EBioMedicine 3 (2016) 122–134

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Small CD4 Mimetics Prevent HIV-1 Uninfected Bystander CD4 + T Cell Killing Mediated by Antibody-dependent Cell-mediated Cytotoxicity

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ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form 7 December 2015
Accepted 8 December 2015
Available online 9 December 2015

Keywords:
HIV-1
Envelope glycoproteins
gp120
CD4
CD4-bound conformation
Non-neutralizing antibodies
ADCC
CD4-mimetics
Bystander killing

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) infection causes a progressive depletion of CD4 + T cells. Despite its importance for HIV-1 pathogenesis, the precise mechanisms underlying CD4 + T-cell depletion remain incompletely understood. Here we make the surprising observation that antibody-dependent cell-mediated cytotoxicity (ADCC) mediates the death of uninfected bystander CD4 + T cells in cultures of HIV-1-infected cells. While HIV-1-infected cells are protected from ADCC by the action of the viral Vpu and Nef proteins, uninfected bystander CD4 + T cells bind gp120 shed from productively infected cells and are efficiently recognized by ADCC-mediating antibodies. Thus, gp120 shedding represents a viral mechanism to divert ADCC responses towards uninfected bystander CD4 + T cells. Importantly, CD4-mimetic molecules redirect ADCC responses from uninfected bystander cells to HIV-1-infected cells; therefore, CD4-mimetic compounds might have therapeutic utility in new strategies aimed at specifically eliminating HIV-1-infected cells.

1. Introduction

Human immunodeficiency virus (HIV) infection causes the progressive loss of CD4 + T cells, which leads to acquired immunodeficiency syndrome (AIDS) and death. Despite its clinical importance, the precise mechanism(s) underlying CD4 + T cell depletion during HIV-1 infection remains poorly understood (Thomas, 2009; Grossman et al., 2002; Mccune, 2001). HIV replication is known to directly induce the death of infected CD4 + T cells (Hazenberg et al., 2000; Cummins and Badley, 2014) by a plethora of mechanisms, including envelope glycoprotein-mediated cytotoxicity, apoptosis via activation of Caspases and DNA damage responses (Sodroski et al., 1986; Lifson et al., 1986; Cao et al., 1996; Labonte et al., 2003; Cooper et al., 2013; Sainski et al., 2011). The massive depletion of CD4 + T cells in SHIV-infected monkeys has been shown to depend, at least in part, on the membrane-fusing capacity of the viral envelope glycoproteins (Etemad-Moghadam et al., 2001). In addition, HIV-specific CD8 + cytotoxic T cells also play a major role in eliminating HIV-infected cells (Mcmichael and Rowland-Jones, 2001). However, the mechanisms of uninfected bystander CD4 + T cell death and the contribution of this process to HIV-1 pathogenesis are not well understood. It has been described that uninfected CD4 + T cells die from apoptosis induced by over-expression of several death ligands (Cummins and Badley, 2014), activation-induced cell-death caused by a state of hyper-inflammation (Alimonti et al., 2003), direct cytotoxicity mediated by some HIV proteins including Tat, Vpr, Nef and gp120 (Varbanov et al., 2006), and caspase-1-dependent

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http://dx.doi.org/10.1016/j.ebiom.2015.12.004

pyroptosis driven by abortive infections (Cummins and Badley, 2014; Doitsh et al., 2014; Monroe et al., 2014). In fact, killing of uninfected CD4 + T cells was estimated to account for the high rate of turnover of CD4 + T cells in HIV-infected individuals (Mccune et al., 2000; Mohri et al., 2001; Meyaard et al., 1992; Matrajt et al., 2014). However, no particular mechanism has been identified that could account for this short half-life.

The HIV-1 envelope glycoprotein (Env) trimer is derived from proteolytic cleavage of a trimeric gp160 precursor (Allan et al., 1985; Robey et al., 1985) and is composed of gp120 exterior and gp41 transmembrane subunits. The gp120 exterior subunit is retained on the trimer via labile, non-covalent interactions with the gp41 ectodomain. This results in spontaneous dissociation of gp120 from gp41, known as gp120 shedding (Helseth et al., 1991; Yang et al., 2003; Finzi et al., 2010). Accordingly, significant levels of soluble gp120 were found in blood and tissues of HIV-infected individuals (Santosuosso et al., 2009; Oh et al., 1992; Rychert et al., 2010). Interestingly, it has been suggested that shed gp120 bound to the surface of lymphocytes could serve as an effective target for immune destruction by patient antibodies and effector cells and that this could contribute to lymphocyte destruction in HIV-1-infected individuals (Lyerly et al., 1987). During HIV entry, the gp120 glycoprotein is responsible for interactions with the initial receptor, CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). CD4 induces conformational changes in Env that expose the binding site for the chemokine co-receptors (CCR5 or CXCR4) (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). We recently reported that gp120–CD4 interaction in an infected cell also results in exposure of antibody-dependent cell-mediated cytotoxicity (ADCC) epitopes recognized by antibodies present in sera, cervicovaginal fluids and breast milk from HIV-infected individuals. Interestingly, recognition of gp120–CD4 complexes at the surface of infected (Richard et al., 2015; Veillette et al., 2014b, 2015a) or gp120-coated (Batraville et al., 2014; Richard et al., 2014) cells results in ADCC-mediated killing.

Here we investigated whether antibody recognition of shed gp120 bound to the surface CD4 of bystander cells results in ADCC-mediated killing. To this end, we developed a FACS-based assay to measure the relative killing of infected CD4 + T cells versus their uninfected counterparts by ADCC-mediating antibodies and HIV + sera. We observed that HIV-1-infected cells were protected from ADCC, whereas bystander cells were highly sensitive to killing mediated by CD4-induced (CD4i) antibodies and HIV + sera. This difference in susceptibility to ADCC was due to differences in Env conformation in the two contexts. In infected cells, trimeric Env remained in its unbound conformation (i.e., shielding ADCC-mediating epitopes) due to the action of the HIV-1 Vpu and Nef proteins, which limit the surface expression of Env–CD4 complexes (Richard et al., 2015; Veillette et al., 2014b, 2015a, 2016). By contrast, in bystander CD4 + T cells, shed gp120 was bound to CD4 on the cell surface, resulting in a more open Env conformation that exposed ADCC-mediating epitopes. This raises the intriguing possibility that gp120 shedding may represent a viral mechanism to divert cytotoxic immune responses to uninfected cells. Importantly, we demonstrate here that small CD4-mimetic compounds (CD4mc) protect bystander cells by blocking gp120 binding to uninfected CD4 + T cells, while sensitizing HIV-infected cells to ADCC-mediated killing.

2. Materials and Methods

2.1. Cell Lines and Isolation of Primary Cells

293T human embryonic kidney (obtained from ATCC), CEM.NKr cells (obtained from Dr. David Evans, Harvard Medical School) and primary cells were grown as previously described (Richard et al., 2010; Veillette et al., 2014b). CD4 T lymphocytes were purified from resting PBMCs by negative selection and activated as previously described (Richard et al., 2015).

2.2. Viral Production, Infections, Ex Vivo Amplification and Detection of Infected Cells

Vesicular stomatitis viruses G (VSVG)-pseudotyped viruses allowed equivalent levels of infection for the different viruses used, including the D368R Env variant, and were produced and titrated as previously described (Veillette et al., 2015a). Viruses were then used to infect CEM.NKr cells or primary CD4 + T cells from healthy donors by spin infection at 800 g for 1 h in 96-well plates at 25 °C. In order to expand endogenously-infected CD4 + T cells, primary CD4 + T cells were isolated from PBMCs obtained from viremic HIV-1-infected individuals. Purified CD4 + T cells were activated with PHA-L at 10 μg/ml for 36 h and then cultured for 6 to 8 days in RPMI-1640 complete medium supplemented with rIL-2 (100 U/ml).

Detection of GFP + or p24 + infected cells was performed as previously described (Richard et al., 2015). Cells infected with the SHIV-CH505 virus were stained intracellularly for SIVmac p27 using the Cytofix/Cytoperm Fixation/Permeabilization Kit, followed by a 2F12 anti-p27 mAb primary staining (10 μg/ml) and a goat anti-mouse Alexa Fluor 647 secondary antibody staining (1:250 dilution). The percentage of infected cells (GFP +, p24 + or p27 + cells) was determined by gating the living cell population based on the viability dye staining (Aqua Vivid, Invitrogen).

