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Rôle de la conformation des glycoprotéines de l'enveloppe du VIH-1 dans la réponse cytotoxique cellulaire dépendante des anticorps et impact des protéines virales Nef et Vpu

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Rôle de la conformation des glycoprotéines de l'enveloppe du VIH-1 dans la réponse cytotoxique cellulaire dépendante des anticorps et impact des protéines virales Nef et Vpu

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RÉSUMÉ

Alors que d'énormes efforts sont mis de l'avant pour mettre en place des stratégies thérapeutiques contre l'infection au VIH-1, il est nécessaire de mieux cerner les déterminants viraux qui aideront à l'efficacité de celles-ci. En ce sens, une volumineuse littérature scientifique suggère que les anticorps contre le VIH-1 possédant une capacité à induire une réponse effectrice dépendante de leur portion Fc puissent jouer un rôle important dans la prévention de l'infection et dans la progression de la maladie. Cependant, peu d'information est disponible concernant les déterminants reconnus par ces anticorps et comment le virus s'en protège. Le but des travaux présentés dans cette thèse est donc d'élucider les mécanismes viraux contrôlant la reconnaissance des cellules infectées par ces anticorps capables d'induire une réponse effectrice. De par les corrélats de protection identifiés au cours de l'essai vaccinal RV144, les travaux présentés ici se concentrent sur la réponse cytotoxique dépendante des anticorps (ADCC), puisqu'il s'agit d'une réponse effectrice suggérée pour avoir joué un rôle dans la protection observée dans le RV144, seul essai vaccinal anti-VIH à avoir démontré un certain degré de protection. De plus, plusieurs anticorps capables d'induire cette réponse contre le VIH sont connus pour reconnaître les glycoprotéines de surface du virus (Env) dans une conformation dite ouverte, c'est-à-dire la conformation adoptée lors de la liaison d'Env avec son récepteur CD4 (épitopes CD4i). Nous avons mis au point deux techniques *in vitro* permettant d'étudier ces changements de conformation ainsi que leur impact sur la réponse ADCC.

Les techniques mises au point, un ÉLISA sur base cellulaire pour mesurer les changements de conformation d'Env ainsi que la mesure de la réponse ADCC par cytométrie en flux, nous ont permis de démontrer comment le virus empêche l'exposition des épitopes d'Env CD4i. L'activité simultanée des protéines accessoires virales Nef et Vpu sur le retrait du récepteur CD4 de la surface des cellules infectées et l'inhibition du facteur de restriction Tétherine / BST-2 par Vpu contrôlent à la fois les niveaux d'Env et de CD4 à la surface cellulaire et donc modulent l'interaction Env-CD4 et ultimement la susceptibilité à la réponse ADCC contre les épitopes CD4i reconnus par des anticorps hautement prévalents lors de l'infection au VIH. Également, nous démontrons comment de petits composés mimant la

liaison de CD4 sur Env sont capables de forcer l'exposition des épitopes CD4i, même en présence des protéines Nef et Vpu, et donc d'augmenter la susceptibilité des cellules infectées à la réponse ADCC. Une autre découverte présentée ici est la démonstration que la portion soluble d'Env produite par les cellules infectées peut interagir avec le récepteur CD4 des cellules non-infectées avoisinantes et induire leur reconnaissance et élimination par la réponse ADCC contre Env.

Somme toute, la modulation de la réponse ADCC par l'interaction Env–CD4 représente un important pilier de la relation hôte – pathogène du VIH-1 de la perspective des réponses Fc-dépendantes. Les travaux présentés dans cette thèse ont le potentiel d'être utilisés dans l'élaboration de nouvelles stratégies antivirales tout en élargissant les connaissances fondamentales de cette interaction hôte – pathogène.

