown to be important for CD4 interaction (27) but also to confer strong resistance to the BMS-599793 entry inhibitor (36). To evaluate whether His 375 influences the recognition of infected cells by ADCC-mediating antibodies and HIV$^+$ sera, His 375 in the CRF01_AE$_{703357}$ strain was replaced by a serine (H375S). This strain was identified in the RV144 cohort and submitted to GenBank.

CRF01_AE IMCs were used to infect primary CD4$^+$ T cells from healthy uninfected donors. The impact of residue 375 on Env conformation was evaluated with CD4i 17b and A32 antibodies as well as sera from clade B- and CRF01_AE-infected individuals. The nature of the residue at position 375 did not affect CD4 downregulation (Fig. 4A) or Env expression, as evaluated by the binding of the 10e8 antibody (Fig. 4B). In contrast to the limited recognition of clade B, C, and D HIV-1-infected cells by A32, 17b, and antibodies within HIV$^+$ sera (Fig. 2) (13, 18, 20, 22, 37), cells infected with wild-type CRF01_AE Env (H375) were better recognized by both CD4i monoclonal antibodies (MAbs) as well as polyclonal sera (Fig. 4C to F). Strikingly, the replacement of His 375 by a serine residue (H375S) significantly decreased the recognition of infected cells by these ligands (Fig. 4). Overall, these results suggest that the recognition of wild-type

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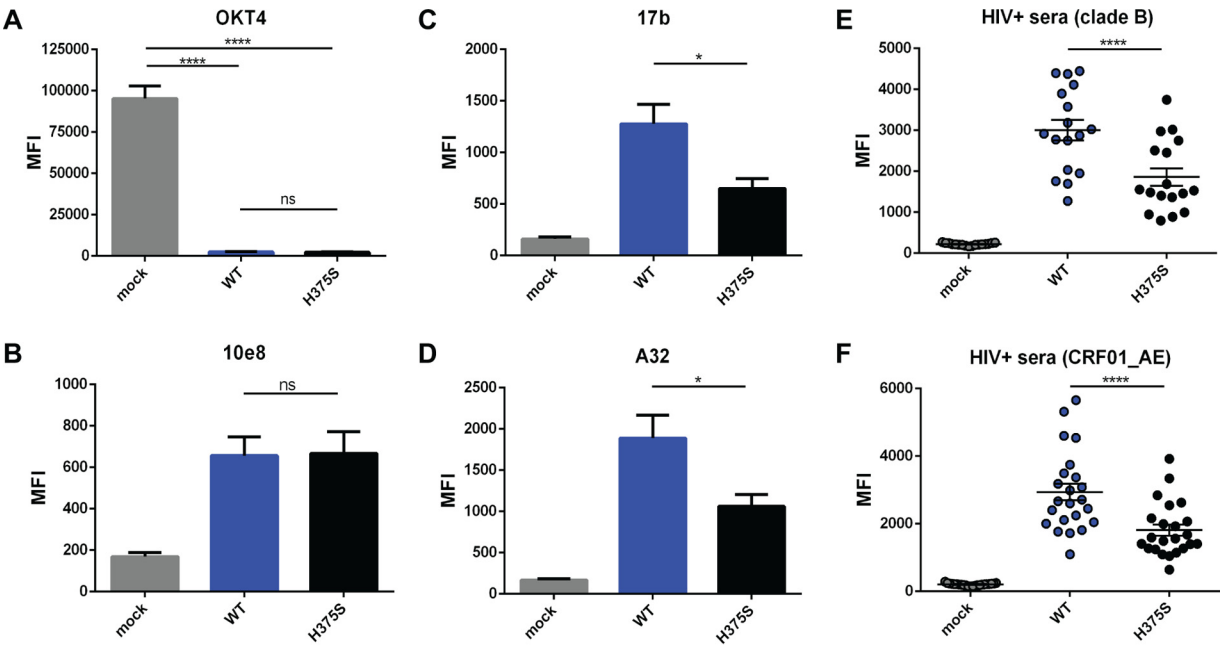

