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A Highly Conserved Residue of the HIV-1 gp120 Inner Domain Is Important for Antibody-Dependent Cellular Cytotoxicity Responses Mediated by Anti-cluster A Antibodies

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Previous studies have shown that sera from HIV-1-infected individuals contain antibodies able to mediate antibody-dependent cellular cytotoxicity (ADCC). These antibodies preferentially recognize envelope glycoprotein (Env) epitopes induced upon CD4 binding. Here, we show that a highly conserved tryptophan at position 69 of the gp120 inner domain is important for ADCC mediated by anti-cluster A antibodies and sera from HIV-1-infected individuals.

Human immunodeficiency virus type 1 (HIV-1) infection elicits a potent B cell response resulting in the production of antibodies (Abs) against the envelope glycoproteins (Env), which are exposed at the surface of viral particles and infected cells (1). We recently reported that these antibodies have the potential to eliminate HIV-1-infected cells by mediating antibody-dependent cellular cytotoxicity (ADCC) (2, 3). We found that these nonneutralizing CD4-induced (CD4i) ADCC-mediating antibodies are present in sera (2, 4), breast milk (4), and cervicovaginal lavage fluid (3, 4) of HIV-1-infected individuals and preferentially target Env in its CD4-bound "open" conformation. However, in order to evade ADCC responses, HIV-1 has developed a highly sophisticated strategy to keep Env at 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 amount of Env (via Vpu-mediated BST-2 downregulation) and CD4 at the cell surface (2, 5–7). In addition, decreased amounts of Env at the cell surface due to efficient internalization also help the virus to avoid ADCC responses (8). In agreement with the necessity for HIV-1 to avoid exposing Env in the CD4-bound conformation, we recently showed that forcing Env to adopt this conformation with small CD4 mimetics (CD4mc) sensitizes HIV-1-infected cells to ADCC mediated by sera, breast milk, and cervicovaginal fluids from HIV-1-infected subjects (4).

Previous studies showed that the human monoclonal antibody (MAb) A32 targets an ADCC epitope commonly detected by antibodies present in sera from HIV-1-infected individuals (2, 5, 9, 10). Accordingly, an A32 Fab fragment blocked the majority of ADCC-mediating antibody (Ab) activity in plasma from chronically HIV-1-infected individuals (9). A subsequent study showed that the majority of ADCC responses were targeted against the gp120 core but not its variable regions V1, V2, V3, and V5 (2). Here, we evaluated the ADCC-mediating capacity of a panel of human antibodies targeting several well-defined epitopes in gp120 and gp41 and sera from randomly selected chronically HIV-1 clade B-infected individuals (HIV^+ sera).

We infected CEM.NKr cells with a panel of HIV-1 NL4.3–green fluorescent protein (GFP) constructs containing the ADA-Env and either wild-type or defective *nef* and *vpu* genes, as described previously (2, 5). Furthermore, we examined a well-characterized infectious molecular HIV-1 clone constructed from a transmitted/founder (T/F) virus (CH77) (11–14) containing intact or defective *nef* and *vpu* genes. Two days postinfection, the cells were evaluated for cell surface levels of CD4 and stained with HIV^+ sera or anti-Env antibodies targeting well-known epitopes in gp120, gp41, or both (Fig. 1A and Table 1). Nef and Vpu are known to synergistically decrease cell surface levels of CD4 (2, 3). Accordingly, defects in both genes impaired the ability of HIV-1 to downregulate CD4 to extents that were not achieved by either *nef* or *vpu* alone. The highest surface CD4 levels were observed for cells infected with virus lacking intact *nef* and *vpu* genes and containing a mutation of D368R in Env that abrogates its interaction with CD4 (15, 16) (Fig. 1A; Table 1). The latter observation is in agreement with the notion that Env-CD4 interaction plays a role in CD4 downregulation (17, 18). HIV^+ sera and the anti-cluster A antibodies (these antibodies target conformational CD4i epitopes mapped to the C1-C2 regions of gp120 [10, 19, 20]) recognized wild-type-infected cells with low efficiency (Fig. 1C and D). Our results are in agreement with previous reports indicating that the highly conserved region recognized by anti-cluster A antibodies is