Mots-clés : VIH-1, ADCC, Env, CD4, anticorps, Vpu, Nef

ABSTRACT

While huge efforts are put forth to develop therapeutic strategies against HIV-1 infection, it is necessary to better understand the viral determinants that will help the effectiveness of these approaches. Moreover, a voluminous scientific literature suggests that antibodies against HIV-1 that have the ability to induce an Fc-mediated effector response can play an important role in the prevention and control of the disease. However, little information is available regarding the determinants recognized by these antibodies and how the virus protects itself from this response. The aim of the work presented in this thesis is therefore to better elucidate the viral mechanisms controlling recognition of infected cells by antibodies capable of inducing effector responses. In regards to the correlates of protection identified in the RV144 vaccine trial, the work presented here focuses on antibody-dependant cellular cytotoxicity (ADCC), since this effector response was suggested to have played a role in the protection observed in the RV144 trial. In addition, many antibodies that induce this response against HIV are known to recognize the virus surface glycoprotein (Env) in an open conformation, that is to say, the conformation adopted by the binding of Env with the CD4 receptor (CD4i epitopes). We have developed two *in vitro* techniques to study these conformational changes and their impact on ADCC responses.

The techniques developed, a cell-based ELISA to measure Env conformational changes and the measure of ADCC responses by flow cytometry, allowed us to demonstrate how the virus prevents exposure of Env CD4i epitopes. The simultaneous activity of viral accessory proteins Vpu and Nef on the removal of CD4 from the surface of infected cells and the Vpu-mediated inhibition of the restriction factor Tetherin / BST-2 control both Env and CD4 levels at the cell surface, thus modulating Env-CD4 interaction. This ultimately results in a decrease in the susceptibility of infected cells to ADCC responses against CD4i epitopes recognized by antibodies that are highly prevalent during HIV infection. Also, we demonstrate how using small compounds mimicking the CD4 binding of Env forces the exposure of CD4i epitopes, even in the presence of Nef and Vpu proteins, and therefore increases the susceptibility to ADCC responses against infected cells. Another discovery is presented here that demonstrates how the soluble portion of Env produced by infected cells can interact with

the CD4 receptor on the bystanders non-infected cells and induce their recognition and elimination by ADCC responses against Env.

Overall, the modulation of ADCC responses by Env-CD4 interaction is an important pillar of HIV-1 host – pathogen interaction from the perspective of Fc effector functions. The work presented in this thesis has the potential to be used in the development of new antiviral strategies while expanding the fundamental understanding of HIV-1 host - pathogen interactions.

Keywords : HIV-1, ADCC, Env, CD4, antibodies, Vpu, Nef

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ADCC	Réponse cytotoxique dépendante des anticorps (*Antibody-Dependent Cellular Cytotoxicity*)
ADN	Acide désoxyribonucléique
AP-1	Protéine adaptatrice 1 (*Adapter Protein 1*)
AP-2	Protéine adaptatrice 2 (*Adapter Protein 2*)
APOBEC3G	Polypeptide 3G d'apolipoprotéine B catalytique à l'activité d'édition de l'ARNm (*Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G*)
ARN	Acide ribonucléique
ARNm	Acide ribonucléique messager
ARNt(Lys3)	ARN de transfert 3 pour la lysine
ARN	Acide ribonucléique
ATR	*Ataxia telangiectasia-mutated and Rad3-related*
AZT	Azidothymidine
B-COP	*Coatomer protein complex subunit beta*
CA	Capside virale
CD	Cluster de différentiation
CD4i	Induits par CD4
CD4mc	Composés mimant la liaison de CD4 à Env
CDC	*Center for Disease Control and Prevention*
CDK9	Protéine kinase-9 cycline-dépendante
CDR	*Complementarity Determining Regions*
CMH-I	Complexe majeur d'histocompatibilité de classe I
CMV	Cytomégalovirus
CMVs	Corps multivésiculaires
CRF	Formes circulantes recombinantes (*Circulating Recombinant Forms*)
CRM1	*Chromosome maintenance gene 1*
CTL	Lymphocyte T cytotoxique (*Cytotoxic T lymphocyte*)