FIG 4 The Phe 43 cavity modulates the recognition of CRF01_AE HIV-1-infected primary CD4$^+$ T cells by CD4i antibodies and HIV$^+$ sera. Shown are data for cell surface staining of primary CD4$^+$ T cells that were either mock infected or infected with the WT or the H375S variant of CRF01_AE transmitted-founder HIV-1 (IMC 703357) with anti-CD4 antibody OKT4 (A), conformation-insensitive Env-specific antibody 10e8 (B), CD4i antibodies 17b (C) and A32 (D), and sera from individuals infected with HIV-1 clade B (E) or HIV-1 CRF01_AE (F). Shown are the MFIs obtained in at least 4 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using an unpaired two-tailed *t* test for MAbs or a paired two-tailed *t* test for HIV$^+$ sera (*, $P < 0.05$; ****, $P < 0.0001$; ns, nonsignificant).

CRF01_AE HIV-1 by CD4i antibodies and antibodies within HIV$^+$ sera is enhanced by the Phe 43 cavity-filling histidine residue at 375. H375 functions as a proxy of small CD4mcs, which were recently shown to push Env into the CD4-bound conformation and sensitize HIV-1-infected cells to ADCC (18).

The Phe 43 cavity modulates recognition of CRF01_AE HIV-1-infected cells by serum antibodies isolated from RV144 vaccinees. A correlation between high levels of ADCC-mediating antibodies and HIV-1 acquisition in the RV144 vaccine trial was suggested for a subset of individuals with low plasma IgA anti-Env antibody levels (26, 28). ADCC-mediating MAbs targeting multiple epitopes were isolated from memory B cells of RV144 vaccine recipients (26, 38). Importantly, we demonstrated that the vaccine-induced ADCC-mediating antibodies directed against A32-blockable conformational epitopes poorly recognize Env on the surface of HIV-1-infected cells (clade B) unless the CD4 receptor was present on the surface of infected cells. This was achieved by deleting the *nef* and *vpu* genes (14). Since the predominantly circulating strain in Thailand is a CRF01_AE HIV-1 strain, which has a histidine at residue 375, we asked whether these antibodies, thought to have protected against HIV-1 transmission in RV144 recipients, recognized CRF01_AE HIV-1-infected cells in a manner dependent on Env residue 375. Wild-type (H375) or H375S mutant CRF01_AE IMCs were used to infect primary CD4$^+$ T cells from healthy uninfected donors. Figure 5 shows that 11/12 (91.7%) A32-blockable antibodies induced by the RV144 vaccine recognized CRF01_AE HIV-1-infected cells expressing wild-type Env H375 significantly better than they recognized cells infected with the H375S counterpart.

Residue 375 modulates ADCC responses against CRF01_AE HIV-1-infected cells by CD4i antibodies, antibodies within HIV$^+$ sera, and antibodies from RV144 vaccinees. To assess whether the recognition of CRF01_AE HIV-1-infected cells by A32, antibodies within HIV$^+$ sera, and antibodies from RV144 vaccinees correlated with ADCC responses, primary CD4$^+$ T cells were infected as described above, and ADCC was measured as described in Materials and Methods. Figure 6 shows that wild-type (H375) CRF01_AE HIV-1-infected cells were readily susceptible to ADCC mediated by A32 and

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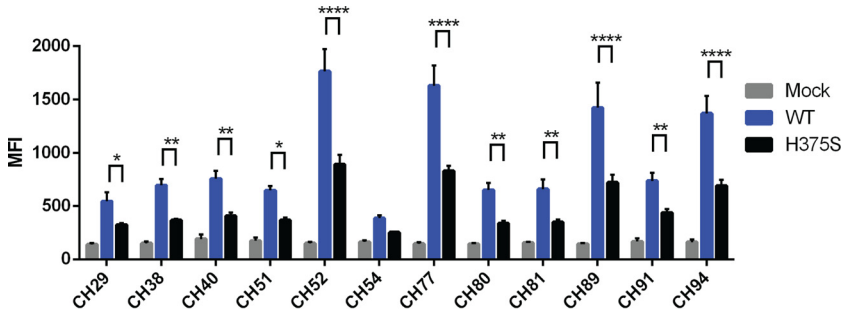