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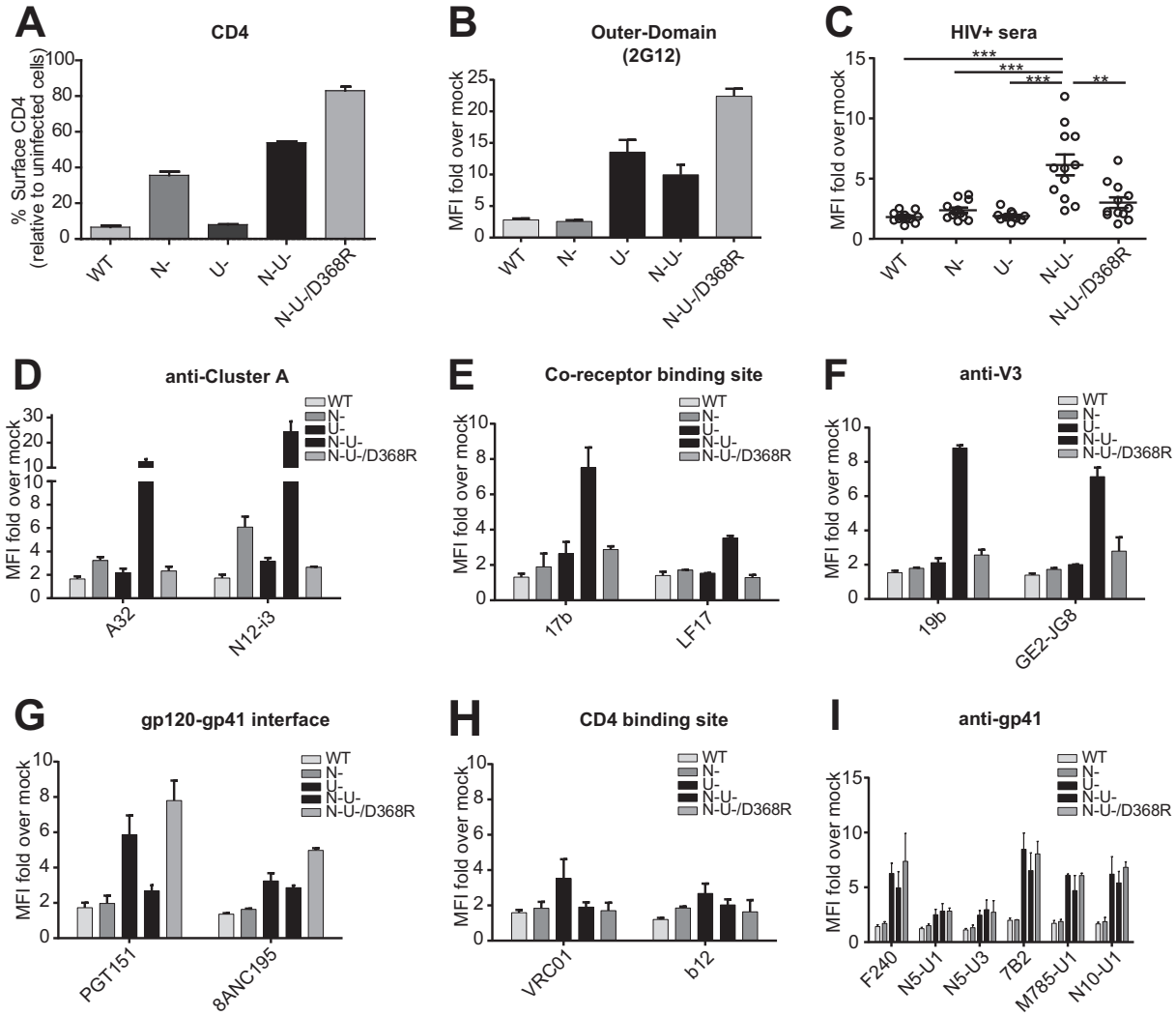

FIG 1 Effect of Nef, Vpu, and Env-CD4 interaction on recognition of infected cells by HIV$^+$ sera and a panel of monoclonal antibodies. CEM.NKr cells infected with a panel of vesicular stomatitis virus glycoprotein-pseudotyped NL4.3–GFP ADA viruses expressing wild type (WT) or a CD4-binding site (D368R) Env variant, lacking Nef (N$^-$), Vpu (U$^-$), or both Nef and Vpu (N$^-$ U$^-$), were stained at 48 h postinfection with an anti-CD4 antibody (OKT4) (A); 1 μg/ml of the CD4-independent outer domain recognizing 2G12 antibody (B); a 1/1,000 dilution of sera from 12 HIV-1-infected individuals (HIV$^+$ sera) (C); or 1 μg/ml of anti-cluster A (A32 and N12-i3) (D), anti-CoRBS (17b and LF17) (E), anti-V3 (19b and GE2-JG8) (F), anti-gp120-gp41 interface (PGT151 and 8ANC195) (G), anti-CD4-binding site (VRC01 and b12) (H), and anti-gp41 (F240, N5-U1, N5-U3, 7B2, M785-U1, and N10-U1) (I) antibodies and then fluorescently labeled with an Alexa Fluor 647-conjugated anti-human IgG secondary Ab. Shown is the fold increase of staining relative to mock for all tested sera and antibodies. Data shown are the results of 3 different experiments, and error bars depict the standard errors of the means. Statistical significance was tested using paired one-way analyses of variance (**, $P < 0.01$; ***, $P < 0.001$). MFI, mean fluorescence intensity.