2.3. Antibodies and Sera

The following Abs were used as first Ab for cell-surface staining: 1 μg/ml mouse anti-CD4 mAb OKT4 (14-0048-82; eBiosciences), 5 μg/ml human anti-HIV-1 Env mAbs 2G12 (AB002; Polymun), PGT-151 (kindly provided by P. Poignard, The Scripps Research Institute, La Jolla, CA), A32, C11, 7B2 (kindly provided by J. Robinson, Tulane University, New Orleans), F240, M785-U1 and N10-U1 (kindly provided by G. K. Lewis, University of Maryland), whereas 1 μg/ml either goat anti-mouse Alexa Fluor-594, goat anti-human Alexa Fluor 647 or goat anti-human Alexa Fluor 594 mAbs (Invitrogen) was used as a secondary Ab, and AquaVivid (Invitrogen) was used as a viability dye. The anti-SIVmac p27 antibody was purified from SIVmac p27 Hybridoma (55-2F12, NIH AIDS Reagent) (Higgins et al., 1992) using Protein A-Sepharose beads (GE, USA) according to the manufacturer's protocol.

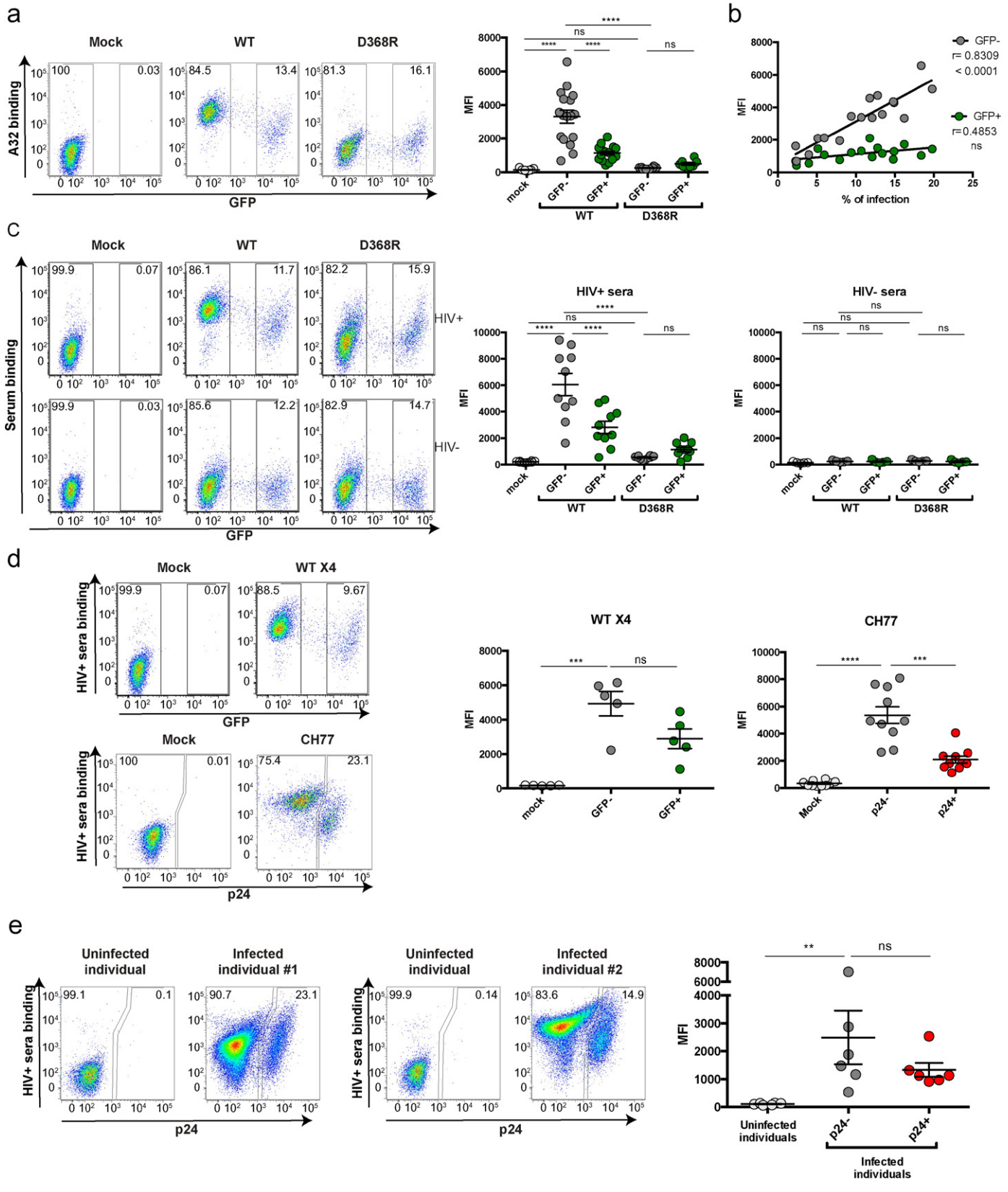
Sera from HIV-infected and healthy donors were collected, heat-inactivated and conserved as previously described. Written informed consent was obtained from all study participants [the Montreal Primary HIV Infection Cohort (Fontaine et al., 2009, 2011) and the Canadian Cohort of HIV Infected Slow Progressors (Peretz et al., 2007; Kamya et al., 2011; International et al., 2010)], and research adhered to the ethical guidelines of CRCHUM and was reviewed and approved by the CRCHUM institutional review board (ethics committee). All sera 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 for each experiment.

2.4. Plasmids and Site-directed Mutagenesis

pNL43-ADA(Env)-GFP.IRES.Nef proviral vectors (*wt* or expressing the D368R Env variant) and the VSV G-encoding plasmid (pSVCMV-IN-VSV-G) were previously described (Veillette et al., 2015a). The pNL43-GFP.IRES.Nef provirus encoding the NL43 X4-tropic Env was generated by inserting the NL43 Env into pNL43-ADA(Env)-GFP.IRES.Nef using SalI and BamHI restrictions sites, as previously described (Veillette et al., 2014b). The plasmid encoding the HIV-1 transmitted founder (T/F) IMC CH77 was previously described (Ochsenbauer et al., 2012; Bar et al., 2012; Parrish et al., 2013; Fenton-May et al., 2013; Richard et al., 2015).

2.5. Flow Cytometry Analysis of Cell-surface Staining and ADCC Responses

Cell-surface staining was performed as previously described (Richard et al., 2015; Veillette et al., 2015a). Binding of HIV-1-infected cells by either sera (1:1000 dilution) or mAbs (5 μg/ml) was performed 48–72 h after in vitro infection or at 6–8 days post activation for endogenously-infected ex-vivo-amplified cells, at 37 °C. Samples were analyzed on a LSRII cytometer (BD Biosciences, Mississauga, ON, Canada) and data analysis was performed using FlowJo vX.0.7 (Tree Star, Ashland, OR, USA).



Measurement of serum-mediated ADCC was performed with a previously described assay (Veillette et al., 2014b; Richard et al., 2014) after 48–72 h of in vitro infection using a 1:1000 final concentration of serum or 5 μg/ml mAbs and an Effector: Target ratio of 10:1 (1×10^6 PBMC:1×10^5 CEM.NKr cells). The percentage of cytotoxicity was calculated with the following formula for either infected or uninfected cells populations: (relative cell counts in Targets plus Effectors) − (relative cell counts in Targets plus Effectors plus A32 or serum) / (relative cell counts in Targets), as described (Richard et al., 2014, 2015; Veillette et al., 2015a).

2.6. Co-culture Assay

Activated primary CD4 T cells were stained with the eFluor-450 cell marker (1:500 dilution, eBiosciences) for 15 min at room temperature and washed twice with complete RPMI-1640 media before being co-cultured with autologous infected cells (in vitro infection for 72 h) at a ratio of 1 fresh cell to 2 infected cells in the presence or absence of 10 μg/ml VRC01 Fab or 50 μM of the CD4-mimetic compound JP-III-48 (Fig. S1) (Richard et al., 2015). Co-cultures were performed with or without transwells (3 μm pore size, Corning, NY, USA), infected cells were loaded in the upper chamber of the transwell and the uninfected cells were placed in the bottom chamber. For every time point of the co-culture assays, transwells were carefully removed and discarded where applicable and aliquots of the co-cultured cells were distributed in 96-well V-bottom plates and surface stained as described above.

