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The HIV-1 gp120 CD4-bound conformation is preferentially targeted by ADCC-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 HIV-1 and 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 a decreased HIV-1 acquisition risk. These antibodies were recently 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 therefore were proposed to play a role in reducing the susceptibility of infected cells to ADCC mediated by this class of antibodies. Here we report a 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.

64 **Introduction**

65

66 The IgG class of antibodies (Abs) can mediate cellular cytotoxic effector
67 functions such as Ab-dependent cell-mediated cytotoxicity (ADCC), viral inhibition
68 (ADCVI) or phagocytosis (ADCP). These immune responses are driven by the
69 engagement of the Ab Fc region with a family of proteins, known as Fcγ receptors
70 (FcγR), at the surface of effector immune cells (1). In the case of ADCC, crosslinking of
71 the FcγRIII (CD16) leads to the activation of the ITAM-containing associated subunits
72 CD3ζ and/or FcεRIγ which promotes the effector cells (e.g. NK cells, macrophages or
73 neutrophils) to perform a cytotoxic attack on the target cell (2, 3). Interestingly, there is
74 increasing evidence that ADCC plays a role in protecting from or controlling different
75 viral infections (4-6). Accordingly, Fc-mediated effector functions were reported to
76 correlate with decreased viral loads or rate of disease progression in both HIV-1 and SIV
77 infections (7-14). Additionally, it was recently suggested that ADCC could apply a
78 significant immune pressure on HIV-1 (15), which further supports a role for this effector
79 function *in vivo*. Analysis of the correlates of protection in the RV144 vaccine trial
80 suggested that increased ADCC activity was linked with decreased HIV-1 acquisition
81 (16). Interestingly, Abs with potent ADCC activity were isolated from some RV144
82 vaccinees (17). However, little is known regarding the cellular and viral determinants that
83 govern the susceptibility of HIV-1-infected cells to ADCC killing by Abs normally
84 elicited during the course of HIV-1 infection.

85

We recently reported that HIV-1 envelope (Env) interaction with the CD4 receptor at the surface of infected cells was critical for efficient ADCC activity mediated by monoclonal Abs (mAbs) targeting CD4-induced (CD4i) Env epitopes (18). Our initial findings were recently corroborated by others (19). Importantly, we reported that multiple mAbs with potent ADCC activity isolated from RV144 vaccinees also recognized Env CD4i epitopes in a manner similar to the well-characterized inner domain recognizing A32 Ab (18, 20). Studies by other groups suggested that Abs recognizing variable region 1 and 2 (V1V2) elicited in some RV144 vaccinees could mediate Fc effector functions (21, 22). However, little is known as to the relative contribution of 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 ATCC and NIH AIDS Research and Reference Reagent Program, respectively) were grown at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) containing 10 % fetal bovine serum (Sigma) and 100 µg/ml of penicillin-streptomycin (Wysent). CEM.NKr cells (obtained from Dr. David Evans, Harvard Medical School) were grown at 37 °C and 5 % CO_2 in RPMI-1640 (Invitrogen) containing 10 % fetal bovine serum and 100 µg/ml primocine (Invitrogen).

Ficoll density gradient isolated and cryopreserved human PBMCs from healthy donors were thawed and kept at 37 °C and 5 % CO_2 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 pNL43-ADA-GFP.IRES.Nef proviral vector (23). To generate *env* mutants, the SalI – BamHI fragment of pNL43-ADA-GFP.IRES.Nef was subcloned in a pUC19 intermediate before being subjected to site-directed mutagenesis using the QuickChange II XL protocol (Stratagene). The mutated insert was then cloned back into pNL43-ADA-GFP.IRES.Nef. Mutations in *nef* were introduced by a two-step PCR strategy using primers having 18 nucleotides overlaps and cloned back into the proviral construct using XhoI and NcoI restriction sites. All mutations were confirmed by Sanger DNA sequencing. The codon-

optimized pcDNA3.1-HIV-1_{YU2} ΔV1V2V3V5 expression construct was made by replacing the sequence encoding 124-198 from the V1/V2 loop with a sequence encoding a GG linker and the sequence encoding 302-323 from the V3 loop with a sequence encoding a GGSGSG linker (24). The ΔV5 was made by replacing residues 460-465 by a GSG linker into pcDNA3.1-HIV-1_{YU2} ΔV1V2V3.