buried inside the Env trimer, where it is not readily accessible for binding in the ligand-free closed state (10, 21–27). Accordingly, anti-cluster A and HIV$^+$ sera recognized more efficiently cells infected with a virus lacking Nef and Vpu and hence exposing Env at higher levels and in its CD4-bound conformation (2, 5, 28). Nevertheless, our results are also consistent with previous reports indicating that anti-cluster A antibodies, such as A32, can recognize a large proportion of cells infected with a wild-type virus (9, 29, 30). Indeed, A32 recognized ~32% of pNL4.3-ADA- and ~54% of CH77 wild-type-infected cells (Fig. 2). However, the intensity of recognition (i.e., the amount of antibody binding per cell) was dramatically increased for cells presenting Env in its CD4-bound conformation (i.e., Nef$^-$ Vpu$^-$ virus-infected cells), as previously reported (2–5, 28, 31). Similarly to anti-cluster A antibodies, coreceptor binding site (CoRBS) (17b and LF17) (Fig. 1E) as well as anti-V3 antibodies (19b and GE2-JG8) (Fig. 1F) recognized cells infected with Nef$^-$ Vpu$^-$ HIV-1 most efficiently. This suggests that their epitope was formed upon Env-CD4 interaction and that they all belong to the CD4i family of antibodies. We noted, however, that the overall recognition of CoRBS and anti-V3 Abs was lower than that observed for HIV$^+$ sera and anti-cluster A Abs.

In the absence of Vpu, there is more Env at the cell surface, as measured by the CD4-independent outer domain recognizing 2G12 antibody (Fig. 1B), likely due to tetherin/BST-2 trapping of viral particles. Cells infected with viruses lacking Nef and Vpu but containing the D368R mutation in Env that impairs CD4 binding (2, 4, 5, 15, 16) were poorly recognized by

TABLE 1 Recognition of infected cells by a panel of anti-gp120 and anti-gp41 antibodies

		Staining with antibody[b]:																
		Anti-cluster A		gp120-gp41 interface		CD4-binding site		Coreceptor binding site		Anti-V3		Anti-gp41						
pNL4.3-ADA	CD4 (%)[a]	A32	N12-i3	PGT151	8ANC195	VRC01	b12	17b	LF17	19b	GE2-JG8	F240	N5-U1	N5-U3	7B2	M785-U1	N10-U1	Outer domain (2G12)
WT[c]	6.5	1.6	1.7	1.7	1.4	1.6	1.2	1.3	1.4	1.5	1.4	1.4	1.2	1.1	2.0	1.7	1.7	2.8
N$^-$	35.7	3.2	6.1	2	1.6	1.8	1.9	1.9	1.7	1.8	1.7	1.7	1.5	1.3	2.0	1.9	1.9	2.4
U$^-$	7.9	2.2	3.2	5.9	3.2	3.5	2.7	2.6	1.5	2.1	2.0	6.3	2.5	2.5	8.5	6.1	6.2	12.9
N$^-$ U$^-$	53.8	12.7	25.8	2.7	2.9	1.9	2	7.5	3.5	8.8	7.1	5.0	2.8	3.0	6.5	4.7	5.4	9.5
N$^-$ U$^-$/D368R	83.1	2.3	2.7	7.8	5	1.7	1.6	2.9	1.3	2.6	2.8	7.4	2.8	2.7	8.1	6.1	6.8	21.5

[a] Relative percentage of surface CD4 on infected cells compared to that on uninfected cells.
[b] Staining (fold mean fluorescence intensity) of infected cells over that of uninfected mock cells.
[c] WT, wild type.

HIV$^+$ sera and anti-cluster A, anti-coreceptor binding site, and anti-V3 antibodies (Fig. 1C to F). When we tested PGT151 and 8ANC195, two antibodies that recognize the interface between gp120 and gp41 (32, 33), we observed a different phenotype. Both antibodies efficiently recognized cells infected with a virus lacking Vpu or expressing Env D368R but not a virus lacking Vpu and Nef (Fig. 1G). 8ANC195 and PGT151 have been shown to bind to CD4-bound Env (34), but in our system, CD4