2.7. Statistical Analyses

Statistics were analyzed using GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA). Every data set was tested for statistical normality and this information was used to apply the appropriate (parametric or nonparametric) statistical test. p Values < 0.05 were considered significant; significance values are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3. Results

3.1. ADCC-mediating Antibodies Recognize Uninfected Bystander Cells

Env interaction with the CD4 receptor at the surface of infected cells is critical for efficient ADCC activity mediated by monoclonal antibodies targeting CD4i Env epitopes (Veillette et al., 2014b) or by sera from HIV-1-infected individuals (Richard et al., 2015; Veillette et al., 2015a). HIV-1 has evolved a sophisticated mechanism to avoid ADCC by decreasing the overall amount of cell-surface Env via Vpu-mediated BST-2 downregulation (Veillette et al., 2014b; Arias et al., 2014; Alvarez et al., 2014) and Nef and Vpu-mediated CD4 downregulation from the cell surface (Veillette et al., 2014b, 2015a). To determine whether uninfected CD4 + T cells could be recognized by these antibodies, we infected primary CD4 + T cells with a previously-reported wild-type (wt) HIV-1 strain that encodes all accessory proteins as well as a *gfp* reporter gene and an R5-tropic (ADA) envelope (Veillette et al., 2014b). Thus, in this system, GFP + cells are HIV-1-infected. Seventy-two hours post-infection, cells were incubated with A32, a well-known ADCC-mediating CD4i antibody that recognizes an epitope located on the gp120 inner domain Layers 1 and 2. The A32 epitope is occluded in the unbound Env trimer but becomes exposed upon CD4 binding (Veillette et al., 2014b). As previously reported, mock-infected cells were not recognized by A32. The GFP + cells infected with wild-type HIV-1 were recognized inefficiently by A32; previous studies showed that this poor recognition is due to efficient down-modulation of CD4, which permits Env to remain in its unbound conformation (Veillette et al., 2014b). In contrast, uninfected (GFP −) CD4 + T cells were efficiently recognized by the A32 antibody (Fig. 1A). We hypothesized that the exposure of CD4i epitopes on the GFP − cells was due to the binding of shed gp120 to cell-surface CD4. To test this possibility, we infected cells with an Env variant that contained an alteration in the CD4 binding site (D368R) that abrogates CD4 binding (Richard et al., 2014; Veillette et al., 2014a, 2015a). Interestingly, for the D368R variant, neither infected (GFP +) nor uninfected (GFP −) cells were recognized by A32 (Fig. 1A). These data support a model in which gp120 shed from the infected cell binds uninfected CD4 + T cells, resulting in gp120 conformational changes that expose CD4i epitopes, including those recognized by A32. Importantly, 2G12, an antibody that binds the outer domain of the gp120 and is not affected by gp120–CD4 interaction (Veillette et al., 2014b), did recognize D368R-infected (GFP +) cells, indicating that the lack of recognition observed with A32 was not due to decreased expression of the Env D368R variant (Fig. S2). As expected, we observed a strong positive correlation between the levels of infection and recognition of uninfected bystander (GFP −) CD4 + T cells by A32, (Fig. 1B), suggesting that bystander cell recognition by A32 depends on the amount of productive infection.

We next evaluated sera from HIV-1-infected and uninfected individuals (Supplemental Table 1). Similar to the results obtained with the A32 antibody, more uninfected cells (GFP −) were recognized by HIV + sera than infected (GFP +) cells (Fig. 1C, upper panels). Sera from uninfected individuals did not recognize infected (GFP +) or uninfected (GFP −) cells (Fig. 1C, lower panels). To evaluate whether this phenotype was related to the viral strain used, we infected cells with a transmitted/founder HIV-1 (CH77) as well as an X4-tropic virus (Figs. 1D and S3). In both cases, uninfected bystander cells were efficiently recognized by HIV + sera (Fig. 1D). To rule out any contribution of the method used to infect the cells, we purified primary CD4 + T cells from six viremic HIV-1-infected individuals. Cells were activated with PHA and then cultured in the presence of rIL-2. Six to eight days after activation, viral replication was measured by intracellular p24 staining and Env detected at the surface of p24 + cells with autologous sera. Of note, infected (p24 +) cells presented significantly lower amounts of CD4 at their surface compared to uninfected bystander (p24 −) cells, suggesting that these individuals have viruses coding for functional Nef and Vpu proteins (Fig. S4). Under these conditions, recognition of uninfected (p24 −) cells isolated from HIV + donors was significantly higher than cells from an uninfected individual (Fig. 1E). We note that in some patients, uninfected bystander (p24 −) cells were better recognized than infected (p24 +) cells; however, this was not always the case. This variation could be due to the concentration of CD4-induced antibodies present in the sera. Of note, we obtained similar results using a SHIV infectious molecular clone (Fig. S5). Thus, independently

Fig. 1. ADCC-mediating antibodies and sera from HIV-1-infected individuals efficiently recognize uninfected bystander CD4 + T cells. Cell-surface staining of primary CD4 + T cells either mock-infected or infected with NL4-3 GFP ADA based virus, either wild-type (wt) or expressing D368R Env with (a,b) A32 mAbs or (c) sera from 10 HIV-1-infected individuals and sera from 5 uninfected individuals. Shown in (a,c) are (left) dot blots depicting representative staining and (right) mean fluorescence intensities (MFI) obtained for multiple stainings. (b) Correlation between the levels of infection and recognition of uninfected bystander (GFP −) (depicted in gray) and infected (GFP +) CD4 + T cells (depicted in green) by the A32 mAb. (d) Cell-surface staining of primary CD4 + T cells infected with an X4-tropic virus (NL4-3 GFP) and a primary HIV-1 isolate (CH77) with sera from 10 HIV-1-infected individuals. Shown in (left) are dot blots depicting representative staining and (right) MFI for all tested sera. (e) Cell-surface staining of primary CD4 + T cells isolated from 6 HIV-1-infected individuals after activation with autologous sera. Shown in (left) and (center) are dot blots depicting representative staining obtained for two different HIV-1-infected individuals, and (right) Median Fluorescence Intensity (MFI) values obtained with all individuals. Error bars indicate the mean ± SEM. Statistical significance was tested using (a,c) an ordinary one-way ANOVA test with a Holm–Sidak post-test, (d) an unpaired *t* test, (e) a Kruskal–Wallis with a Dunn's post-test and (b) a Spearman rank correlation ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns: not significant).

of the viral strain or tropism, uninfected CD4 T cells present in an HIV-1 culture are efficiently recognized by ADCC-mediating Abs.

3.2. Shed gp120 is the Major Source of Env at the Surface of Bystander CD4 + T Cells

Theoretically, in addition to shed soluble gp120, gp120 detected at the surface of bystander cells could result from the attachment of defective viral particles or from abortive infections. To evaluate the relative contribution of viral particles compared to shed gp120, we infected primary CD4+ T cells and determined Env recognition on infected (GFP+) and uninfected (GFP−) cells with a panel of anti-Env (anti-gp120 and anti-gp41) antibodies. Unlike gp120, the gp41 transmembrane glycoprotein cannot be shed; therefore, gp41 detection on uninfected bystander CD4T+ cells could result from the attachment of viral particles. As shown in Fig. 2, CD4i Abs A32 and C11 (an antibody that binds to the gp120 β-sandwich and N- and C-termini) (Robinson et al., 1992; Moore et al., 1994) recognized bystander (GFP−) significantly better than infected (GFP+) cells whereas the CD4-independent 2G12 antibody recognized both cell populations equivalently. This is consistent with a model in which the lack of CD4 at the cell surface of infected cells allows Env to remain in its unbound conformation; by contrast, in uninfected bystander cells, which have higher levels of CD4 (Fig. S4), gp120 samples the CD4-bound conformation, exposing CD4i epitopes. Interestingly, we observed that a panel of anti-gp41 antibodies or antibodies targeting the interface between the gp120 and gp41 (PGT151) recognized infected cells more efficiently than bystander cells (Figs. 2 and S6). These results suggest that the majority of Env detected on bystander cells originates from shed gp120.