Sera from HIV-infected individuals

Informed consent was obtained from all study participants (the Montreal Primary HIV Infection Cohort (25, 26) and the Canadian Cohort of HIV Infected Slow Progressors (27-29)) and research adhered to the ethical guidelines of CRCHUM. Sera was collected during Ficoll isolation of PBMCs and conserved at -80 °C. Sera aliquots were heat-inactivated for 30 min at 56 °C and stored at 4 °C until ready to use in subsequent experiments. A random number generator (GraphPad QuickCalcs) was used to randomly select a number of sera from each cohort.

Purification of recombinant HIV-1 gp120 glycoproteins

FreeStyle 293F cells (Invitrogen) were grown in FreeStyle 293F medium (Invitrogen) to a density of 1×10^6 cells / ml at 37 °C with 8 % CO_2 with regular agitation (125 rpm). Cells were transfected with a pCDNA3.1 plasmid encoding codon-optimized His_6-tagged wild-type or mutant HIV-1 YU2 gp120 using the 293Fectin reagent, as directed by the manufacturer (Invitrogen). One week later, cells were pelleted and discarded. The supernatants were filtered (0.22-µm-pore-size filter) (Corning), and the gp120 glycoproteins were purified by nickel affinity columns, according to

manufacturer instructions (Invitrogen). The gp120 preparations were dialyzed against PBS and stored in aliquots at −80 °C. To assess purity, recombinant proteins were loaded on SDS-PAGE polyacrylamide gels and stained with Coomassie blue.

Cell-based ELISA

Detection of trimeric Env at the surface of HOS cells was performed by cell-based ELISA, as previously described (18, 30, 31). Briefly, HOS cells were seeded in 96-well plates (2 x 10^4 cells per well) and transfected the next day with a cytoplasmic-tail deleted HIV-1 Env_{YU2} variant alone or together with a human CD4 expressor using standard polyethylenimine transfection method. Two days later, transfected cells were washed and then incubated with 1:1000 dilutions of sera from HIV-infected or 1 µg/mL of relevant mAbs. Env-specific IgGs were detected using an HRP-conjugated anti-human IgG-specific secondary Ab (Pierce) with a TriStar LB 941 luminometer (Berthold Technologies).

Viral productions and infections

Vesicular stomatitis virus G (VSVG)-pseudotyped NL4.3 GFP-encoding ADA-based viruses were produced as previously described (18). Briefly, our panel of pNL4.3-GFP-ADA-based HIV-1 proviral vectors and VSVG-encoding plasmid were co-transfected in 293T cells by standard calcium phosphate transfection. Two days after transfection, cell supernatants were harvested, clarified by slow-speed centrifugation (5 min at 1200 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 TCID50 procedure using TZM-bl cells (32). Viruses were then used to infect approximately 20 % to 30 % of CEM.NKr 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.NKr cells were incubated for 20 min at room temperature 48 h post-infection with 1 µg/ml OKT4 (anti-CD4 Ab, 14-0048-82, eBiosciences), 2 µg/ml BST-2 (sc-99191, Santa Cruz), 1 µg/ml 2G12 (AB002, Polymun), 1 µg/ml A32 Ab (kindly given by Dr J. Robinson) or a 1:1000 final concentration of serum from participants 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.

Measurement of serum- and A32-mediated ADCC was performed with a previously described assay (18, 33). Briefly, CEM.NKr infected cells were stained with viability (AquaVivid; Invitrogen) and cellular (cell proliferation dye eFluor670; eBiosciences) markers and used as target cells. PBMC effectors cells, stained with another cellular marker (cell proliferation dye eFluor450; eBiosciences), were then mixed at an effector/target (E/T) ratio of 10:1 in 96-well V-bottom plates (Corning). A 1:1000

final concentration of serum or 5 µg/ml of the A32 mAb was added to appropriate wells. Co-cultures were centrifuged for 1 min at 300 g and incubated at 37 °C for 5-6 h before being fixed in a 2 % PBS-formaldehyde solution containing $5x10^4$/ml flow cytometry particles (AccuCount Blank Particles, 5.3 µm; Spherotech). Samples were analyzed on an LSRII cytometer (BD Biosciences) and acquisition was set to acquire 1000 particles, which allows the calculation of relative cell counts. Data analysis was performed using FlowJo vX.0.7 (Tree Star). The percentage of cytotoxicity was calculated with the following formula: (relative count of GFP^+ cells in Targets plus Effectors) - (relative count of GFP^+ cells in Targets plus Effectors plus A32 or serum) / (relative count of GFP^+ cells in Targets) according to our previously described gating strategy (18, 33).

For serum adsorption and gp120 competition assays, sera dilutions from HIV-1 infected individuals were pre-incubated for 30 min at room temperature with purified soluble D368R gp120 dV1V2V3V5 at a concentration of 83.3pmol / µl of serum. This concentration was determined by assaying the dose-dependent reduction in gp120-coated cells staining (Suppl. Figure 4), as previously reported (33).