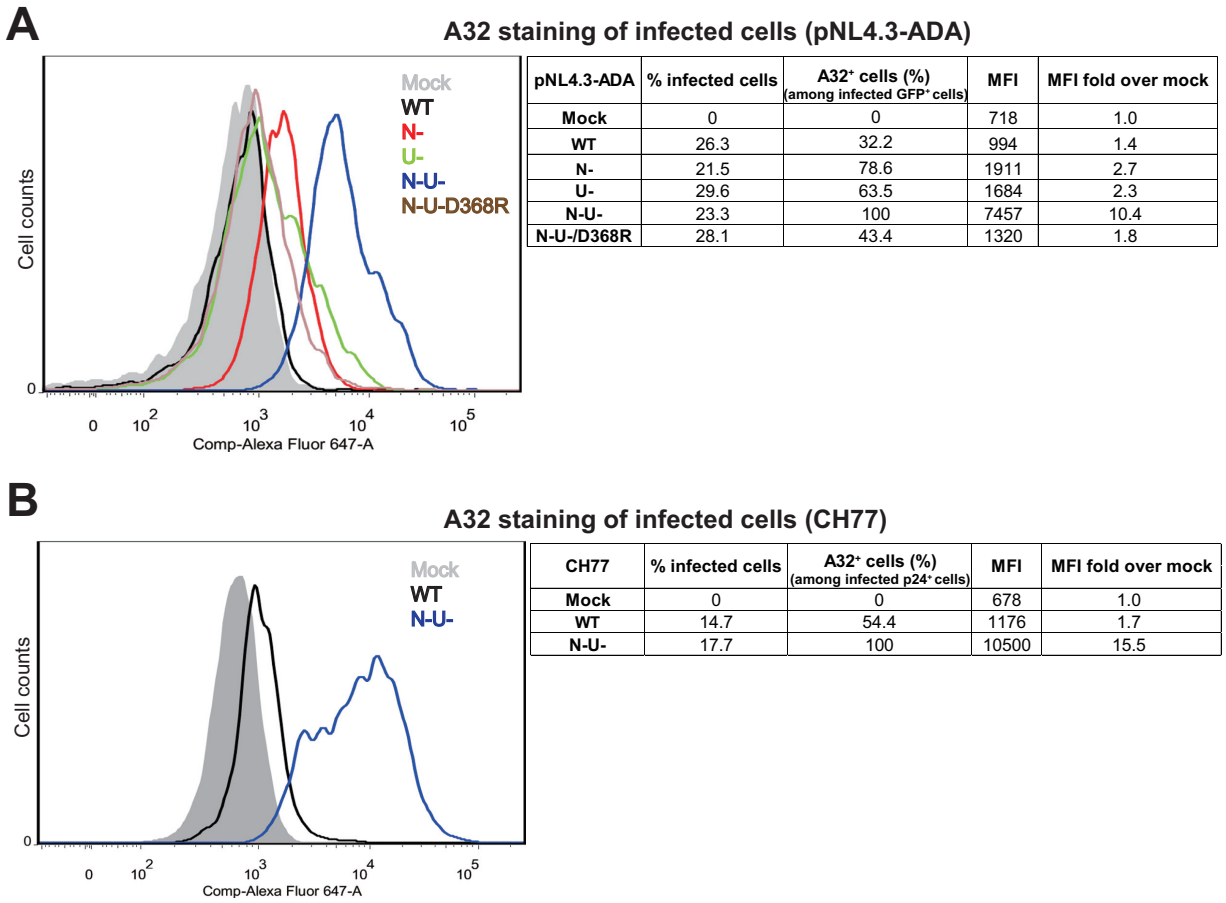

FIG 2 Recognition of HIV-1-infected cells by the anti-cluster A32 antibody. CEM.NKr cells infected with a panel of vesicular stomatitis virus glycoprotein-pseudotyped NL4.3–GFP ADA viruses expressing wild type (WT) or a CD4-binding site (D368R) Env variant, lacking Nef (N$^-$), Vpu (U$^-$), or both Nef and Vpu (N$^-$ U$^-$) (A), or with vesicular stomatitis virus glycoprotein-pseudotyped primary T/F CH77 infectious molecular clone (B) were stained at 48 h postinfection with the anti-cluster A A32 antibody (1 μg/ml) and then fluorescently labeled with an Alexa Fluor 647-conjugated anti-human IgG secondary Ab. Histograms depicting representative staining of infected (GFP$^+$ [A] or p24$^+$ [B]) cells are shown. Right panels show the percentages of infected cells, the percentages of infected (GFP$^+$ [A] or p24$^+$ [B]) cells that were recognized by A32, and the mean fluorescence intensity (MFI) of these cells. Mean fluorescence intensity of infected cells over that of mock-infected cells is shown in the last column.