3.3. Productively Infected Cells are the Source of gp120 Found on Uninfected Bystander CD4 + T Cells

Following Env detection on uninfected bystander cells over time, we observed an increased recognition by anti-gp120 Abs over the course of the infection, which could be explained by the increased percentage of infected cells; recognition by anti-gp41 antibodies minimally increased over the same time period (Fig. 3A and B). These results are consistent with our conclusion that the recognition of bystander cells by anti-gp120 antibodies results from soluble gp120 originating from productively infected cells. To confirm this, we designed a co-culture system that allowed the distinction of productively-infected cells from uninfected cells. Briefly, primary CD4+ T cells were infected with our GFP-reporter wild-type virus. The cell proliferation dye eFluor-450 was

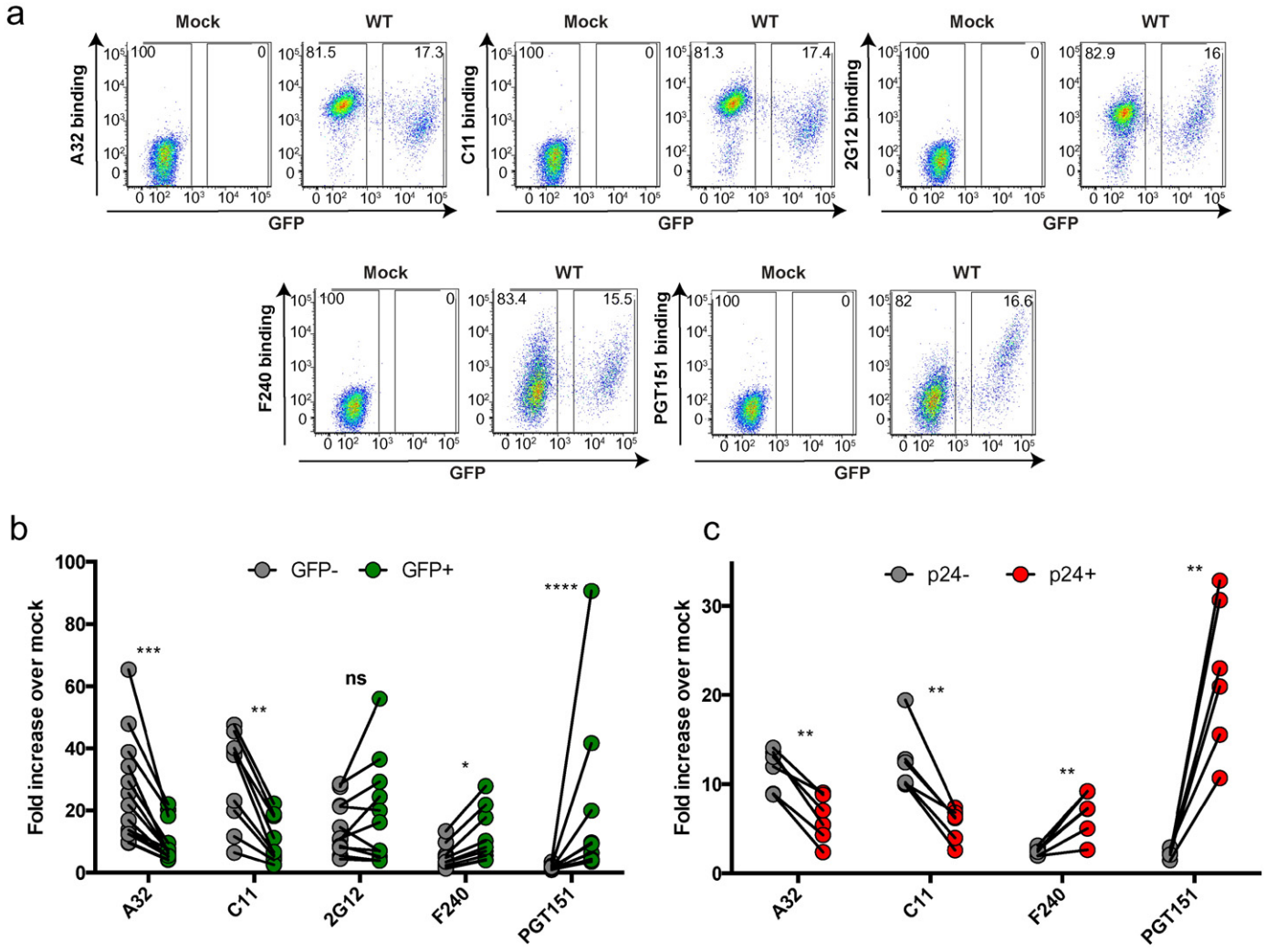


Fig. 2. Shed gp120 represents the major source of Env present on the surface of uninfected bystander cells. (a,b) Cell-surface staining of primary CD4+ T cells infected with the NL4-3 GFP ADA wt virus with anti-Env Abs (A32, C11, 2G12, F240 and PGT151). Shown in (a) are dot blots depicting a representative staining obtained 72 h post-infection. (b) Quantification of data presented in A as fold binding over mock. (c) Quantification of cell-surface staining of primary CD4+ T cells infected with CH77 with anti-Env Abs (A32, C11, F240 and PGT151), as fold binding over mock. Statistical significance was tested using the Mann–Whitney *t* test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant).

used to stain uninfected autologous CD4 + T cells that were then added to the infected cells (eFluor-450 − cells). The detection of autologous uninfected bystander CD4 + T cells, designated as eFluor-450 + GFP − cells (Fig. 3C, top left squares), by anti-Env Abs was then evaluated by FACS (Fig. 3C and 3D). Similar to the results in Fig. 2, uninfected bystander cells were recognized very efficiently by anti-gp120 Abs but not by anti-gp41 Abs, confirming that the major source of bound gp120 originated from shed gp120 and not from viral particles (Fig. 3). As expected, no recognition of uninfected bystander cells was observed for cells co-cultured with mock-infected cells. In this assay, bystander eFluor-450+ cells were not directly infected; gp120 was detected on these cells only after a 24-h co-culture with productively infected cells. These results support a model in which productively-infected cells are the main source of shed gp120 that becomes bound to bystander CD4 + T cells.

3.4. Cell-to-cell Contact Does not Contribute to gp120 Attachment to Bystander CD4 + T Cells

HIV-1 spreads not only by cell-free virus infection but also by cell-to-cell transmission through the virological synapse (Jolly et al., 2004; Jolly and Sattentau, 2004). However, it is not known whether this mechanism is also involved in gp120 acquisition by bystander CD4 + T cells. To explore the contribution of cell-to-cell transmission to bystander gp120 coating, we added a transwell component to the co-culture system described above. Primary CD4 + T cells were infected with wild-type GFP-reporter virus and then added to the top chamber and co-cultured with uninfected CD4 + T cells pre-stained with eFluor-450 for 48 h. This system minimizes cell-to-cell contact and therefore any gp120 detected in eFluor-450-stained bystander cells must result from shed gp120. As shown in Fig. 4, the use of transwell chambers decreased infection and replication of HIV-1 in eFluor-450-stained autologous primary CD4 + T cells. This was expected since under these co-culture conditions, virological synapse formation and thus transmission between infected and eFluor-450-stained uninfected cells is significantly reduced; (Jolly et al., 2004; Jolly and Sattentau, 2004). However, gp120 coating of eFluor-450-stained bystander cells was unaffected (Fig. 4), indicating that cell-to-cell contact and virological synapse formation is not necessary for gp120 coating of uninfected bystander cells.

3.5. Recognition of gp120 on the Surface of Bystander CD4 + T Cells Results in ADCC-mediated Killing

To evaluate whether recognition of uninfected bystander CD4 + T cells by ADCC-mediating antibodies and sera from HIV-1-infected individuals translated into elimination by ADCC, CEM.NKr cells infected with NL4-3 ADA GFP wt virus were used as target cells, while PBMCs from healthy HIV-negative individuals were used as effector cells. As reported for primary CD4 + T cells, uninfected CEM.NKr cells were efficiently recognized by A32 mAbs and HIV + sera in the context of R5-tropic, X4-tropic or primary HIV-1 isolate viruses (Fig. S6 and data not shown). The total number of ADCC-mediated killing (either infected (GFP +) or uninfected bystander (GFP −)) was measured by a FACS-based assay that uses a fixed number of flow-cytometry particles to normalize cell counts (Richard et al., 2014). Consistent with their enhanced recognition by ADCC-mediating antibodies and sera from HIV-1-infected individuals, uninfected (GFP −) bystander CD4 + T cells were significantly more sensitive to ADCC killing mediated by both A32 or HIV + sera than infected (GFP +) cells or mock-infected cells (Fig. 5). Analysis of the absolute number of cells being killed by ADCC indicates that uninfected bystander (GFP −) cells accounted for more than 83% (in the case of A32) and 93% (for HIV + sera) of the cells killed (Fig. S7). As expected, pre-incubation of target cells with an A32 Fab fragment protected uninfected bystander cells from ADCC-mediated killing mediated by the A32 Abs (Fig. 5C). Altogether, these results suggest that, in this system, uninfected bystander CD4 + T cells are eliminated by ADCC more efficiently than HIV-1-infected CD4 + T cells.