FIG 5 The Phe 43 cavity modulates the recognition of CRF01_AE HIV-1-infected primary CD4$^+$ T cells by antibodies isolated from RV144 vaccinees. Shown are data for cell surface staining of primary CD4$^+$ T cells that were either mock infected or infected with the WT or an H375S mutant of CRF01_AE transmitted-founder HIV-1 (IMC 703357) with A32-blockable MAbs isolated from RV144 vaccinees (CH29, CH38, CH40, CH51, CH52, CH54, CH77, CH80, CH81, CH89, CH91, and CH94). Shown are the MFIs obtained in at least 5 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using an unpaired two-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

antibodies within HIV$^+$ sera from individuals infected with clade B and CRF01_AE HIV-1 strains. This result contrasts with the relatively poor ADCC activity observed for these antibodies against primary CD4$^+$ T cells infected with wild-type (S375) HIV-1 strains from clades B, C, and D (Fig. 3). ADCC responses directed against CRF01_AE HIV-1-infected cells depended on the presence of His 375, as cells infected with the H375S virus variant were significantly less sensitive to ADCC. Similar results were observed when ADCC experiments were done using the majority of RV144 anti-cluster A antibodies (CH29, CH38, CH40, CH51, CH52, CH54, CH77, CH80, and CH94) (Fig. 7).

DISCUSSION

Renewed interest in the Fc-mediated functions of antibodies such as ADCC stems in part from observed correlations with the control or prevention of HIV-1 infection. Fc-mediated effector functions were found to inversely correlate with viral loads or disease progression in simian immunodeficiency virus (SIV)-infected macaques (39–41) as well as in HIV-1-infected individuals (42–46). Furthermore, analysis of correlates of immune protection in the recent RV144 vaccine trial suggested that high levels of ADCC-mediating Abs correlated with decreased HIV-1 acquisition when combined with low plasma IgA anti-Env antibody levels (28, 47). Moreover, the CD4-bound conformation of Env was recently shown to be a major target of ADCC-mediating antibodies present in the sera of HIV-1-infected individuals (13). Accordingly, here we report that the size of the residue located within the Phe 43 cavity of Env modulates the