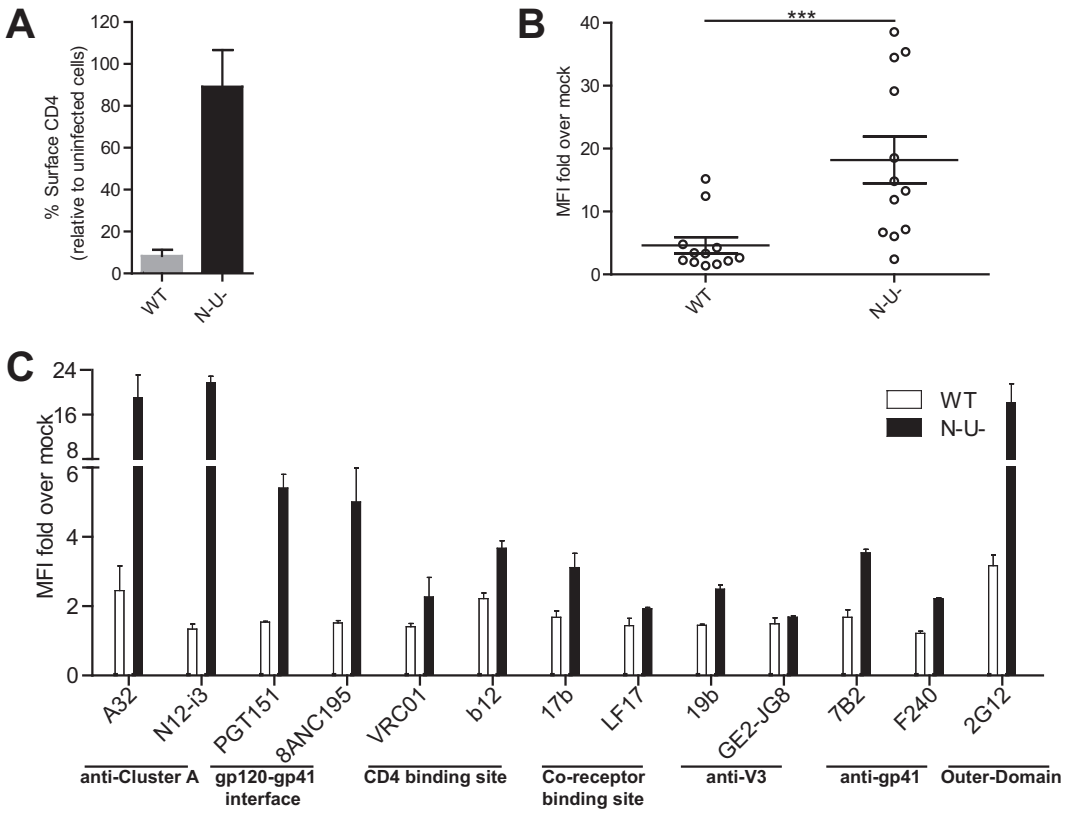

FIG 3 Effect of Nef and Vpu on recognition of cells infected with a primary isolate by HIV$^+$ sera and a panel of monoclonal antibodies. (A and B) CEM.NKr cells infected with vesicular stomatitis virus glycoprotein-pseudotyped T/F CH77 expressing wild type (WT) or lacking Nef and Vpu (N$^-$ U$^-$) were stained at 48 h postinfection with an anti-CD4 antibody (OKT4) (A) or a 1/1,000 dilution of sera from 12 HIV-1-infected individuals (HIV$^+$ sera) (B). (C) CH77-infected cells were also stained with a panel of anti-gp120 and -gp41 antibodies (1 μg/ml) and then fluorescently labeled with an Alexa Fluor 647-conjugated anti-human IgG secondary Ab. Data shown are the results of 3 independent experiments, with medians ± interquartile ranges. Statistical significance was tested using paired one-way analyses of variance (***, $P < 0.001$).

and Env are interacting on the same membrane and CD4 domains D3 and D4 may block access of these Abs to their epitopes on Env, which are located underneath the CD4-binding site (CD4BS).

CD4-binding site (CD4BS) antibodies (VRC01 and b12) better recognized cells infected with a Vpu$^-$ virus (Fig. 1H). This recognition was diminished by deleting Nef. In the absence of Nef, there is more CD4 at the cell surface interacting with Env (5) and therefore occluding the CD4BS. The D368R variant abrogated recognition by VRC01 and b12, as expected due to the importance of D368 for their interaction (35). Anti-gp41 antibodies (F240, N5-U1, N5-U3, 7B2, M785-U1, and N10-U1) behaved in a completely different manner; their recognition was enhanced by deletion of *vpu* independently of the presence of Nef and the ability of Env to interact with CD4 (Fig. 1I). Thus, this panel of anti-gp41 antibodies recognizes epitopes that are not greatly affected by CD4 binding.

We extended these findings to primary viruses by infecting CEM.NKr cells with the T/F CH77 isolate encoding either wild-type or no Nef and Vpu proteins. As expected, efficient CD4 downregulation was observed only for wild-type CH77 (Fig. 3A). Recognition of CH77-infected cells by HIV$^+$ sera and anti-cluster A, anti-gp120-gp41 interface, anti-CD4BS, anti-CoRBS, anti-V3, and anti-gp41 antibodies was similar to that of pNL4.3-ADA-infected cells. All ligands, particularly HIV$^+$ sera and anti-cluster A antibodies, recognized cells infected with *nef*- and *vpu*-deletion viruses more efficiently than wild-type-infected cells (Fig. 3B and C).

