The HIV-1 gp120 CD4-Bound Conformation Is Preferentially Targeted by Antibody-Dependent Cellular Cytotoxicity-Mediating Antibodies in Sera from HIV-1-Infected Individuals

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ABSTRACT

Recent studies have linked antibody Fc-mediated effector functions with protection or control of human immunodeficiency type 1 (HIV-1) and simian immunodeficiency (SIV) infections. Interestingly, the presence of antibodies with potent antibody-dependent cellular cytotoxicity (ADCC) activity in the Thai RV144 vaccine trial was suggested to correlate with decreased HIV-1 acquisition risk. These antibodies recently were found to recognize HIV envelope (Env) epitopes exposed upon Env-CD4 interaction. CD4 downregulation by Nef and Vpu, as well as Vpu-mediated BST-2 antagonism, were reported to modulate exposure of those CD4-induced HIV-1 Env epitopes and were proposed to play a role in reducing the susceptibility of infected cells to ADCC mediated by this class of antibodies. Here, we report the high prevalence of antibodies recognizing CD4-induced HIV-1 Env epitopes in sera from HIV-1-infected individuals, which correlated with their ability to mediate ADCC responses against HIV-1-infected cells, exposing these Env epitopes at the cell surface. Furthermore, our results indicate that Env variable regions V1, V2, V3, and V5 do not represent a major determinant for ADCC responses mediated by sera from HIV-1-infected individuals. Altogether, these findings suggest that HIV-1 tightly controls the exposure of certain Env epitopes at the surface of infected cells in order to prevent elimination by Fc-effector functions.

IMPORTANCE

Here, we identified a particular conformation of HIV-1 Env that is specifically targeted by ADCC-mediating antibodies present in sera from HIV-1-infected individuals. This observation suggests that HIV-1 developed sophisticated mechanisms to minimize the exposure of these epitopes at the surface of infected cells.

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inner domain CD4i to the overall Fc-mediated effector functions present in sera from HIV-1-infected individuals.

MATERIALS AND METHODS

Cells. 293T human embryonic kidney and HOS cell lines (obtained from the ATCC and NIH AIDS Research and Reference Reagent Program, respectively) were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Sigma) and 100 µg/ml of penicillin-streptomycin (Wysent). CEM.NK cells (obtained from David Evans, Harvard Medical School) were grown at 37°C and 5% CO2 in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum and 100 µg/ml of primocine (Invitrogen).

Ficol density gradient isolated and cryopreserved human peripheral blood mononuclear cells (PBMCs) from healthy donors were thawed and kept at 37°C and 5% CO2 in RPMI 1640 containing 10% FBS and 100 µg/ml penicillin-streptomycin for at least 16 h before subsequent experiments.

Plasmids and site-directed mutagenesis. Mutations were introduced into the previously described pNL4.3-ADA-GFP.IRES.Nef.Nef proviral vector (23). To generate env mutants, the Sall-BamHI fragment of pNL4.3-ADA-GFP.IRES.Nef was subcloned in a pUC19 intermediate before being subjected to site-directed mutagenesis using the QuikChange II XL protocol (Stratagene). The mutated insert was then cloned back into pNL4.3-ADA-GFP.IRES.Nef. Mutations in nef were introduced by a two-step PCR strategy using primers having 18-nucleotide overlaps and cloned back into the proviral construct using XhoI and NcoI restriction sites. All mutations except the mutations G162D, V300P, and Y331C were conserved at 37°C and 5% CO2 in RPMI 1640 containing 10% FBS and 100 µg/ml penicillin-streptomycin for at least 16 h before subsequent experiments.

Measurement of serum- and A32-mediated ADCC was performed with a previously described assay (18, 33). Briefly, CEM.NK-infected cells were stained with viability (AquaVivid; Invitrogen) and cellular (cell proliferation dye eFluor670; eBioscience) markers and used as target cells. PBMC effectors cells, stained with another cellular marker (cell proliferation dye eFluor450; eBioscience), were then mixed at an effector/target ratio of 10:1 in 96-well plates (Corning). A 1:1,000 final dilution of serum from patients in PBS. Cells were then washed once with PBS and stained with 1 µg/ml goat anti-mouse and anti-rabbit (Alexa Fluor 594; Invitrogen) or anti-human (Alexa Fluor 647; Invitrogen) secondary Abs for 15 min in PBS. After one more PBS washing, cells were fixed in a 2% PBS-formaldehyde solution.

