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A B S T R A C T
Human immunodeficiency virus type 1 (HIV-1) has evolved a sophisticated strategy to conceal conserved epitopes of its envelope glycoproteins (Env) recognized by antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies. These antibodies, which are present in the sera of most HIV-1-infected individuals, preferentially recognize Env in its CD4-bound conformation. Accordingly, recent studies showed that small CD4-mimetics (CD4mc) able to “push” Env into this conformation sensitize HIV-1-infected cells to ADCC mediated by HIV + sera. Here we test whether CD4mc also expose epitopes recognized by anti-cluster A monoclonal antibodies such as A32, thought to be responsible for the majority of ADCC activity present in HIV + sera and linked to decreased HIV-1 transmission in the RV144 trial. We made the surprising observation that CD4mc are unable to enhance recognition of HIV-1-infected cells by this family of antibodies in the absence of antibodies such as 17b, which binds a highly conserved CD4-induced epitope overlapping the co-receptor binding site (CoRBS). Our results indicate that CD4mc initially open the trimeric Env enough to allow the binding of CoRBS antibodies but not anti-cluster A antibodies. CoRBS antibody binding further opens the trimeric Env, allowing anti-cluster A antibody interaction and sensitization of infected cells to ADCC. Therefore, ADCC responses mediated by cluster A antibodies in HIV-positive sera involve a sequential opening of the Env trimer on the surface of HIV-1-infected cells. The understanding of the conformational changes required to expose these vulnerable Env epitopes might be important in the design of new strategies aimed at fighting HIV-1.

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1. Introduction

Several lines of evidence support a role for antibody-dependent cellular cytotoxicity (ADCC) in controlling human immunodeficiency virus infection.
type 1 (HIV-1) infection and replication (Alpert et al., 2012; Banks et al., 2002; Baum et al., 1996; Chung et al., 2011; Forthal et al., 1999; Mabuka et al., 2012; Sun et al., 2011; Williams et al., 2015). Analysis of the correlates of protection in the RV144 vaccine trial suggested that decreased HIV-1 acquisition was linked to increased ADCC activity in protected vaccinees (Haynes et al., 2012).

Accordingly, potent ADCC-mediating antibodies (Abs) targeting anti-cluster A epitopes were isolated from some RV144 vaccinees (Bonfigli et al., 2012) and were shown to preferentially recognize the HIV-1 envelope glycoproteins (Env) in their CD4-bound conformation (Veillette et al., 2014b). The CD4-bound Env conformation is also recognized by non-neutralizing CD4-induced (CD4i) ADCC-mediating antibodies present in sera (Veillette et al., 2015; Richard et al., 2015; Richard et al., 2016; Williams et al., 2015), breast milk (Richard et al., 2015) and cervicovaginal lavages (Batraville et al., 2014; Richard et al., 2015) of HIV-1-infected individuals. These antibodies represent a significant portion of the anti-Env Abs elicited during natural HIV-1 infection (Veillette et al., 2015; Decker et al., 2005). However, to limit the exposure of CD4-bound Env on the surface of infected cells, HIV-1 evolved sophisticated mechanisms to efficiently internalize Env (Von Bredow et al., 2015), to counteract the host restriction factor BST-2 with the viral Vpu protein (Arias et al., 2014; Alvarez et al., 2014; Veillette et al., 2014b), and to downregulate CD4 by Nef and Vpu (Veillette et al., 2015; Veillette et al., 2014b). The requirement to evade ADCC provides one explanation for why the vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, which limit the exposure of CD4 Env epitopes on the surface of infected cells.

In agreement with the requirement for HIV-1 to avoid exposing the CD4-bound conformation of Env, we recently showed that forcing Env to adopt this conformation with small CD4-mimetics (CD4mc) sensitizes HIV-1-infected cells to ADCC mediated by non-neutralizing Abs present in sera, breast-milk and cervicovaginal fluids from HIV-1-infected subjects (Richard et al., 2015). These non-neutralizing ADCC-mediating Abs target gp120 epitopes that overlap the epitope recognized by the anti-cluster A A32 Ab (Ferrari et al., 2011; Guan et al., 2013; Arias et al., 2014; Alvarez et al., 2014; Veillette et al., 2014b), and to downregulate CD4 by Nef and Vpu (Veillette et al., 2015; Veillette et al., 2014b). The requirement to evade ADCC provides one explanation for why the vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, which limit the exposure of CD4 Env epitopes on the surface of infected cells.