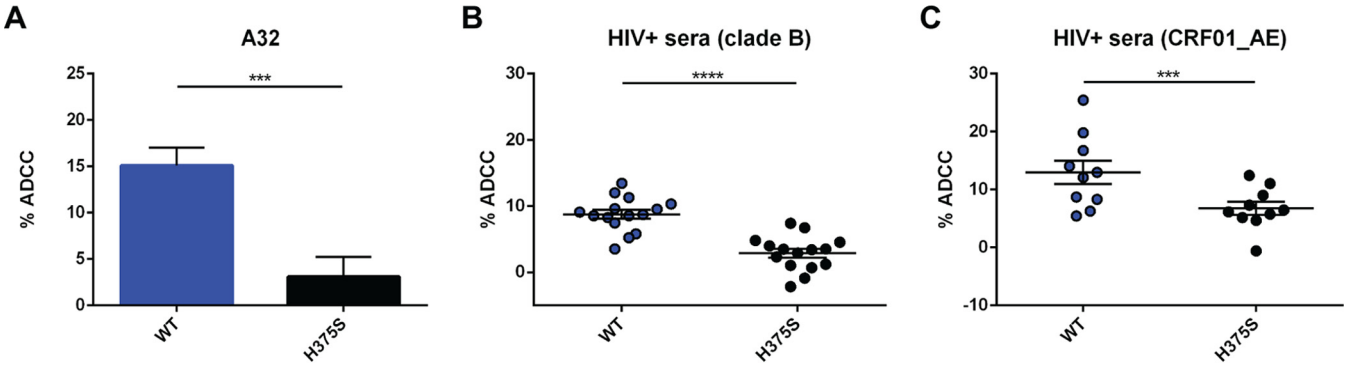

FIG 6 Histidine 375 modulates ADCC responses against CRF01_AE HIV-1-infected cells mediated by the A32 antibody and HIV$^+$ sera. Primary CD4$^+$ T cells infected with WT and H375S mutant CRF01_AE transmitted-founder HIV-1 (IMC 703357) were used as target cells and autologous PBMCs were used as effector cells in our FACS-based ADCC assay, as described in Materials and Methods. Shown are the percentages of ADCC activity by A32 (A) or by antibodies in sera from HIV-1 clade B-infected (B) or CRF01_AE HIV-1-infected (C) individuals. These results are representative of data from at least 4 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using an unpaired two-tailed *t* test for A32 or a paired two-tailed *t* test for HIV$^+$ sera (***, $P < 0.001$; ****, $P < 0.0001$).

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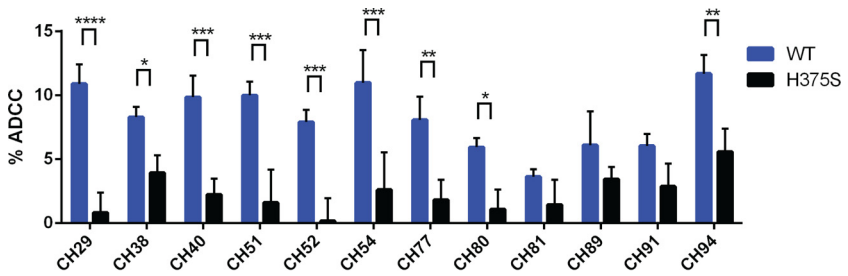

FIG 7 Histidine 375 modulates ADCC responses against CRF01_AE HIV-1-infected cells mediated by antibodies isolated from RV144 vaccinees. Primary CD4$^+$ T cells infected with the WT or the H375S variant of CRF01_AE transmitted-founder HIV-1 (IMC 703357) were used as target cells, with autologous PBMCs as effector cells, in our FACS-based ADCC assay. Shown are the percentages of ADCC activity in the presence of the A32-blockable monoclonal antibodies (CH29, CH38, CH40, CH51, CH52, CH54, CH77, CH80, CH81, CH89, CH91, and CH94) isolated from RV144 vaccinees. These results are representative of those obtained in at least 5 independent experiments. Error bars indicate means ± standard errors of the means. Statistical significance was tested by using an unpaired two-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

recognition and killing of HIV-1-infected cells by antibodies within HIV$^+$ sera and ADCC-mediating monoclonal antibodies, including those isolated from RV144 vaccinees. Altogether, our results suggest a model (Fig. 8) where the conformation spontaneously sampled by Env at the surface of infected cells has a significant impact on ADCC susceptibility. Env with an empty Phe 43 cavity are protected from such responses due to the adoption of a conformation similar to that of the unliganded closed state (state 1) and the ability of Nef and Vpu to downregulate CD4 from the cell surface (14). This state 1 conformation of Env could be modulated by CD4mcs (18) or by naturally occurring Phe 43 cavity-filling residues, which shift Env conformation toward the CD4-bound state. The CD4-bound Env conformation efficiently exposes anti-cluster A epitopes, known to be recognized by antibodies present in the majority of HIV-1-