Viral production and infection. Vesicular stomatitis virus G (VSV-G)-psuedotyped NL4.3 green fluorescent protein (GFP)-encoding ADA-Env-based viruses were produced as previously described (18). Briefly, our panel of pNL4.3-ADA-GFP-ADA-based HIV-1 proviral vectors and VSV-G-encoding plasmid were cotransfected in 293T cells by standard calcium phosphate transfection. Two days after transfection, cell supernatants were harvested, clarified by low-speed centrifugation (5 min at 1,200 rpm), and concentrated by ultracentrifugation for 1 h at 4°C at 143,260 × g over a 20% sucrose cushion. Pellets were harvested in fresh RPMI, and aliquots were stored at −80°C until use. Viral preparations were normalized before infection according to reverse transcriptase activity or using a standard 50% tissue culture infectious dose (TCID50) procedure using TZM-bl cells (32). Viruses were then used to infect approximately 20% to 30% of CEM.NK cells by spin infection at 800 × g for 1 h in 96-well plates at 25°C.

Flow cytometry analysis of cell surface staining, ADCC responses, and binding competition assays. For cell surface staining, infected or mock-infected CEM.NK cells were incubated for 20 min at room temperature 48 h postinfection with 1 µg/ml OKT4 (anti-CD4 Ab; 14-0048-82; ebioscience), 2 µg/ml BST-2 (sc-99191; Santa Cruz), 1 µg/ml 2G12 (AB0002; Polymun), 1 µg/ml A32 Ab (kindly given by J. Robinson), or a 1:1,000 final dilution of serum from patients in PBS. Cells were then washed once with PBS and stained with 1 µg/ml goat anti-mouse and anti-rabbit (Alexa Fluor 594; Invitrogen) or anti-human (Alexa Fluor 647; Invitrogen) secondary Abs for 15 min in PBS. After one more PBS washing, cells were fixed in a 2% PBS-formaldehyde solution.

RESULTS

Env-C4D interaction enhances recognition of HIV-1-infected cells by sera from HIV-1-infected individuals. HIV-1 accessory proteins Nef and Vpu are known to modulate cell surface levels of CD4 (34, 35). In addition to its role in CD4 degradation, Vpu also antagonizes a restriction factor, Tetherin/BST-2, which normally inhibits retroviral release (36, 37). Viruses lacking Vpu remain trapped at the cell surface, resulting in an accumulation of exposed Env (18, 19). Therefore, Nef and Vpu can independently modulate Env-C4D interaction at the surface of infected cells through...
CD4 and BST-2 downregulation (18). Accordingly, we recently reported that cells infected with viruses defective for both Nef and Vpu present enhanced levels of CD4 and Env at the cell surface, resulting in the exposure of Env CD4i epitopes (18). To address whether these epitopes were recognized at the surface of infected cells by sera from HIV-1-infected individuals, we infected CEM.NKr cells with a panel of VSV-G-pseudotyped NL4.3 GFP ADA-Env-based viruses expressing the wild type (wt) or a CD4-binding site (D368R) Env variant, lacking Nef (Nef−) or expressing a Nef variant (L166A-L168A) defective for CD4 downregulation (34) (NefAA) or lacking Vpu (Vpu−) or both Nef and Vpu (Nef− Vpu−), were stained at 48 h postinfection with sera from 30 HIV-infected individuals and then fluorescently labeled with an Alexa Fluor 647-conjugated anti-human IgG secondary Ab. (A) Histograms depicting representative staining of infected (GFP+) cells by serum from one HIV+ and one HIV− donor. (B) Fold increase of staining relative to mock infection for all tested sera. Data shown are the results from two different experiments, and error bars depict the standard errors of the means (SEM). Statistical significance was tested using paired one-way analyses of variance (ANOVA) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