Interestingly, when we analyzed the ability of HIV$^+$ sera and different antibodies described above to mediate ADCC with our previously described fluorescence-activated cell sorting (FACS)-based ADCC assay (4, 31), we observed that, in addition to HIV$^+$ sera, only the anti-cluster A antibodies mediated potent ADCC against pNL4.3-ADA- or CH77-infected cells (Fig. 4). However, this was only the case when *nef* or *nef* and *vpu* genes were deleted. HIV$^+$ sera and anti-cluster A antibodies did not mediate potent ADCC against cells infected with wild-type viruses. Moreover, while the ability of HIV$^+$ sera and anti-cluster A antibodies to mediate ADCC correlated with their recognition of infected cells (Fig. 4 and data not shown), this was not the case for the rest of the antibodies (data not shown). In fact, none of the anti-gp41 antibodies tested in this study mediated efficient ADCC compared to A32 (Fig. 4D and E), indicating that recognition of infected cells by a given antibody does not necessarily translate into potent ADCC.

To investigate which region of the gp120 was targeted by ADCC-mediating Abs present in HIV$^+$ sera, we used our previously described antibody competition assay using purified soluble $gp120_{Yu2}$ lacking variable regions V1, V2, V3, and V5 with the D368R mutation (ΔV1V2V3V5/D368R) (2). As a control, we also