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

Vesicular stomatitis viruses G (VSVG)-pseudotyped viruses were produced and titrated as described (Veillette et al., 2015). Viruses were 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 7 days in RPMI-1640 complete medium supplemented with rIL-2 (100 U/ml) (obtained from the NIH AIDS Reagent Program, Cat# 136).

2.3. CD4-mimetics

The mini-protein M48U1 was produced and purified as previously described (Martin et al., 2003). The CD4-mimetic small molecules JP-Ill-48 and BNM-III-170 were synthesized as described (Melillo et al., 2016). The compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, aliquoted, and stored at −20 °C. Each compound was then diluted to 50–100 μM in PBS for cell-surface staining or in RPMI-1640 complete medium for ADCC assays.

2.4. Antibodies and Sera

The anti-cluster A mAbs A32, C11 and N5-i5 were conjugated with Alexa-Flour 647 probe (Thermo Fisher Scientific, Cat# A20186) as per the manufacturer instructions and used for cell-surface staining of HIV-1-infected primary CD4+ T cells at 0.35 μg/ml. The following anti-Env mAbs were used in combination of anti-cluster A mAbs during cell-surface staining: 48d, 412d, 19b (kindly provided by Dr. J. Robinson), VRC01, b12 (NIAID Reagent, Cat# 12033 and 2640), CH58 (kindly provided by Dr. B. Haynes), C2 and the Fab fragments, Fab’ fragments or full 17b and N12-i2 mAbs. Goat anti-human Alexa Fluor-647 mAbs (Thermo Fisher Scientific, Cat# A-21445 RRID:AB_2535862) were used as secondary Abs for sera binding and AquaVivid (Thermo Fisher Scientific, Cat# L43957) as a viability dye.

Sera from HIV-infected (Supplemental Table 1) 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., 2011; Fontaine et al., 2009) 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). Research adhered to the standards indicated by the Declaration of Helsinki. All sera were heat-inactivated for 30 min at 56 °C and stored at 4 °C until ready to use in subsequent experiments. A random number generator (GraphPad, QuickCalcs) was used to randomly select a number of sera for each experiment.

2.5. Plasmids

pNL4-3-ADA(Env)-GFP.IRES.Nef proviral vector was previously described (Veillette et al., 2015). The plasmid encoding the HIV-1 transmitted founder (T/F) IMC CH58 was previously described (Ochsner et al., 2012; Bar et al., 2012; Parrish et al., 2013; Fenton-May et al., 2013; Richard et al., 2015).

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

Cell-surface staining was performed as previously described and MFI histograms shows signal on live infected populations (Richard et al., 2015; Veillette et al., 2015). Binding of HIV-1-infected cells by HIV + sera (1:1000 dilution) or Alexa-Flour 647-conjugated anti-cluster A Abs A32, C11 and N5-i5 was performed 48 h after in vitro infection or 7 days post-activation for endogenously-infected (clade B) ex vivo-amplified cells, in presence of CD4mc JP-Ill-48 (50 μM), BNM-III-170 (50 μM), M48U1 (100 nM) or with equivalent volume of vehicle (DMSO). Binding of HIV-1-infected cells with Alexa-Flour 647-conjugated anti-cluster A Abs A32, C11 and N5-i5 was performed alone or in combination with different mAbs or Fab fragments (5 μg/ml), or in combination with HIV+ or HIV- sera (1:1000 dilution), with or without Fab fragments of 17b or N12-i2 (5 μg/ml). Detection of GFP+ or p24+ infected cells was performed as described (Richard et al., 2015). The percentage of infected cells (GFP+ or p24+ cells) was determined by gating the living cell population based on the viability dye staining (Aqua Vivid, Thermo Fisher Scientific, Cat# L43957). 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 ADCC-mediated killing was performed with a previously described assay (Richard et al., 2015). Briefly, primary CD4+ T cells infected for 48 h with the CH58 T/F virus were incubated with autologous PBMC (Effector: Target ratio of 10:1) in presence of A32 (0.3125, 0.625, 1.25 or 2.5 μg/ml, kindly provided by Dr. G. Ferrari) and 17b (5 μg/ml) or 17b Fab fragments alone or in combination, or with HIV + sera (1:1000), in presence of CD4mc JP-III-48, BNM-III-170 or with equivalent volume of vehicle (DMSO). The percentage of cytoxicity was calculated as described (Richard et al., 2015).