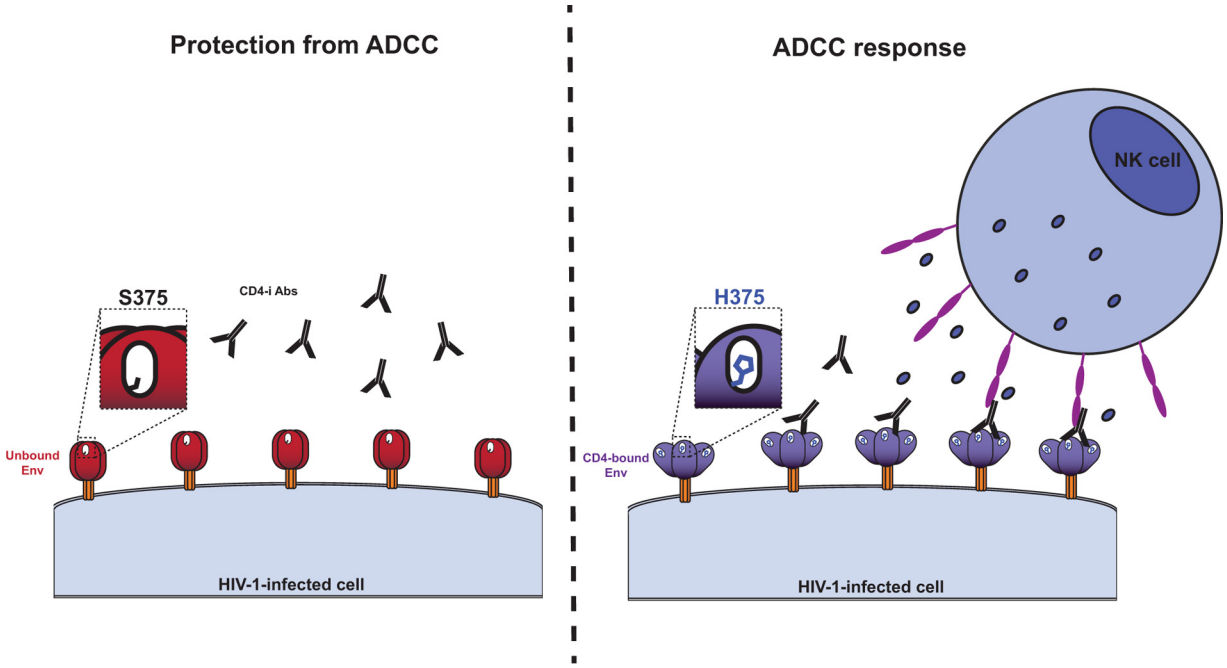

FIG 8 The Phe 43 cavity modulates HIV-1 Env conformation and ADCC responses. Flanking the Phe 43 cavity, the identity of residue 375 modulates the transition of Env to the CD4-bound conformation. The presence of a serine residue at this position keeps Env in its unliganded, closed state (state 1), thus preventing the exposure of highly conserved epitopes recognized by anti-cluster A antibodies. The presence of a larger residue within the cavity, such as the naturally occurring histidine at position 375, shifts Env conformation to a state closer to the CD4-bound state, thus more readily exposing cluster A epitopes and enhancing the susceptibility of infected cells to ADCC mediated by antibodies in HIV$^+$ sera.

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infected individuals and to mediate potent ADCC responses (13, 14, 18, 20–26). Therefore, our results raise the intriguing possibility that histidine 375 present in the predominant strain replicating in Thailand might have contributed to the efficacy of the trial by spontaneously exposing epitopes recognized by ADCC-mediating antibodies elicited by the RV144 vaccine regimen.

Since the first emergence of CRF01_AE HIV-1 strains in Africa and subsequent spread to Asia, where it established a major epidemic, this virus has become the dominant strain in many Asian countries, including southern China and Thailand (48–50). Why CRF01_AE HIV-1 was able to spread so rapidly is unclear at the moment, but high viral loads and short survival times have been associated with CRF01_AE infection compared with the values seen for HIV-1 infections in the Western world (51–53). Additional work is needed to assess the evolutionary selective pressures that resulted in the fixation of a histidine residue at position 375 in this rapidly spreading viral strain. Substitutions of large residues like histidine at this position have been shown to result in SHIVs that bind rhesus macaque CD4 and replicate in monkeys more efficiently (12). Apparently, the advantages of the S375W change in specific hosts (rhesus monkeys and some southern Chinese and Thai individuals) outweigh negative consequences such as enhanced susceptibility of HIV-1-infected cells to ADCC responses. Our results warrant further studies that assess potential host factors that drive HIV-1 variation in the Phe 43 cavity. The importance of the variation in residue 375 to susceptibility to the protective efficacy of HIV-1 vaccine-induced antibody responses also merits additional investigation.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis. Individual Env mutations were introduced in the previously described pNL43-ADA(Env)-GFP.IRES.Nef proviral vector (14) by using the QuikChange II XL site-directed mutagenesis protocol (Stratagene). The plasmids encoding SHIV clade C strain CH505, SHIV clade D strain 191859, and their variants were previously described (12). The sequence of HIV-1 CRF01_AE transmitted-founder (T/F) clone 703357 was derived by using a single-genome amplification (SGA) strategy. The entire DNA sequence including both long terminal repeats (LTRs) was cloned into pUC57 to generate a full-length infectious molecular clone (FLIMC) (GenBank accession numbers JX448154 and JX448164). Single mutations were then introduced into FLIMC 703357.