3.6. CD4mc Block Recognition of Bystander CD4 + T Cells by ADCC-mediating Antibodies

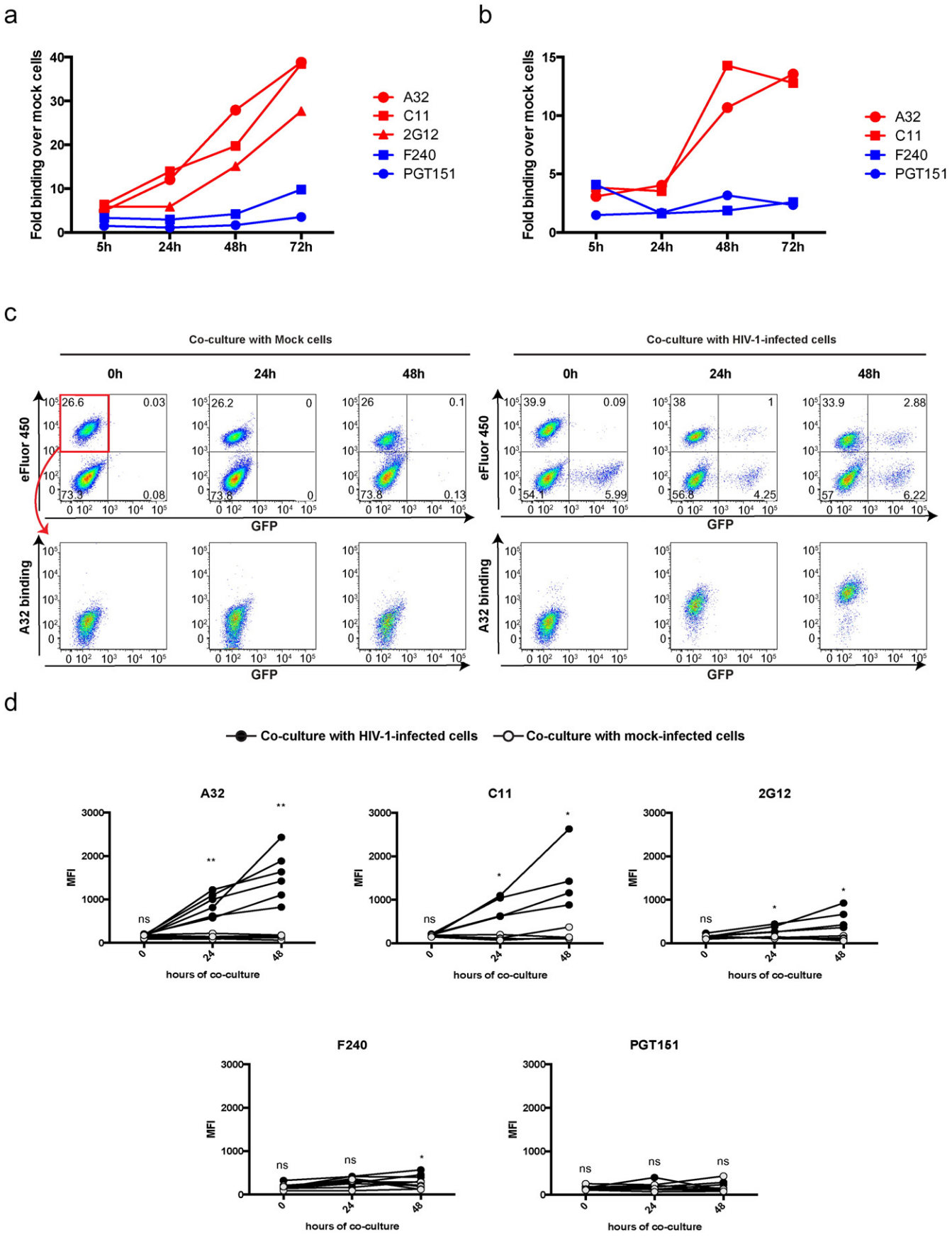
Since soluble gp120 binds uninfected bystander CD4 + T cells through CD4 interaction (Fig. 1A and C), we asked whether ligands targeting the gp120 CD4-binding site could decrease gp120 interaction with bystander cells. To this end, a Fab fragment of a very potent and broadly-neutralizing CD4-binding site antibody (VRC01) (Wu et al., 2010; Zhou et al., 2010) or a small CD4-mimetic compound (CD4mc), JP-III-48 (Fig. S1), was added at the time of co-culture between infected and eFluor-450-stained bystander cells. JP-III-48 belongs to the CD4mc family, the prototype of which, NBD-556, was found in a screen for molecules able to block gp120–CD4 binding (Zhao et al., 2005); JP-III-48 has been previously shown to sensitize HIV-1-infected cells to ADCC (Richard et al., 2015). Interestingly, both ligands that interact near the CD4-binding site of gp120 were able to drastically reduce gp120 interaction with uninfected bystander CD4 + T cells by HIV + sera and A32 (Figs. 6A–B and S8). However, only JP-III-48 was able to expose CD4i epitopes recognized by HIV + sera on infected cells (Fig. 6C), in agreement with recent findings (Richard et al., 2015). Differences in this regard between VRC01 Fab and JP-III-48 are likely due to the fact that VRC01 does not induce Env conformational changes that result in the exposure of Env CD4i epitopes (Zhou et al., 2010), whereas JP-III-48 does (Madani et al., 2014; Richard et al., 2015). Therefore, small CD4mc such as JP-III-48, while blocking gp120 coating and recognition of bystander CD4 + T cells by ADCC-mediating antibodies, expose HIV-1-infected cells to ADCC-mediating killing.

4. Discussion

HIV-1 infection causes a massive depletion of CD4 + T cells. This decrease results from direct viral replication and subsequent death of infected cells but also from indirect mechanisms that result in the killing of uninfected, bystander, CD4 + T cells (Cummins and Badley, 2014; Mccune, 2001; Mccune et al., 2000). This was not only observed in HIV-infected humans but also in SHIV-infected macaques (Matrajt et al., 2014).

Here we investigated whether ADCC contributes to bystander CD4 + T cell killing in a cell culture model. We observed that while HIV-1-infected cells are only inefficiently detected by ADCC-mediating antibodies and HIV + sera, uninfected bystander CD4 + T cells are highly recognized by these antibodies; importantly, this translates into ADCC-mediated killing. The differences in antibody recognition result from distinct Env conformations, which are exposed depending on the ability of HIV-1 to remove CD4 from the cell surface. In the absence of CD4 on the surface of infected cells, Env remains in its unbound conformation and does not present epitopes recognized by CD4i ADCC-mediating antibodies (Veillette et al., 2014b). However, when shed gp120 binds the CD4 receptor on the surface of bystander CD4 + T cells, the bound glycoprotein samples the CD4-bound conformation and exposes ADCC-mediating epitopes. This is independent from viral coreceptor usage and was observed using a variety of HIV-1 variants including one X4, one R5, one transmitted/founder virus and a SHIV infectious molecular clone. The latter observation indicates that SHIV-infected macaques could represent an animal model to study ADCC-mediated bystander CD4 + T cell depletion in vivo (Matrajt et al., 2014). Altogether, our data suggest the possibility that recognition of bystander CD4 + T cells by ADCC-mediating antibodies is a conserved mechanism used by HIV-1 to divert ADCC responses to uninfected cells, resulting in the protection of HIV-1-infected cells from ADCC and other Fc-mediated effector functions (Veillette et al., 2015b).