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. CD4mc Fail to Enhance Recognition of HIV-1-infected Cells by ADCC-mediating Anti-cluster A Abs

We recently reported that small CD4mc enhance the recognition of HIV-1-infected cells by autologous and heterologous sera in both in vitro and ex vivo experiments (Richard et al., 2015; Lee et al., 2015). Accordingly, primary CD4+ T cells infected with a pHLA.3 full-length HIV-1 molecular clone coding for the primary ADA Env and a GFP reporter gene (NL4.3 ADA GFP) were better recognized by HIV + sera after the addition of rationally-designed small CD4mc compounds (JP-III-48, BNM-III-170) (Melillo et al., 2016) or CD4-mimetic peptides (M48U1) (VanHerrewege et al., 2008) (Fig. 1A). These molecules engage gp120 within the Nhex43 cavity (Madani et al., 2008; Melillo et al., 2016) and can act as CD4 agonists, inducing thermodynamic changes in the Env trimer similar to those observed upon membrane CD4 binding (Schon et al., 2006; Lalonde et al., 2012). As previously reported (Ding et al., 2016; Richard et al., 2015; Veillette et al., 2015), Env present on the surface of infected cells with a wild-type (wt) virus is poorly recognized by HIV-1 + sera (Fig. 1A, C). This is due to efficient CD4 downregulation by the virus; Env cannot engage with CD4 and therefore remains in the unbound conformation, preventing CD4 epitope exposure (Veillette et al., 2015). Axially conjugated Alexa-Fluor 647 Fab (Ferrari et al., 2011), the Fab fragment from the 17b antibody, which marks the CD4 epitope, was not recognized by HIV-1-infected cells by these antibodies in the presence of all three CD4mc (Fig. 1A, Supplemental Fig. 1). Importantly, similar results were observed with the unmodified clinically-relevant primary transmitted/founder (T/F) virus CH58 (CH58 T/F) and endogenously-infected ex-vivo-amplified primary CD4 + T cells from HIV-1-infected individuals (Fig. 2), indicating that the phenomenon is not restricted to laboratory-adapted viruses. These results suggest that some antibodies present in the sera of HIV-1-infected individuals facilitate the exposure of anti-cluster A epitopes upon CD4mc addition.

3.3. CoRBS Abs Allow Recognition of HIV-1-infected Cells by Anti-cluster A Abs

In the same report describing the ADCC blocking capacity of the A32 Fab (Ferrari et al., 2011), the Fab fragment from the 17b antibody, which belongs to the co-receptor binding site (CoRBS) family of anti-Env antibodies (Wyatt et al., 1995), was able to block the ADCC activity in approximately 60% of the HIV + sera tested. In some cases, the blocking effect was comparable to that obtained with the A32 Fab (Ferrari et al., 2011). To explore the possibility that CoRBS antibodies present in sera from HIV-1-infected individuals facilitate Env recognition by anti-cluster A antibodies in the presence of CD4mc, we added the Fab fragments of 17b or another CoRBS antibody, N5-i5 (Guan et al., 2013), to 10 HIV + sera. In all the HIV + sera tested, addition of CoRBS Fab fragments decreased the recognition of cells infected with CH58 T/F viruses (Fig. 3 A–B) and endogenously-infected ex-vivo-amplified primary CD4 + T cells from three HIV-1-infected individuals (Fig. 3D) by Alexa-Fluor 647-conjugated A32 Abs (A32-AF647). These observations suggest that CoRBS antibodies facilitate the access of anti-cluster A antibodies to trimeric Env in the presence of CD4mc.
To test this conclusion directly, we co-incubated primary CD4+ T cells infected with NL4.3 ADA GFP or CH58 T/F viruses in the presence or absence of CD4mc with the full 17b antibody and evaluated A32-AF647 and C11-AF647 recognition of infected cells. Incubation with the 17b antibody was sufficient to allow recognition of HIV-1-infected cells by these two anti-cluster A antibodies (Fig. 4A–C). Importantly, while this was recapitulated by the 17b Fab'2 fragment, the 17b Fab fragment was unable to do so (Fig. 4D). This was reiterated with a
different CoRBS (N12-i2) antibody and Fab fragments (Supplemental Fig. 2), indicating that the bivalent recognition of Env by CoRBS antibodies may be required to facilitate anti-cluster A antibody binding in the presence of CD4mc. Of note, similar to the CD4mc JP-III-48, soluble CD4 (sCD4) alone was unable to facilitate A32 recognition of CH58 T/F-infected primary CD4+ T cells (Supplemental Fig. 3A).
Interestingly, the 17b CoRBS antibody allowed recognition of CH58 T/F-infected primary CD4+ T cells by the A32 antibody in the presence of JP-III-48, but not in the presence of sCD4 (Supplemental Fig. 3B). This is in agreement with our previous observations indicating that CD4mc but not sCD4 sensitize primary CD4+ T cells infected with primary HIV-1 viruses to ADCC (Richard et al., 2015).
To extend these observations to additional CoRBS antibodies, we evaluated the ability of well-established CoRBS antibodies (48d, 412d) or a newly-isolated CoRBS antibody from a clade-B HIV-1-infected individual (C2, Supplemental Fig. 4) to allow A32-AF647 or C11-AF647 binding. All tested CoRBS antibodies facilitated A32-AF647 and C11-AF647 binding upon CD4mc addition (Fig. 4 A–C). We also evaluated whether this activity was shared by antibodies with different Env specificities. We tested anti-V3 (19b), anti-V2 (CH58) and anti-CD4 binding
site (VRC01, b12) antibodies. These antibodies did not facilitate A32 recognition of infected cells in the presence of CD4mc (Fig. 4 A–B). These observations suggest that antibodies that recognize CD4-induced epitopes near the CoRBS specifically enhance the recognition of the HIV-1 Env trimer by anti-cluster A antibodies in the presence of CD4mc. It remains possible that antibodies with different Env specificities, present in HIV+ sera, might also contribute to the exposure of epitopes recognized by anti-cluster A antibodies.