Results

Env – CD4 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 indirectly modulate Env – CD4 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 NL4.3-GFP ADA-Env encoding either wild-type (wt) or defective Nef and Vpu accessory proteins as previously described (18). Two days post-infection, infected cells were stained with sera from HIV-infected individuals and then fluorescently labeled (Figure 1). Interestingly, as we previously reported for the ADCC-mediating A32 Ab (18), we observed that a threshold of both CD4 and Env must be reached at the cell surface and, in addition, Env must be able to engage with CD4 in order for Env to be detected by A32 or sera from HIV-1-infected individuals (Figures 1, 2 and supplemental Figure 1 and 2). The majority of the sera recognized more efficiently cells infected with a virus lacking Nef and Vpu than its wild-type counterpart. Cells infected

with viruses lacking Nef and Vpu (i.e., presenting high levels of CD4 and Env at the cell surface, Figure 2A and Suppl. Figure 2) but where the ability of Env to interact with CD4 was dramatically decreased by a CD4-binding site mutation (D368R) (38, 39) were poorly recognized by sera from HIV-1-infected individuals. Of note, decreased recognition of the D368R Env variant by sera from HIV-1-infected individuals was not due to decreased levels of CD4 and/or Env at the surface of HIV-1-*nef*-*vpu*- -infected cells (Suppl. Figure 2).

The impact of Env – CD4 interaction on the binding of sera IgGs from 163 HIV-infected individuals was also evaluated by cell-based ELISA, as previously-described (18, 31, 40). Briefly, HOS cells were transfected with a cytoplasmic-tail deleted HIV-1 Env_{YU2} variant alone or with a human CD4 expressor, as reported (18, 40). Two days later, transfected cells were washed and then incubated with 1:1000 dilutions of sera from HIV-infected individuals. Env-specific IgGs were detected using an anti-human IgG-specific secondary Ab. Interestingly, recognition of the Env trimer by sera from all the clinical categories tested was significantly increased by co-expression of CD4 while the outer-domain recognizing Ab 2G12 was not affected (Suppl. Figure 3), suggesting that sera from HIV-1-infected individuals contain a significant portion of CD4i Abs, as previously proposed (41).

The ability of sera from HIV-infected individuals to mediate ADCC requires Env – CD4 interaction

We previously described that Env – CD4 interaction modulates 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 remains unknown. We therefore 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 above, we measured serum-mediated ADCC with our previously-described FACS-based ADCC assay (18, 33). We thus determined the ability of 48 randomly-chosen sera (comprising those tested in Figure 1B) from HIV-infected individuals to mediate ADCC against infected cells (Figure 3). Strikingly, while cells infected with wild-type (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 (Figure 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 (Figure 3B), suggesting that 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 (Figure 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 on HIV+-sera-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, 42). To investigate whether, aside from gp120 CD4-induced antibodies, epitopes recognized by anti-gp41, variable regions or quaternary-dependent antibodies contributed to sera-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, the variable region 4 (V4) could not be removed without impacting the structural integrity of the protein (data not shown and(43)). Pre-incubation 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 sera Abs (Suppl. Figure 4). 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 Nef-Vpu-infected cells (Figure 4A) and correlated with decreased ADCC activity (Figure 4B), indicating that a recombinant protein lacking the V1V2V3V5 variable regions is

296 sufficient to absorb the majority of ADCC activity present in sera from HIV-1-infected
297 individuals.
298

299 **Discussion**

300

301 Renewed interest on Fc-mediated functions such as ADCC stems in part from
302 correlations in controlling or preventing HIV-1 infection. Fc-mediated effector functions
303 were found to inversely correlate with viral loads or decreased disease progression in
304 SIV-infected macaques (7-9) as well as in HIV-1-infected individuals (10-14).
305 Furthermore, analysis of immune protection correlates in the recent RV144 vaccine trial
306 suggested that high levels of ADCC mediating Abs correlated with decreased HIV-1
307 acquisition when combined with low plasma IgA anti-Env Ab levels (16, 44) thus
308 warranting further studies on the viral determinants modulating ADCC.