CTS	Signal central de terminaison (*Central Termination Signal*)
DART	*Dual-Affinity Re-Targeting molecule*
Env	Glycoprotéine d'enveloppe virale
ERAD	Système de dégradation de protéines associé au ER (*ER-Associated protein Degradation*)
ESCRT-I	*Endosomal Sorting Complex 1 Required for Transport*
ESCRT-III	*Endosomal Sorting Complex 3 Required for Transport*
Fc	Fragment cristallisable
FcR	Récepteur Fc
FDA	*Food and Drug Administration*
FS	Signal de changement de cadre de lecture (*FrameShifting motif*)
Gag	*Group-Specific Antigen*
GPI	Glycosylphosphatidilinositol
HR1	*Helical heptad Repeat 1*
HTLV-III	Virus T-lymphotropique humain de type III (*Human T-lymphotropic virus III*)
Ig	Immunoglobuline
IN	Intégrase
INs	Motif d'instabilité d'ARN
ITAM	*Immunoreceptor Tyrosine-based Activation Motif*
LEDGF	*Lens Epithelium-Derived Growth Factor*
LPS	Lipopolysacharride
LTR	*Long Terminal Repeat*
MA	Matrice
mAb	Anticorps monoclonal (*monoclonal antibody*)
MLV	Virus de la leucémie murine
MPER	*Membrane Proximal External Region*
MSM	Homme ayant des relations sexuelles avec des hommes (M*en having Sex with Men*)
NC	Nucléocapside
Nef	*Negative factor*
NES	Signal d'exportation nucléaire (*Nuclear Export Signal*)

NFAT	*Nuclear Factor of Activated T cells*
NK	*Natural Killer*
NLS	Signal de localisation nucléaire (*Nuclear Localisation Signal*)
NRTI	Inhibiteur nucléosidique de la transcriptase inverse (*Nucleoside Reverse-Transcriptase Inhibitor*)
NNRTI	Inhibiteur non nucléosidique de la transcriptase inverse (*Non-Nucleoside Reverse-Transcriptase Inhibitor*)
OMS	Organisation Mondiale de la Santé
ORF	Cadre ouvert de lecture (*Open Reading Frame*)
PA	Signal de polyadénylation
PAK2	Kinase Ser/Thr activée par la protéine 21 (*p21-activated kinase*)
PBS	Site de liaison à l'amorce (*Primer Binding Site*)
PIC	Complexe de pré-intégration (*Pre-Integration Complex*)
Pol	Polymérase
PPT	Tract de polypurine (*PolyPurine Tract*)
PPTc	Tract central de polypurine (*central PolyPurine Tract*)
PR	Protéase
RE	Réticulum endoplasmique
RRE	Élément de réponse à Rev (*Rev-responsive element*)
RT	Transcriptase inverse (*Reverse transcriptase*)
sCD4	CD4 soluble
SCF	Complexe E3 ubiquitine ligase Skp1/Cullin 1/F-box
SERINC3	*SERine INCorporator 3*
SERINC5	*SERine INCorporator 5*
SIDA	Syndrome d'immunodéficience acquise
TAR	Élément de réponse de trans-activation (*Trans-Activating Response element*)
TEF β	Facteur d'élongation de la transcription beta
Tsg101	*Tumour Susceptibility Gene 101*
Vif	*Virus Infectivity Factor*
VIH-1	Virus de l'immunodéficience humaine de type 1
VIH-2	Virus de l'immunodéficience humaine de type 2

VIS	Virus d'immunodéficience simienne
VLP	Particule ressemblant à un virus (*Viral-Like Particle*)
Vpr	*Viral Protein R*
VprBP	*Vpr Binding Protein*
Vpu	*Viral Protein U*

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CHAPITRE I

INTRODUCTION

Les contacts inter-espèces animales, bien qu'essentiels à la vie sur terre et à l'évolution, mènent parfois à la transmission d'agents infectieux vers un nouvel hôte mésadapté à combattre ce nouveau pathogène. Un exemple adéquat, qui récemment était présent dans notre quotidien grâce aux médias, est la crise causée par le virus Ébola en Afrique de l'Ouest. Cependant, l'un de ces agents nouvellement transmis entre espèces est moins affiché dans notre quotidien : le virus de l'immunodéficience humaine de type 1 (VIH-1). Néanmoins, les plus récentes données de l'Organisation mondiale de la santé (OMS) démontrent qu'il y a encore annuellement plus de 2 millions de personnes nouvellement infectées par le VIH-1 (1). Prises dans leur ensemble, les données sont encore plus alarmantes, alors que plus de 35 millions d'individus vivent avec cette infection chronique et que le taux de mortalité, bien que réduit depuis quelques années, est en augmentation dans certaines régions du globe.