Cell lines and isolation of primary cells. 293T human embryonic kidney cells were obtained from the ATCC and were grown as previously described (14). PBMCs from healthy donors were obtained under research regulations approved by the Centre de Recherche du CHUM (CRCHUM); written informed consent was obtained from each individual. $CD4^+$ T cells were purified from rested PBMCs by negative selection and activated as previously described (18).

Virus production and infections. In order to achieve the same level of infection among the different mutants tested, vesicular stomatitis virus G (VSVG)-pseudotyped HIV-1 clones were produced, as previously described (14). This was necessary since mutations at residue 375 were shown to affect CD4 binding and infectivity in CRF01_AE strains (27). Briefly, proviral vectors and a VSVG-encoding plasmid were cotransfected into 293T cells by standard calcium phosphate transfection. Two days after transfection, cell supernatants were harvested, clarified by low-speed centrifugation (5 min at 1,500 rpm), and concentrated by ultracentrifugation for 1 h at 4°C at 100,605 $\times$ *g* over a 20% sucrose cushion. Pellets were resuspended in fresh RPMI, and aliquots were stored at −80°C until use. Viruses were then used to infect ~10 to 15% of primary $CD4^+$ T cells by spinoculation at 800 $\times$ *g* for 1 h in 96-well plates at 25°C.

Antibodies and sera. The gp120 outer-domain-specific 2G12 and the anti-gp41 10e8 antibodies were obtained from the NIH AIDS and Research and Reference Reagent Program. Anti-gp120 cluster A antibody A32 and coreceptor binding site antibody 17b were previously reported (14, 24, 54, 55). A32-blockable conformational epitope ADCC-mediating antibodies isolated from recipients of the RV144 ALVAC-HIV AIDSVAX B/E vaccine (CH29, CH38, CH40, CH51, CH52, CH54, CH77, CH80, CH81, CH89, CH91, and CH94) were previously described (14, 26). Sera from HIV-infected individuals (Table 1) were collected, heat inactivated, and stored as previously described (18). Written informed consent was obtained from all study participants (the Montreal Primary HIV Infection Cohort [56, 57] and the Canadian Cohort of HIV Infected Slow Progressors [58–60]), and research adhered to the ethical guidelines of the CRCHUM and was reviewed and approved by the CRCHUM institutional review board (ethics committee). Additional serum samples were acquired from HIV-1-infected individuals from Thailand who were enrolled in the HIV STAR study, as previously described (61) (Table 2). The Thai Ministry of Public Health and local ethics committees approved the collection of these samples. All donors provided informed consent. Research adhered to the standards indicated by the Declaration of Helsinki. All sera were heat inactivated for 30 min at 56°C and stored at 4°C until they were ready for use in subsequent experiments. A random-number generator (QuickCalcs; GraphPad) was used to randomly select a number of serum samples for each experiment.