3.4. CoRBS Abs Are Required for CD4mc to Sensitize Infected Cells to ADCC Mediated by Anti-cluster A Abs

To evaluate whether the enhanced recognition of HIV-1-infected cells by anti-cluster A antibodies in presence of CD4mc and CoRBS mAbs resulted in ADCC killing, we infected primary CD4+ T cells with CH58 T/F virus and evaluated their susceptibility to ADCC mediated by autologous PBMCs using a previously-described FACS-based assay (Richard et al., 2014; Richard et al., 2015). As expected, A32 and 17b alone or in combination were unable to mediate ADCC (Fig. 5). Recognition of HIV-1-infected cells by the CoRBS 17b antibody upon CD4mc addition (Supplemental Fig. 5) did not translate into enhanced ADCC killing (Fig. 5). This is in agreement with recent reports indicating that CoRBS antibodies do not mediate efficient ADCC, despite good recognition of target cells when Env samples the CD4-bound conformation (Ding et al., 2016; Guan et al., 2013). However, when the anti-cluster A A32 and CoRBS 17b antibodies were added, at different concentrations, in conjunction with CD4mc JP-III-48 or BNM-III-170, we observed a marked increase in the killing of HIV-1-infected cells (Fig. 5 and Supplemental Fig. 6), reminiscent of the CD4mc sensitization of infected cells to ADCC mediated by HIV+ sera (Richard et al., 2015) (Fig. 5B). Importantly, this response was only observed when the full 17b antibody but not its Fab fragment was added in combination with A32 in the presence of CD4mc (Fig. 5B). Thus, CD4mc and CoRBS antibodies participate in the formation of a suitable target for ADCC responses mediated by anti-cluster A antibodies.

4. Discussion

HIV-1 has evolved several mechanisms to avoid the robust humoral response elicited against its envelope glycoproteins, including sequence-variable loops, extensive glycosylation, and conformational masking of vulnerable epitopes (Kwong et al., 2002; Wyatt et al., 1998; Wyatt and Sodroski, 1998). While the majority of the antibodies elicited during natural infection are strain-specific neutralizing or non-neutralizing antibodies (reviewed in Kwong and Mascola, 2012) and thought to play only a minimal role in controlling viral replication, recent studies showed that these antibodies can exert a constant selection pressure through their weakly neutralizing activities and thus drive viral evolution (Moody et al., 2015). In addition to their non- or weakly-neutralizing activities, these antibodies possess Fc-mediated effector functions with the potential to eliminate HIV-1-infected cells through several immune responses, including ADCC. The analysis of the correlates of protection in the RV144 vaccine trial suggesting that decreased HIV-1 acquisition was linked to increased ADCC activity in protected vaccinees (Haynes et al., 2012) spurred a renewed interest in these Fc-mediated functions (Chung et al., 2015; Ackerman et al., 2016; Williams et al., 2015; Veillette et al., 2014b; Ding et al., 2016; Richard et al., 2015; Veillette et al., 2015; Richard et al., 2016; Ferrari et al., 2011; Von Bredow et al., 2016). These ADCC-mediating antibodies appear to preferentially target Env in its CD4-bound conformation (Ding et al., 2016; Veillette et al., 2015). In other words, these antibodies primarily recognize Env epitopes exposed upon interaction with the viral receptor CD4. To avoid exposing these epitopes at the surface of infected cells, HIV-1 limits the amount of Env and CD4 present at the cell surface (reviewed in Veillette et al., 2016).