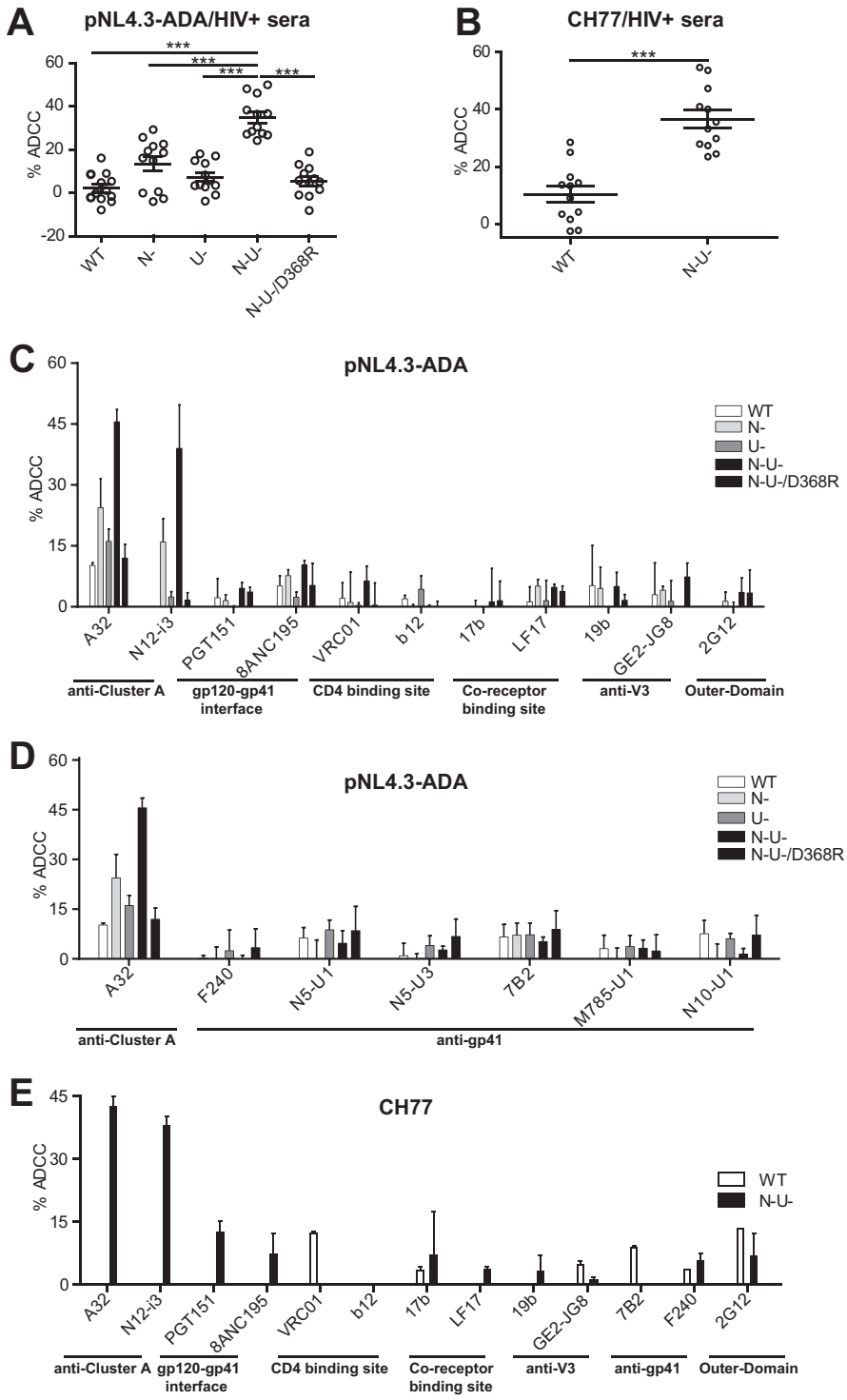

FIG 4 Effect of Nef, Vpu, and Env-CD4 interaction on ADCC responses mediated by HIV$^+$ sera and a panel of monoclonal antibodies. (A) CEM.NKr cells infected with a panel of vesicular stomatitis virus glycoprotein-pseudotyped NL4.3–GFP ADA viruses expressing wild type (WT) or a CD4-binding site (D368R) Env variant, lacking Nef (N$^-$), Vpu (U$^-$), or both Nef and Vpu (N$^-$ U$^-$), were used at 48 h postinfection as target cells in our FACS-based ADCC assay (5) to determine their susceptibility to ADCC mediated by a 1/1,000 dilution of sera from 12 HIV-1-infected individuals. (B) The susceptibility of vesicular stomatitis virus glycoprotein-pseudotyped T/F CH77-infected cells expressing wild type (WT) or lacking Nef and Vpu (N$^-$ U$^-$) to ADCC mediated by the same panel of HIV$^+$ sera was also evaluated. (C and D) The susceptibility of pNL4.3-ADA-infected cells to ADCC mediated by 5 μg/ml of anti-gp120 (anti-cluster A antibodies A32 and N12-i3; anti-CD4-binding site antibodies VRC01 and b12; anti-CoRBS antibodies 17b and LF17; anti-V3 antibodies 19b and GE2-JG8; anti-outer domain antibody 2G12) or anti-gp120-gp41 (PGT151 and 8ANC195) (C) or anti-gp41 (F240, N5-U1, N5-U3, 7B2, M785-U1, and N10-U1) (D) antibodies was also evaluated. (E) Susceptibility of CH77-infected cells to anti-gp120 and anti-gp41 antibodies. Peripheral blood mononuclear cells from healthy donors were used as effector cells. Data shown are the results of 3 independent experiments, with medians ± interquartile ranges. Statistical significance was tested using paired one-way analyses of variance (***, $P < 0.001$).