Several studies have previously shown that in-vitro-coated gp120–CD4 + T cells are susceptible to ADCC killing (Richard et al., 2014;


a
Fold binding over mock cells
40
30
20
10
0
5h
24h
48h
72h
A32
C11
2G12
F240
PGT151
b
Fold binding over mock cells
15
10
5
0
5h
24h
48h
72h
A32
C11
F240
PGT151
c
Co-culture with Mock cells
Co-culture with HIV-1-infected cells
0h
24h
48h
eFluor 450
GFP
A32 binding
26.6
0.03
73.3
0.08
26.2
0
73.8
0
26
0.1
73.8
0.13
39.9
0.09
54.1
5.99
38
1
56.8
4.25
33.9
2.88
57
6.22
d
Co-culture with HIV-1-infected cells
Co-culture with mock-infected cells
A32
C11
2G12
F240
PGT151
MFI
hours of co-culture
ns
*
**

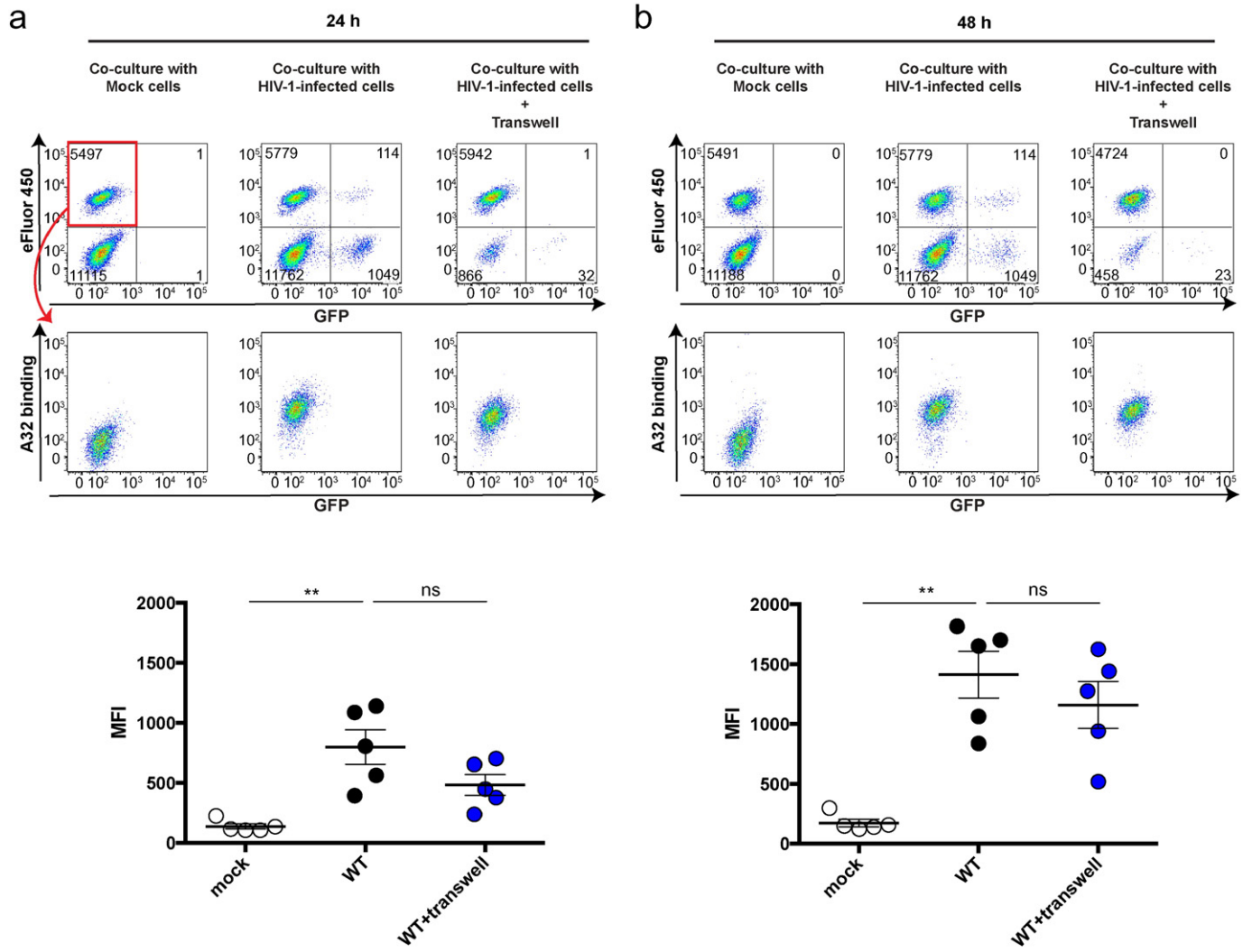


Fig. 4. Cell-to-cell transmission is not required for the presence of gp120 on the surface of bystander cells. Uninfected cells, stained with the cellular dye eFluor 450, were co-cultured with unstained autologous mock-infected cells or with HIV-1-infected cells, either in the context or not of a transwell system. The ability of the anti-Env Ab A32 to recognize uninfected bystander cells, designated as eFluor-450 + GFP − cells, was evaluated by FACS after (a) 24 h and (b) 48 h of co-culture. Shown in the top panels are the gating strategy and a representative staining obtained with the A32 Ab. Shown in the bottom panels are the quantification of the results obtained with 5 donors. Error bars indicate the mean ± SEM. Statistical significance was tested using a Kruskal–Wallis with a Dunn's post-test (**$p < 0.01$, ns: not significant).

Batraville et al., 2014; Baum et al., 1996; Bonsignori et al., 2012; Ferrari et al., 1994, 2011; Gooneratne et al., 2015; Hober et al., 1995; Lambotte et al., 2009, 2013; Lin et al., 1998). However, in the majority of these studies, the amount of gp120 used to coat target CD4+ T cells was well above the maximum amount of gp120 found at the surface of infected cells (approximately 50 ng of gp120/million CEM-NKr cells) (Richard et al., 2014) and the estimated quantities of soluble gp120 present in blood from HIV-1-infected individuals (Rychert et al., 2010; Santosuosso et al., 2009). Here we did not use recombinant gp120; instead, we infected cells and showed that the quantity of gp120 naturally shed from these cells is sufficient to coat bystander cells. Under these conditions, the amount of gp120 present on the surface of bystander cells was sufficient to sensitize them to ADCC enabled by ADCC-mediating antibodies and sera from HIV-1-infected but not from uninfected individuals. Importantly, we analyzed the source of the gp120 present on the surface of bystander cells using a panel of anti-Env antibodies (both anti-gp120 and anti-gp41 antibodies). This analysis suggested that the majority of gp120 bound on the surface of bystander cells resulted from gp120 shedding from productively HIV-1-infected cells rather than attached virions. Of note, cell-to-cell contact between productively-HIV-1-infected cells and bystander cells did not appear to contribute to gp120 coating on bystander cells, reinforcing the notion that shedding is the main mechanism contributing to the attachment of gp120 to the surface of bystander CD4+ T cells.

How might ADCC contribute to CD4+ T-cell destruction in vivo, particularly in light of differences in the tissue distribution of CD4+ T cells

Fig. 3. Shed gp120 from productively infected cells is the major source of gp120 present on the surface of uninfected bystander CD4+ T cells. Detection of uninfected bystander primary CD4+ T cells infected with (a) NL4-3 GFP ADA wt virus or (b) CH77, by anti-gp120 Abs (A32, C11, 2G12; depicted in red) and anti-gp41 (F240) and anti-gp120-gp41 Abs (PGT151; depicted in blue) over the course of the infection. The data presented are representative of the results obtained in cells from at least two donors. (c,d) Uninfected cells, stained with the cellular dye eFluor 450, were co-cultured with unstained autologous mock-infected or HIV-1-infected cells. The ability of anti-Env Abs (A32, C11, 2G12, F240 and PGT151) to recognize uninfected bystander cells, designated as eFluor-450 + GFP − cells, was evaluated by FACS at 0, 24 and 48 h of co-culture. Shown in (c) are the gating strategy and a representative staining obtained with the A32 Ab. Shown in (d) are the quantification of the results obtained with at least 4 donors with all tested anti-Env Abs. Statistical significance was tested using the Mann–Whitney *t* test (*$p < 0.05$, **$p < 0.01$, ns: not significant).