The impact of Env-CD4 interaction on the binding of serum IgGs from 163 HIV-infected individuals was also evaluated by cell-based ELISA, as previously described (18, 30, 31). Briefly,
infected cells to ADCC by CD4i Abs (18). However, whether this is the case for sera from a large number of HIV-1-infected individuals remains unknown. Therefore, we sought to determine if the overall ADCC activity potential in sera from HIV-infected individuals would be associated with efficient Env-CD4 interaction. Using infected CEM.NKr cells as described above, we measured serum-mediated ADCC with our previously described fluorescence-activated cell sorting (FACS)-based ADCC assay (18, 33). Thus, we determined the ability of 48 randomly chosen sera (including those tested in Fig. 1B) from HIV-infected individuals to mediate ADCC against infected cells (Fig. 3). Strikingly, while cells infected with wt virus were slightly more susceptible to ADCC killing than mock-infected cells, those infected with a virus lacking Nef and Vpu were dramatically more susceptible to ADCC (Fig. 3A). Interestingly, the slight increase in ADCC killing of cells lacking Nef was similar to that obtained with cells infected with a Nef variant (L166A-L168A) unable to downregulate CD4 from the cell surface (34), further stressing the importance of CD4 downregulation to protect infected cells from ADCC. However, the increase in ADCC observed with cells lacking Nef or Vpu alone was significantly lower than ADCC levels reached with cells infected with viruses lacking both accessory proteins. Moreover, the ADCC potential of different sera correlated with their ability to recognize Env at the surface of cells infected with viruses lacking both Nef and Vpu (Fig. 3B), suggesting that the efficient recognition of HIV-1-infected cells by sera is required for their ability to mediate ADCC. Finally, introducing the CD4-binding site D368R mutation in Env dramatically decreased the sensitivity of infected cells to killing by ADCC (Fig. 3A and C), further stressing the importance of Env-CD4 interaction for exposure of epitopes recognized by ADCC-mediating Abs normally elicited in the course of HIV-1 infection.

Antibodies targeting the V1V2V3 and V5 gp120 variable regions do not play a major role in HIV+ serum-mediated ADCC. Recent reports indicate that various vaccine-elicited Abs targeting conserved (CD4i) or variable epitopes (V1V2) as well as rare broadly neutralizing Abs can mediate ADCC against HIV-1-infected cells (17, 18, 21, 22, 41). To investigate whether, aside from gp120 CD4-induced antibodies, epitopes recognized by anti-gp41, variable regions, or quaternary-dependent antibodies contributed to serum-mediated ADCC responses, we designed an antibody competition assay using purified, soluble gp120 lacking variable regions V1, V2, V3, and V5 while presenting a D368R mutation (ΔV1V2V3V5 D368R) making it unable to bind cell surface CD4 (33). Of note, variable region 4 (V4) could not be removed without affecting the structural integrity of the protein (42 and data not shown). The preincubation of sera with either full-length or ΔV1V2V3V5 D368R gp120 recombinant proteins captured anti-Env antibodies and prevented the recognition of gp120-coated cells by serum Abs (see Fig. S4 in the supplemental material). Interestingly, this was also observed at the surface of HIV-1-infected cells where competition with the ΔV1V2V3V5 recombinant gp120 almost completely abrogated cell surface staining of cells infected with virus unable to express Nef and Vpu (Fig. 4A) and correlated with decreased ADCC activity (Fig. 4B), indicating that a recombinant protein lacking the V1V2V3V5 variable regions is sufficient to absorb the majority of ADCC activity present in sera from HIV-1-infected individuals.

FIG 2 Env-CD4 interaction modulates the exposure of the Env ADCC-mediating A32 epitope at the surface of infected cells. CEM.NKr cells infected with a panel of VSV-G-pseudotyped NL4.3 GFP ADA-Env-based viruses expressing the wild type (wt) or a CD4-binding site (D368R) Env variant, lacking Nef (Nef−) or expressing a Nef variant (L166A-L168A) defective for CD4 downregulation (34) (NefAA) or lacking Vpu (Vpu−) or both Nef and Vpu (Nef−Vpu−), were stained at 48 h postinfection for surface CD4 levels (A) or Env A32 epitope exposure (B). Data shown are the results from at least three different experiments, and error bars depict SEM. Statistical significance was tested using paired one-way ANOVA (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