309

310 Recent observations suggested that Vpu antagonism of BST2 was sufficient to
311 protect HIV-infected cells from ADCC (45, 46). In this study, we found no significant
312 increase in the susceptibility of cells infected with a virus lacking Vpu to sera-mediated
313 ADCC (Figure 3). However, in agreement with previous work done with CD4i Abs (18),
314 decreasing Env – CD4 interaction by introducing an Env CD4 binding site mutation
315 (D368R) in the context of a virus lacking both Nef and Vpu was sufficient to
316 dramatically diminish killing of infected cells by sera-mediated ADCC (Figure 3C). This
317 is also supported by our soluble gp120 competition assay suggesting that gp120 CD4i
318 Abs represent the major determinant of sera-mediated ADCC in HIV-1-infected
319 individuals (Figure 4) since this recombinant protein could not absorb Abs directed
320 against the gp41, quaternary-dependent Abs or V1V2V3 and V5 variable regions. In this
321 study we only tested sera from HIV-1-infected individuals (not from vaccinees);

322 therefore, we do not exclude the possibility that additional types of ADCC-mediating Abs
323 such as anti-V1V2, elicited through vaccination, could have therapeutic utility and/or
324 help in preventing infection.

325 Furthermore, stratifying the patients sera in clinical disease progression rates
326 (classic, rapid progressors or long-term non-progressors, as described in (25, 26)) did not
327 reveal any significant differences between them as to the requirement of Env – CD4
328 interaction to promote sera-mediated ADCC against infected cells (Suppl. Table I).
329
330 Recent observations suggested that the angle of approach of the Ab is important
331 in order to mediate ADCC (47). Whether anti-gp120 CD4i Abs bind Env with an angle of
332 approach that promotes the recruitment of Fc-bearing effector cells is unclear but
333 warrants further studies.
334
335 Altogether, these data suggest that ADCC-mediating Abs are elicited in the course
336 of natural HIV-1 infection and that a significant proportion of them require Env to
337 interact with CD4 in order to be effective at mediating ADCC. This supports a major role
338 of CD4i Env epitopes in the susceptibility of infected cells to ADCC mediated by sera
339 from HIV-1-infected individuals. Our data suggest that HIV-1, via Nef and Vpu, tightly
340 controls cell-surface levels of CD4 and Env in order to limit the exposure of potential
341 epitopes recognized by ADCC-mediating Abs elicited in the course of natural HIV-1
342 infection. Therefore, targeting Vpu and Nef ability to downregulate CD4 and BST-2 or
343 strategies aimed at modifying Env conformation to expose CD4i epitopes could

potentially render HIV-1-infected cells susceptible to ADCC and thus have therapeutic utility.

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Figure Legends

Figure 1. Env – CD4 interaction is required for efficient recognition of infected cells by sera from HIV-1-infected individuals. CEM.NKr cells infected with a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing 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 post-infection with sera from 30 HIV-infected individuals and then fluorescently labeled with an Alexa-Fluor-647 conjugated anti-human IgG secondary Ab. Shown in (**A**) are histograms depicting representative staining of infected (GFP+) cells by serum from one HIV- and one HIV+ donor, with panel (**B**) presenting the fold increase of staining relative to mock for all tested sera. Data shown are the results of two different experiments and error bars depict the SEM. Statistical significance was tested using paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Figure 2. Env – CD4 interaction modulates the exposure of 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 expressing 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 48h post-infection for surface CD4 levels (**A**) or Env A32 epitope exposure (**B**). Data shown are the results of at least three different

experiments and error bars depict the SEM. Statistical significance was tested using paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Figure 3. Env – CD4 interaction modulates susceptibility of infected cells to ADCC killing mediated by sera from HIV-1-infected individuals. CEM.NKr cells infected with a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing 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 used at 48h post-infection as target cells in our FACS-based ADCC assay (18) to determine their susceptibility to sera from HIV-1-infected individuals to-mediate cell lysis by PBMCs from healthy donors (**A**). Data shown are the results of three different experiments, with median ± interquartile range. (**B**) A positive correlation was observed between the staining intensity of sera from HIV-1-infected individuals on Nef-Vpu- infected cells and their ability to mediate ADCC. Panel (**C**) shows paired values of ADCC mediated by sera from HIV-1-infected individuals to cells infected by HIV-1 viruses lacking both Nef and Vpu and encoding a wt or D368R Env variant. Statistical significance was tested using (**A**) paired one-way ANOVAs or (**B**) paired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Figure 4. A 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.

663 CEM.NKr cells infected with wt or Nef-Vpu- VSV-G pseudotyped NL4.3 GFP ADA
664 were used at 48h post-infection for surface staining (**A**) or FACS-based ADCC assay (**B**)
665 using sera from HIV-1 infected individuals pre-incubated in absence or presence of 83.3
666 pmol ΔV1V2V3V5 D368R / µl sera for 30 min at room temperature. Data shown are
667 representative of at least two different experiments. Statistical significance was tested
668 using paired one-way ANOVAs (** $p<0.01$, ns, not significant).