1.1 Le VIH-1 et le syndrome de l'immunodéficience acquise

1.1.1 *Classification du virus*

Le VIH-1 appartient à la famille des rétrovirus (*Retroviridae*). Membre du groupe viral VI (*ssRNA-RT*), cette famille de virus comprend des virus qui codent tous pour une transcriptase inverse (*reverse transcriptase*, RT). La RT permet la synthèse d'un génome sous forme d'acide désoxyribonucléique (ADN) bicaténaire à partir d'acides ribonucléiques (ARN) monocaténaires qui sont incorporés dans la particule virale. Les rétrovirus ont la particularité d'intégrer ce génome viral nouvellement synthétisé dans le génome cellulaire de l'hôte. L'organisation génomique des rétrovirus a fait en sorte qu'il est possible de les diviser en deux catégories, les rétrovirus dits simples ou complexes. À la base, le génome de tous les rétrovirus code pour trois gènes principaux : *gag* (*group-specific antigen*), *pol* (polymérase) et

env (enveloppe). Ces trois gènes sont à la fois essentiels et suffisants pour fournir les composantes structurales et enzymatiques nécessaires à la réplication virale. Les rétrovirus simples ne codent que pour ces trois gènes essentiels alors que les rétrovirus complexes possèdent des gènes codant pour des protéines exerçant différents rôles régulateurs au cours de l'infection. Bien que ces protéines soient dites *accessoires* puisqu'elles sont facultatives pour la réplication virale *in vitro*, leur présence est nécessaire à la réplication et au développement de la pathogénicité des rétrovirus complexes *in vivo (2, 3)*. Ces derniers comprennent plusieurs genres dans la famille des rétrovirus, dont les lentivirus auxquels appartiennent le VIH-1, le virus de l'immunodéficience humaine de type 2 (VIH-2) ainsi que le virus de l'immunodéficience simienne (VIS). Les lentivirus sont généralement des pathogènes causant des infections ayant des longues périodes d'incubation asymptomatiques, d'où l'origine de leur nom (*lente* en latin signifie lent) au cours de laquelle se maintient une réplication virale et qui engendre le dysfonctionnement des systèmes immunitaire, hématopoïétique et / ou nerveux.

1.1.2 *Origine du virus*

Au cours des années 1970 et 1980, de nouveaux portraits cliniques ont rendu perplexe la communauté médicale. De multiples individus autrement en santé se présentaient pour évaluation et traitements cliniques avec des symptômes rappelant une dysfonction immunitaire : adénopathie généralisée, infections rares et normalement opportunistes (pneumonie à *Pneumocystis jiroveci*, rétinite au cytomégalovirus (CMV) ainsi que des méningites cryptococcique) ainsi qu'une plus grande susceptibilité d'avoir des cancers très rares tels que des lymphomes non-Hodgkiniens ou encore un sarcome de Kaposi. En laboratoire, ces données cliniques étaient généralement associées à une diminution importante des lymphocytes T dits positifs pour le cluster de différentiation 4 (CD4+) dans le sang périphérique. Ce n'est cependant qu'en juin 1981 que la première publication décrivant cinq patients avec une immunodéficience sévère a été publiée dans le *Morbidiy and Mortality Weekly Report* par le *Center for Disease Control and Prevention* (CDC) américain (4). Des observations subséquentes ont identifié que certains groupes tels que les hommes ayant des relations sexuelles avec des hommes (*men having sex with men, MSM*) ainsi que les

utilisateurs de drogues injectables et les patients recevant fréquemment des transfusions sanguines étaient plus à risque de présenter un portrait clinique rappelant une immunodéficience sévère. Cependant, ce qui a alerté la communauté médicale et scientifique au fait que ces phénomènes puissent être causés par un agent infectieux jusqu'alors non identifié est que les partenaires sexuels ainsi que les enfants des individus de ces groupes à risque présentaient à leur tour un risque accru (5-8).