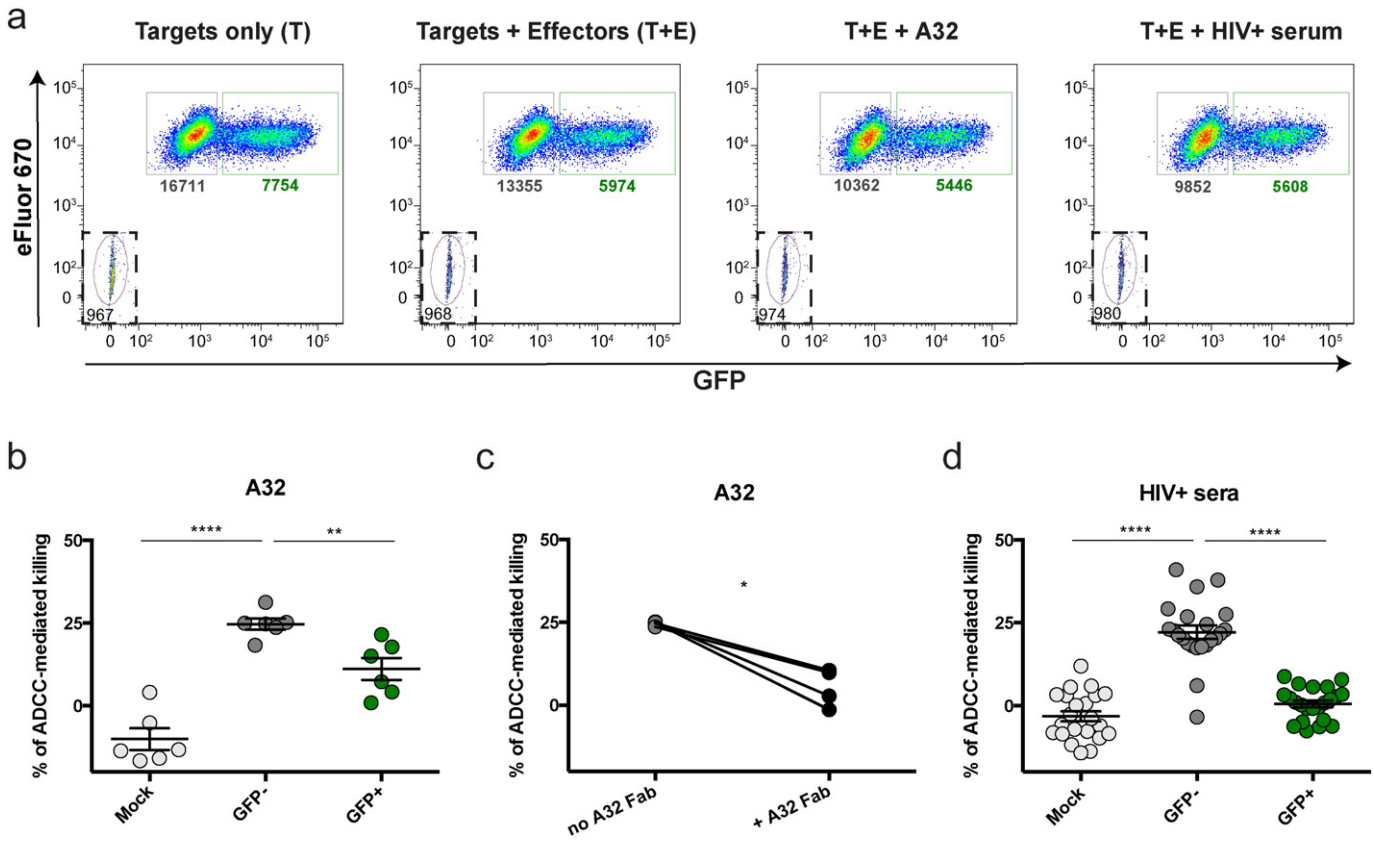


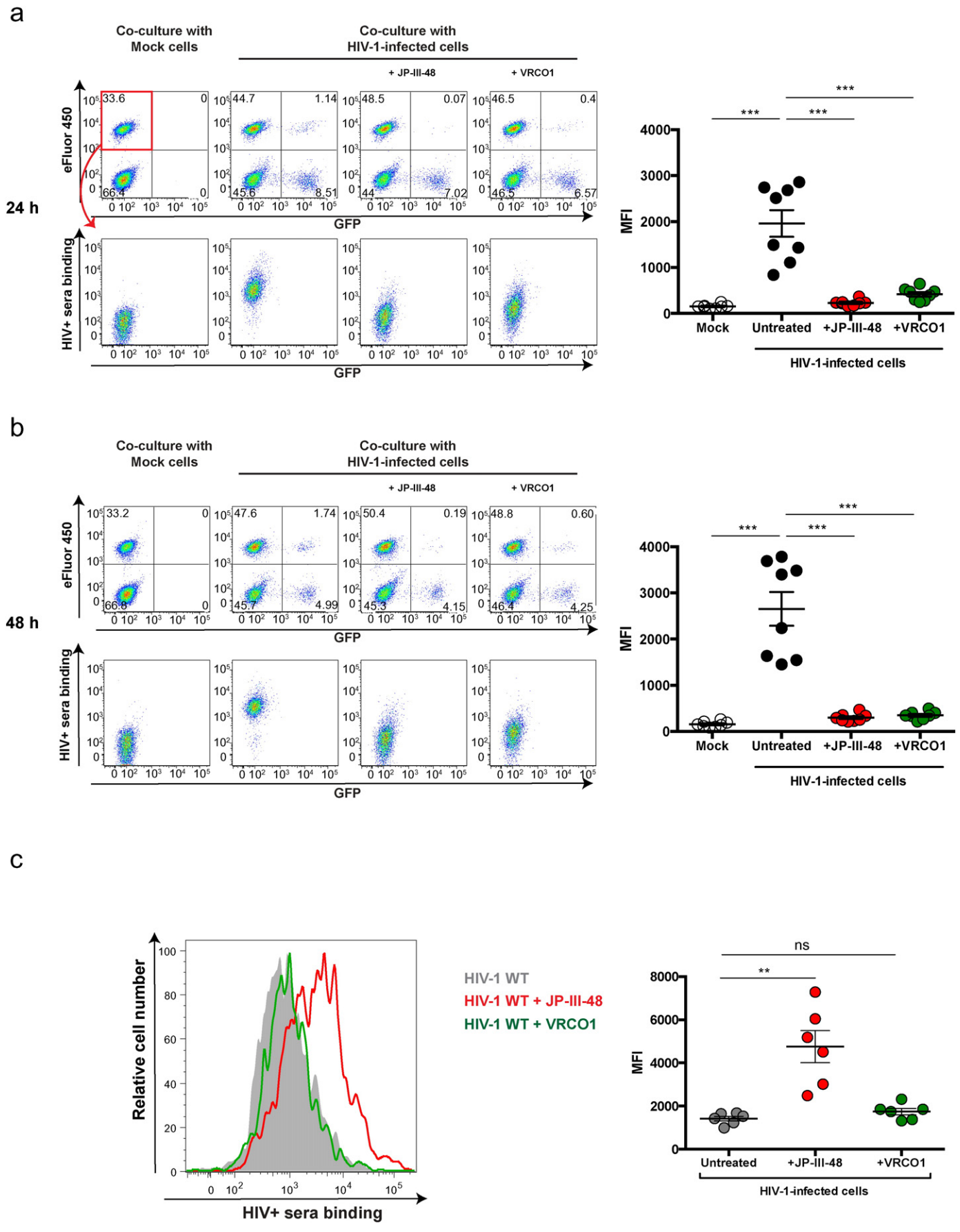
Fig. 5. Uninfected bystander cells can be eliminated by ADCC. CEM.NKr cells infected with the NL4-3 ADA GFP wt virus were used as target cells in our FACS-based ADCC assays. Shown in (a) are dot blots depicting the cell count and flow cytometry particle count (bottom left) of a representative ADCC killing experiment with A32 mAb and serum from an HIV-1-infected individual. (b) Percentage of ADCC killing obtained with the A32 mAb in 6 independent experiments. (c) Percentage of ADCC-mediated killing of uninfected bystander (GFP−) cells with the A32 mAb in the presence or absence of the A32 Fab fragment. (d) Percentage of ADCC-mediated killing obtained with sera from 22 HIV-1-infected individuals. Error bars indicate the mean ± SEM. Statistical significance was tested using (b,d) an ordinary one-way ANOVA test with a Holm–Sidak post-test or (c) a Mann–Whitney *t* test (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

and natural killer (NK) cells, the mediators of ADCC? HIV-1 replication primarily happens in lymphoid tissues, where up to 95% of CD4 + T cells reside (Pantaleo et al., 1994), yet only a small fraction of ADCC-competent $CD56^{dim}CD16+$ NK cell population is distributed within lymph nodes (Luteijn et al., 2011). We hypothesize that NK cells contribute to bystander killing during the transit of CD4 + T cells between lymph nodes, the lymphatic system and the blood, where $CD56^{dim}CD16+$ NK cells are present in large quantities and could mediate efficient ADCC against gp120-coated cells (Artis and Spits, 2015; Fauci et al., 2005). Supporting this hypothesis, an inverse correlation between the percentage of resting CD4 + T cells presenting gp120 at their surface and the number of CD4 + T cells in the blood was recently reported (Suzuki et al., 2014). Moreover, an association between T cell dysfunction and the presence of IgG at the surface of CD4 + T cells in infected individuals was previously reported (Daniel et al., 1996).