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TABLE 1 Characteristics of HIV-infected serum donors

Donor	CD4 count (no. of cells/mm³)	Viral load (no. of copies/ml)
1	410	260
2	692	50
3	680	633
4	620	40
5	580	1,477
6	670	58,475
7	592	13,520
8	810	42
9	780	949
10	1,307	3,387
11	780	905
12	800	7,206
13	747	3,098
14	920	277
15	790	22,045
16	1,009	67
17	830	674
18	1,029	3,779
19	647	961
20	420	1,112
21	460	180,763

Anti-CD4 monoclonal antibody OKT4 (BioLegend) binds to the D3 domain of CD4 and was used to measure cell surface levels of CD4, as described previously (14). Goat anti-mouse and anti-human antibodies coupled to Alexa Fluor 647 (Invitrogen) were used as secondary antibodies in flow cytometry experiments.

Flow cytometry analysis of cell surface staining and ADCC responses. Cell surface staining was performed as previously described, and mean fluorescence intensity (MFI) histograms show signals on live infected populations (13, 18). Binding of HIV-1-infected cells by HIV$^+$ sera (1:1,000 dilution) or anti-CD4 (OKT4) or anti-Env MAbs (5 μg/ml) was performed 48 h after *in vitro* infection. Detection of green fluorescent protein-positive (GFP$^+$), p24$^+$, or p27$^+$ infected cells was performed as described previously (37). The percentage of infected cells (i.e., GFP$^+$, p24$^+$, or p27$^+$ cells) was determined by gating the living cell population based on viability dye staining (Aqua Vivid, catalog number L43957; Thermo Fisher Scientific). Samples were analyzed on an LSRII cytometer (BD Biosciences, Mississauga, ON, Canada), and data analysis was performed by using FlowJo v10.0.7 (Tree Star, Ashland, OR, USA).

Measurement of ADCC-mediated killing was performed by using a previously described assay (18). Briefly, primary CD4$^+$ T cells infected for 48 h with the different molecularly cloned viruses described above were incubated with autologous PBMCs (effector-to-target cell ratio of 10:1) in the presence of

TABLE 2 Characteristics of HIV-1-infected Thai serum donors

Donor	CD4 count (cells/mm³)	Viral load (copies/ml)
1	436	29,600
2	200	87,300
3	775	<40
4	266	43,700
5	119	9,260
6	369	<50
7	505	2,470
8	208	<50
9	690	<50
10	358	<50
11	76	14,000
12	610	<40
13	519	<50
14	362	1,450
15	282	<50
16	488	67
17	180	<50
18	448	<40
19	424	61
20	273	<50
21	519	<50
22	388	<50
23	413	<40

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A32, RV144 antibodies (5 μg/ml), or HIV$^+$ sera (1:1,000). The percentage of cytotoxicity was calculated as described previously (18).

Statistical analyses. Statistics were analyzed by using GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA). *P* values of <0.05 were considered significant; significance values are indicated in the figure legends.

ACKNOWLEDGMENTS

We thank Elizabeth Carpelan for help with manuscript preparation. We thank Dominique Gauchat from the CRCHUM Flow Cytometry Platform for technical assistance and Mario Legault for cohort coordination and clinical samples.

This work was supported by CIHR Foundation grant 352417, by amfAR innovation grant 109343-59-RGRL with support from FAIR to A.F., and by the FRQS AIDS and Infectious Diseases Network. A.F. is the recipient of a Canada research chair on retroviral entry. D.Z. is the recipient of FRQS master award 30888. M.V. was supported by CIHR doctoral research award 291485. J.R. is the recipient of CIHR fellowship award 135349. M.S.P. is the recipient of CIHR fellowship award 135451. D.E.K. is supported by a research scholar career award of the Quebec Health Research Fund (FRQS). This study was also supported by CIHR grant MOP93770, NIH grants AI100645 and AI100663, the Center for HIV/AIDS Vaccine Immunology and Immunogen Design (CHAVI-ID), National Institutes of Health grant GM56550, the late William F. McCarty-Cooper, and NIH grant R01AI116274. Our funding sources had no role in data collection, analysis, or interpretation and were not involved in the writing of the manuscript.

The opinions expressed here are those of the authors and should not be construed as official or representing the views of the U.S. Department of Defense or Department of the Army. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. government.

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