The ability of sera from HIV-infected individuals to mediate ADCC requires Env-CD4 interaction. We previously described that Env-CD4 interaction modulates the susceptibility of HIV-1-infected cells to ADCC by CD4i Abs (18). However, whether this was also the case for sera from a large number of HIV-1-infected individuals would be associated with efficient Env-CD4 interaction. Using infected CEM.NKr cells as described above, we measured serum-mediated ADCC with our previously described fluorescence-activated cell sorting (FACS)-based ADCC assay (18, 33). Thus, we determined the ability of 48 randomly chosen sera (including those tested in Fig. 1B) from HIV-infected individuals to mediate ADCC against infected cells (Fig. 3). Strikingly, while cells infected with wt virus were slightly more susceptible to ADCC killing than mock-infected cells, those infected with a virus lacking Nef and Vpu were dramatically more susceptible to ADCC (Fig. 3A). Interestingly, the slight increase in ADCC killing of cells lacking Nef was similar to that obtained with cells infected with a Nef variant (L166A-L168A) unable to downregulate CD4 from the cell surface (34), further stressing the importance of CD4 downregulation to protect infected cells from ADCC. However, the increase in ADCC observed with cells lacking Nef or Vpu alone was significantly lower than ADCC levels reached with cells infected with viruses lacking both accessory proteins. Moreover, the ADCC potential of different sera correlated with their ability to recognize Env at the surface of cells infected with viruses lacking both Nef and Vpu (Fig. 3B), suggesting that the efficient recognition of HIV-1-infected cells by sera is required for their ability to mediate ADCC. Finally, introducing the CD4-binding site D368R mutation in Env dramatically decreased the sensitivity of infected cells to killing by ADCC (Fig. 3A and C), further stressing the importance of Env-CD4 interaction for exposure of epitopes recognized by ADCC-mediating Abs normally elicited in the course of HIV-1 infection.

Antibodies targeting the V1V2V3 and V5 gp120 variable regions do not play a major role in HIV+ serum-mediated ADCC. Recent reports indicate that various vaccine-elicited Abs targeting conserved (CD4i) or variable epitopes (V1V2) as well as rare broadly neutralizing Abs can mediate ADCC against HIV-1-infected cells (17, 18, 21, 22, 41). To investigate whether, aside from gp120 CD4-induced antibodies, epitopes recognized by anti-gp41, variable regions, or quaternary-dependent antibodies contributed to serum-mediated ADCC responses, we designed an antibody competition assay using purified, soluble gp120 lacking variable regions V1, V2, V3, and V5 while presenting a D368R mutation (ΔV1V2V3V5 D368R) making it unable to bind cell surface CD4 (33). Of note, variable region 4 (V4) could not be removed without affecting the structural integrity of the protein (42 and data not shown). The preincubation of sera with either full-length or ΔV1V2V3V5 D368R gp120 recombinant proteins captured anti-Env antibodies and prevented the recognition of gp120-coated cells by serum Abs (see Fig. S4 in the supplemental material). Interestingly, this was also observed at the surface of HIV-1-infected cells where competition with the ΔV1V2V3V5 recombinant gp120 almost completely abrogated cell surface staining of cells infected with virus unable to express Nef and Vpu (Fig. 4A) and correlated with decreased ADCC activity (Fig. 4B), indicating that a recombinant protein lacking the V1V2V3V5 variable regions is sufficient to absorb the majority of ADCC activity present in sera from HIV-1-infected individuals.

DISCUSSION

Renewed interest in Fc-mediated functions, such as ADCC, stems in part from correlations in controlling or preventing HIV-1 in-
Recombinant gp120 protein lacking V1V2V3 and V5 variable regions is sufficient to adsorb the majority of ADCC activity present in sera from HIV-infected individuals. CEM.NKr cells infected with wt or Nef- and Vpu-defective VSV-G-pseudotyped NL4.3 GFP ADA-based viruses were used at 48 h postinfection for surface staining (A) or FACS-based ADCC assay (B) using sera from HIV-1-infected individuals. (C) Paired values of ADCC mediated by sera from HIV-1-infected individuals and those of cells infected by HIV-1 viruses lacking both Nef and Vpu encoding a wt or D368R Env variant. Statistical significance was tested using paired one-way ANOVA (A) or a paired t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001) (C).
elicited in the course of natural HIV-1 infection and that a significant proportion of them require Env to interact with CD4 in order to be effective at mediating ADCC. This supports a major role of CD4-dependent ADCC in the susceptibility of infected cells to ADCC mediated by sera from HIV-1-infected individuals. Our data suggest that HIV-1, via Nef and Vpu, tightly controls cell surface levels of CD4 and Env in order to limit the exposure of potential epitopes recognized by ADCC-mediating Abs elicited in the course of natural HIV-1 infection. Therefore, targeting the ability of Vpu and Nef to downregulate CD4 and BST-2 or strategies aimed at modifying Env conformation to expose CD4 epitopes could render HIV-1-infected cells susceptible to ADCC and have therapeutic utility.

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REFERENCES