In addition to providing a potential mechanism of in vivo CD4 T cell bystander killing, our results also have practical implications. The fact that uninfected bystander cells coated with HIV-1 gp120 expose CD4i epitopes and thus are highly susceptible to ADCC can influence in vitro measurement of ADCC responses. Indeed, in vitro ADCC assays that rely on measurements made on the total cell population or on NK cell activation cannot distinguish ADCC responses targeting HIV-infected cells versus ADCC directed against bystander cells. This could result in an overestimation of the ADCC-mediating activity of CD4i Abs such as A32, which targets gp120-coated bystander cells more efficiently than infected cells.

In addition to its role in sensitizing bystander CD4 + T cells to ADCC, the presence of gp120 at the surface of these cells could also alter different immune functions. The presence of soluble gp120 in infected individuals has been shown to be associated with higher levels of IL-6, IL-10, and TNF-α; gp120 binding to CD4 + T cells modulates the expression of several cytokines, which may contribute to the reduced immune function of CD4 + T cells observed in HIV-1-infected individuals (Guo et al., 2012; Rychert et al., 2010). Moreover, the binding of shed gp120

Fig. 6. CD4-binding site ligands abrogate recognition of bystander CD4 + T cells. Uninfected cells, stained with the cellular dye eFluor 450, were co-cultured with unstained autologous mock-infected cells or with HIV-1-infected cells in the absence or presence of the CD4-mimetic JP-III-48 or VRC01 Fab fragment. The ability of HIV + sera to recognize uninfected bystander cells, designed as eFluor-450 + GFP − cells, was evaluated by FACS after (a) 24 h and (b) 48 h of co-culture. Shown in the top panels are the gating strategy and a representative staining. Shown in the bottom panels are the quantification of the results obtained with 8 HIV + sera using 4 different CD4 + T-cell donors. (c) Staining of primary CD4 T cells infected with the NL4-3 ADA GFP wt virus with sera from HIV-1-infected individuals, in presence of the CD4mc JP-III-48 or VRC01 Fab fragment. Shown on the left are histograms depicting representative staining. Shown in the right panel is the quantification of the results obtained with 6 different HIV + sera. Error bars indicate the mean ± SEM. Statistical significance was tested using a Mann–Whitney *t* test (**$p < 0.01$, ***$p < 0.001$, ns: not significant).


a
Co-culture with Mock cells
Co-culture with HIV-1-infected cells
+ JP-III-48
+ VRCO1
24 h
eFluor 450
GFP
HIV+ sera binding
33.6
0
66.4
0
44.7
1.14
45.6
8.51
48.5
0.07
44
7.02
46.5
0.4
46.5
6.57
MFI
***
Mock
Untreated
+JP-III-48
+VRCO1
HIV-1-infected cells
b
48 h
33.2
0
66.8
0
47.6
1.74
45.7
4.99
50.4
0.19
45.3
4.15
48.8
0.60
46.4
4.25
c
Relative cell number
HIV+ sera binding
HIV-1 WT
HIV-1 WT + JP-III-48
HIV-1 WT + VRCO1
ns
**

to CD4 on uninfected bystander cells and the subsequent presentation of CD4i epitopes likely also generates CD4-induced Abs, which are commonly found in HIV-1 infected individuals. For example, analysis of the frequency of B cells producing anti-Env Abs in HIV-1 acutely infected subjects revealed that >47% of cells produced CD4i Abs (Robinson et al., 2005; Decker et al., 2005). Our results showing ADCC-mediated killing of bystander cells by CD4i antibodies raise the intriguing possibility that in untreated HIV-1-infected individuals, the concentration of these antibodies might influence the rate of CD4 T-cell depletion and disease progression. It is also possible that the presence of other ADCC-mediating antibodies that do not require the CD4-bound conformation of Env to recognize infected cells might help control viral replication and transmission (Milligan et al., 2015; Baum et al., 1996; Forthal et al., 1999; Ljunggren et al., 1990; Mabuka et al., 2012). Finally, it has been shown that NK cells from HIV-infected individuals exhibit an altered phenotype, subset distribution and effector functions (Fauci et al., 2005). It is thus conceivable that recognition of shed gp120 on uninfected bystander cells could contribute to the sustained activation of NK cells and the subsequent perturbation of NK-cell functions. In that context, recent findings suggested that sustained stimulation of NK cells by anti-HIV or anti-CD16 Abs has the potential to impair NK cell function, via attenuation of NKp46-dependent signals (Parsons et al., 2014).

We recently reported that, in addition to their direct antiviral effect (Madani et al., 2008) and sensitization of HIV-1 viral particles to neutralization (Madani et al., 2014), small CD4mc such as JP-III-48 sensitize HIV-1-infected cells to ADCC (Richard et al., 2015). Here we extend the antiviral properties of these compounds to protecting uninfected bystander CD4+ T cells from gp120 attachment and recognition by HIV+ sera and ADCC-mediating Abs. Although antibodies directed at the CD4-binding site could also decrease attachment of gp120 to bystander cells, they do not increase recognition of HIV-1-infected cells by HIV+ sera and therefore do not focus ADCC responses on HIV-1-infected cells like CD4mc. As CD4+ T cells are essential for the immunologic control of HIV, any therapy decreasing the rapid turnover of bystander cells could help preserve CD4+ T cells and therefore enable additional immunological mechanisms and/or curative approaches aimed at eradicating HIV. Therefore, by protecting bystander CD4+ T cells from killing while sensitizing HIV-1-infected cells to ADCC, CD4mc might prove to have therapeutic utility in approaches aimed at specifically eliminating HIV-1-infected cells.

Conflicts of Interest

The authors have no conflicts of interest to report.

Author Contributions

J.R., M.V. and A.F. conceived and designed the experiments; J.R., M.V. S.D, D.Z. and N.A. performed the experiments; J.R., M.V. S.D, D.Z. N.B., M.C., J.S., G.M.S, B.H.H, J.P., J.R.C., B.M., A.B.S, D.E.K and A.F. contributed unique reagents and analyzed the data. J.R., M.V., J.S. and A.F. wrote the paper.

Acknowledgments

We thank Elizabeth Carpelan for the help with manuscript preparation. The authors thank the CRCHUM Flow Cytometry Platform for the technical assistance and Mario Legault for cohort coordination and clinical samples. This work was supported by a Canada Foundation for Innovation Program Leader grant #29866, by CIHR operating grants #119334 and #134117, by a FRQS Establishment of Young Scientist grant #26702 to A.F. and by the FRQS AIDS and Infectious Diseases Network #5227. A.F. is the recipient of a Canada Research Chair on Retroviral Entry #950-229930. M.V. was supported by a CIHR Doctoral Research Award #291485 and J.R. is the recipient of a CIHR Fellowship Award #135349. D.E.K. is supported by a Research Scholar Career Award of the Quebec Health Research Fund (FRQS) #25131. This study was also supported by NIH AI100645, UM AI100663 Center for HIV/AIDS Vaccine Immunology and Immunogen Design (CHAVI-ID), by the National Institutes of Health grant #GM56550 and the late William F. McCarty-Cooper. Our funding sources had no role in data collection, analysis or interpretation, and were not involved in the writing of this manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.12.004.

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