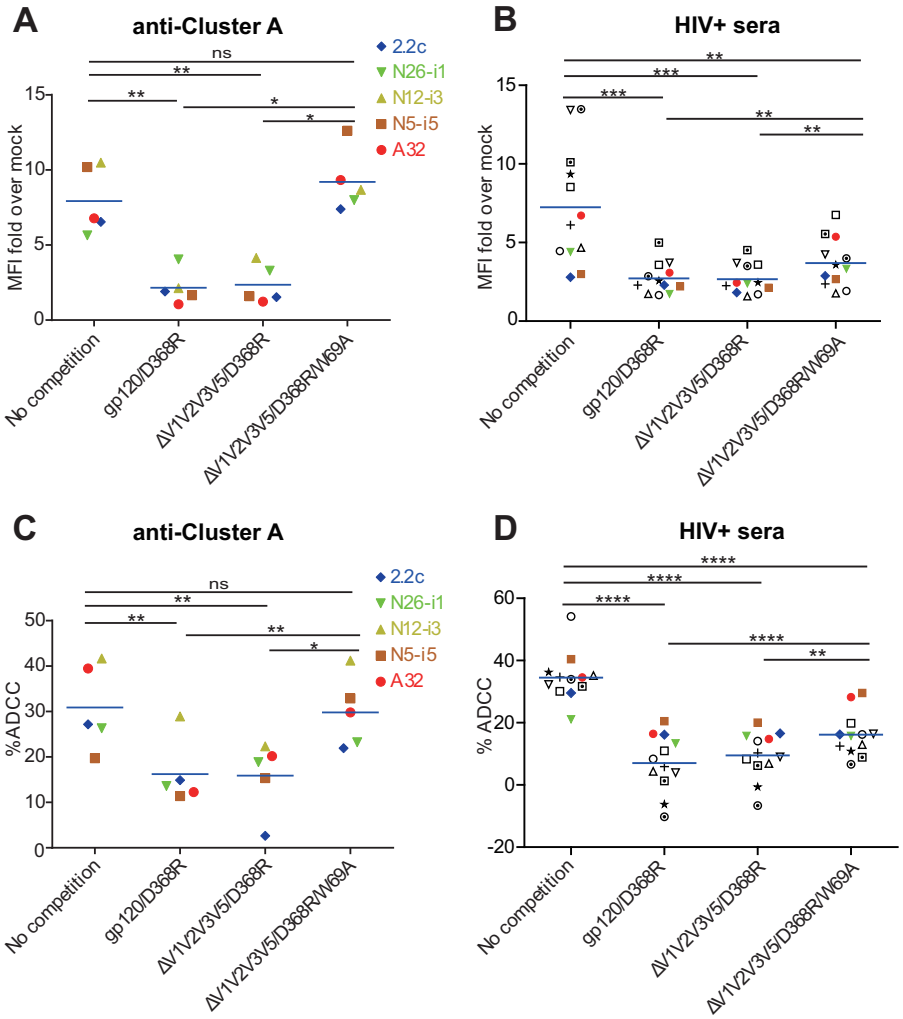

FIG 5 Tryptophan 69 in layer 1 is important for ADCC responses mediated by anti-cluster A antibodies and some HIV$^+$ sera. CEM.NKr cells infected with N$^-$ U$^-$ vesicular stomatitis virus glycoprotein-pseudotyped NL4-3–GFP ADA virus were used at 48 h postinfection for surface staining (A and B) or FACS-based ADCC assay (C and D) using 5 μg/ml of anti-cluster A antibodies (A32, N5-i5, N12-i3, N26-i1, and 2.2c) or a 1/1,000 dilution of sera from 12 HIV-1-infected individuals (HIV$^+$ sera, different color or symbol for each serum sample) in the absence or presence of recombinant gp120/D368R (10 μg/μg of antibody), ΔV1V2V3V5/D368R (6 μg/μg of antibody), or ΔV1V2V3V5/D368R/W69A (6 μg/μg of antibody) for 30 min at room temperature. Data shown are representative of at least 3 independent experiments. Statistical significance was tested using paired one-way analyses of variance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

tested the ability of these recombinant proteins to block recognition of infected cells by five anti-cluster A antibodies (A32, N5-i5, N12-i3, N26-i1, and 2.2c). Our recent structural studies mapped the cluster A epitope region to mobile layers 1 and 2 of the gp120 inner domain of the CD4-triggered gp120 (19, 20, 26). Residues of variable loops and the outer domain of gp120 are not involved in anti-cluster A antibody binding. In addition, although cluster A monoclonal antibodies (MAbs) are capable of binding unliganded gp120, CD4 binding enhances their exposure in the context of full-length gp120 antigen (10, 36, 37). As expected, preincubation of anti-cluster A antibodies (Fig. 5A) or HIV$^+$ sera (Fig. 5B) with either full-length or ΔV1V2V3V5 D368R gp120 recombinant proteins captured anti-Env antibodies and prevented their recognition of infected cells. Decreased recognition correlated with decreased ADCC activity (Fig. 5C and D). These data indicate that antibodies targeting the core of gp120 are responsible for the majority of ADCC responses in HIV$^+$ sera.

Confirming the role of the gp120 inner domain layers in anti-cluster A antibody recognition, a ΔV1V2V3V5/D368R gp120 variant presenting a mutation at a highly conserved residue in the inner domain layer 1, previously shown to be important for anti-cluster A recognition (W69) (5, 19, 26), was unable to efficiently compete for staining or ADCC by anti-cluster A Abs (Fig. 5A and C). W69 is involved in forming the cluster A epitope by stabilizing the layer 1 and 2 interface of the CD4-bound conformation of gp120 (19, 20, 26). Interestingly, preincubation of some but not all HIV$^+$ sera with this recombinant variant was able to decrease recognition and ADCC of HIV-1-infected cells (Fig. 5B and D). Thus, this highly conserved residue in the inner domain of gp120 is important for some but not all of the antibodies mediating ADCC within the polyclonal sera from these HIV-1-infected individuals.

Why does similar binding of infected cells by different classes of antibodies not translate into equivalent ADCC responses? For

example, anti-cluster A and anti-coreceptor binding site antibodies recognize CD4-induced Env epitopes, which become unmasked by the interaction of Env trimers with CD4. Both cluster A and coreceptor binding site region epitopes should persist on infected cell surfaces for similar periods of time, and therefore, both should constitute good targets for ADCC. Why, then, are anti-cluster A antibodies able to mediate potent ADCC responses whereas anti-coreceptor binding site antibodies are not? ADCC is mediated not only by antibody variable region binding to antigen on infected cells but also by the antibody constant region binding to Fc receptors on effector cells, and therefore, even subtle differences in the glycosylation patterns of the Fc portion of these antibodies could affect their ability to mediate ADCC. Nevertheless, our studies suggest that fine specificities among epitope targets at the surface of infected cells might also play a role in determining the potency of the ADCC response. We believe that targeting CD4i conformational, C1-C2 epitopes within the cluster A region, which depend on W69, could allow for an efficient antigen engagement and optimal angle of approach to engage with the Fcγ receptor of the effector cell for effective ADCC immune complex formation, as previously suggested (19, 20). Our results confirm that Nef and Vpu protect HIV-1-infected cells from ADCC but also show that recognition of infected cells by an antibody does not necessarily translate into ADCC. This raises the intriguing possibility that the angle of approach of a given class of antibodies could impact its capacity to mediate ADCC.

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