Small CD4mc with the capacity to "push" Env to the CD4-bound conformation were recently shown to enhance the recognition of HIV-1-infected cells by sera, cervicovaginal lavages and breast milk from HIV-1-infected individuals (Richard et al., 2015). However, the specificity of the antibodies mediating this response in these complex biological fluids was not known. Previous reports indicated that the ADCC activity present in HIV+ sera is mediated by anti-cluster A antibodies (Guan et al., 2013; Ferrari et al., 2011; Ding et al., 2016). Accordingly, competition experiments using a gp120 inner domain recombinant protein exposing only A32-like epitopes showed the importance of A32-like antibodies in ADCC responses mediated by HIV+ sera (Tolbert et al., 2016). We therefore evaluated whether CD4mc were able to sensitize HIV-1-infected cells to ADCC by this family of antibodies. Surprisingly, all CD4mc tested (M48U1, JP-III-48, BNM-III-170) failed to enhance recognition of HIV-1 Env present on the surface of infected cells by anti-cluster A antibodies. Interestingly, we found that HIV+ sera enabled recognition of Env by anti-cluster A antibodies in the presence of CD4mc. Importantly, this depended on the presence of CoRBS.
antibodies. Indeed, 17b and N12-12 Fab fragments were able to negate the effect of HIV + sera on Env recognition by these antibodies. Moreover, several CoRBS antibodies but not anti-V2, anti-V3 or anti-CD4bs antibodies were able to recapitulate the effect of HIV + plasma in the presence of CD4mc; and the CoRBS antibodies facilitated ADCC-mediated killing of HIV-1-infected cells by the anti-cluster A family of antibodies. Interestingly, the Fab 2 but not Fab fragments of CoRBS antibodies were able to recapitulate the effect of full antibodies, indicating that the bivalent recognition of Env by these antibodies is required to facilitate anti-cluster A antibody binding in the presence of CD4mc.

The unexpected requirement for CoRBS antibodies to allow Env recognition by anti-cluster A antibodies in the presence of CD4mc suggests a model for the sequential opening of trimeric Env. In this model (Fig. 6), CD4mc engagement in the Phe43 cavity induces a partial opening of trimeric Env, which is sufficient to expose the CoRBS but not enough to expose inner domain cluster-A epitopes. Interaction of CoRBS antibodies with two gp120 subunits within this CD4mc-sensitized trimer further opens the trimeric Env and exposes highly-conserved epitopes recognized by anti-cluster A antibodies. It is unclear why a bivalent recognition of Env by CoRBS is required to expose anti-cluster A antibodies in the presence of CD4mc. This could be related to a potential asymmetric transition of Env upon CD4mc engagement towards downstream conformational changes, as suggested for CD4 binding (Personal communication, Ma, Mothes, Munro). While in theory CD4mc should induce conformational changes in all EnvVs with an available Phe 43 cavity (the majority of HIV-1 strains), the results presented here have been generated using clade B HIV-1 strains only. How these findings extend to additional HIV-1 strains remains to be evaluated.

We previously reported that HIV-1 avoids exposing Env epitopes of anti-cluster A antibodies through CD4 downregulation by Nef and Vpu accessory proteins (Veillette et al., 2014b; Ding et al., 2016; Veillette et al., 2015; Alsahafi et al., 2016). Our findings herein indicate that several conformational transitions are required to expose these epitopes and suggest that trimeric HIV-1 Env has evolved to minimize their exposure. This represents another example of conformational masking of vulnerable epitopes and helps to explain why the A32 antibody failed to provide sterilizing protection in non-human primates (Santra et al., 2015). Why has HIV-1 evolved multiple mechanisms to avoid anti-cluster A epitope exposure? This highly-conserved region contributes to Env stability (Finzi et al., 2010), so its buried nature may be critical for preserving the global architecture of the HIV-1 Env trimer. This architecture has evolved to minimize the exposure of this and other elements that represent suitable targets for ADCC.

Altogether, our data highlights the difficulty of exposing anti-cluster A epitopes at the surface of infected cells but also indicates that sera from HIV-1-infected individuals have all the components required to do so, provided that the initial opening of the trimer by CD4mc occurs. While it is unknown at the moment whether CD4mc will ultimately do so, provided that the initial opening of the trimer by CD4mc occurs.

Epitopes might expedite the design and application of new strategies aimed at fighting HIV-1.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.09.004.

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Conflicts of Interest
The authors have no conflicts of interest to report.

Author Contributions

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