Deux ans après les premières descriptions de ces nouveaux portraits cliniques, soit en 1983, le pathogène responsable de causer ce qui est maintenant appelé le syndrome de l'immunodéficience acquise (SIDA) a été identifié. Luc Montagnier, Françoise Barré Sinoussi et leurs collègues ont tout d'abord décrit l'isolation d'un pathogène à partir d'un ganglion lymphatique d'un patient présentant une adénopathie généralisée d'origine inconnue mais autrement asymptomatique (9). Des photographies par microscopie électronique ont alors suggéré que ce nouveau pathogène possède des caractéristiques morphologiques propres à la famille des rétrovirus. Peu de temps après, Gallo et collègues ont également annoncé l'isolation d'un rétrovirus d'un patient souffrant du SIDA tout en démontrant que des anticorps ciblant ce nouveau rétrovirus sont présents dans le sérum d'individus atteints du SIDA (10, 11). Ce virus, alors nommé le virus T-lymphotropique humain de type III (HTLV-III), est maintenant mieux connu sous le nom de VIH-1 (12). Quelques années plus tard en 1986, un rétrovirus humain aux propriétés antigéniques et génétiques quelques peu différentes du VIH-1 est identifié chez des patients atteints de SIDA dans certaines régions ciblées d'Afrique de l'Ouest : le VIH de type 2 (VIH-2) (13).

Les rétrovirus sont connus pour présenter une forte diversité génétique (c.f. section 1.1.5 sur le cycle réplicatif viral). L'analyse phylogénétique des souches de VIH-1 isolées à l'échelle mondiale a démontré une diversité extrêmement élevée, impliquant ainsi que le virus circulait dans la population humaine depuis plusieurs années avant sa découverte initiale (14). Ces analyses ont aussi révélé que le VIH-1 se divise en au moins quatre groupes distincts : le groupe M qui est responsable de la majorité de la pandémie, et les groupes N, O et P qui ont une prévalence géographique très limitée (15). Le groupe M est lui-même représenté par différentes clades (A, B, C, D, F, G, H, J et K) et de multiples formes recombinantes

circulantes (*circulating recombinant forms,* CRF) (16). Toutefois, ce n'est qu'après la découverte que des lentivirus similaires à ceux infectant les humains sont également retrouvés chez des primates non-humains qu'il a été proposé que le VIH proviendrait d'un événement zoonotique des singes vers l'homme (17, 18). Puis, finalement, un VIS très similaire au VIH-1 a été identifié chez les chimpanzés (*Pan troglodytes*) (19, 20). Cependant, ce n'est que plusieurs années plus tard qu'il fut confirmé que l'événement zoonotique s'est produit entre l'homme et la sous-espèce animale *Pan troglodytes troglodytes* (*P. t. troglodytes*) autour des années 1920 (21-23). Il est de nos jours accepté que les différents groupes du VIH-1 représentent des événements zoonotiques distincts avec les groupes M et N provenant des chimpanzés *P.t.t.* alors que les groupes O et P proviendraient eux de la transmission du virus de gorilles infectés à l'homme, contribuant ainsi à expliquer les propriétés génétiques, phénotypiques et cliniques distinctes de ces virus (17, 21, 24, 25).

1.1.3 *Structure du virus*

D'une taille variant entre 80 et 120 nm de diamètre, les particules virales du VIH-1 contiennent toutes les protéines virales essentielles à la maturation du virus et à l'infection de nouvelles cellules cibles (Figure 1.1). Au cœur de la particule virale mature, on retrouve deux brins d'ARN monocaténaires de polarité positive qui sont recouverts de protéines de la nucléocapside (NC). La transcriptase inverse (RT), essentielle pour transcrire ces brins d'ARN en ADN pouvant être intégrés dans le génome de la cellule cible et une caractéristique principale des rétrovirus, est également incorporée dans la particule virale (9, 26). La RT reconnaît un complexe formé d'un ARN de transfert (ARNt(Lys3)) spécifiquement incorporé lors de l'assemblage de la particule virale et qui est apparié au site de liaison de l'amorce (*primer binding site*, PBS) de l'ARN génomique viral (27-30). L'intégrase (IN), la protéine Vpr (*viral protein R*) et de faibles quantités des protéines Nef et Vif sont également présentes au cœur de la particule virale (31).

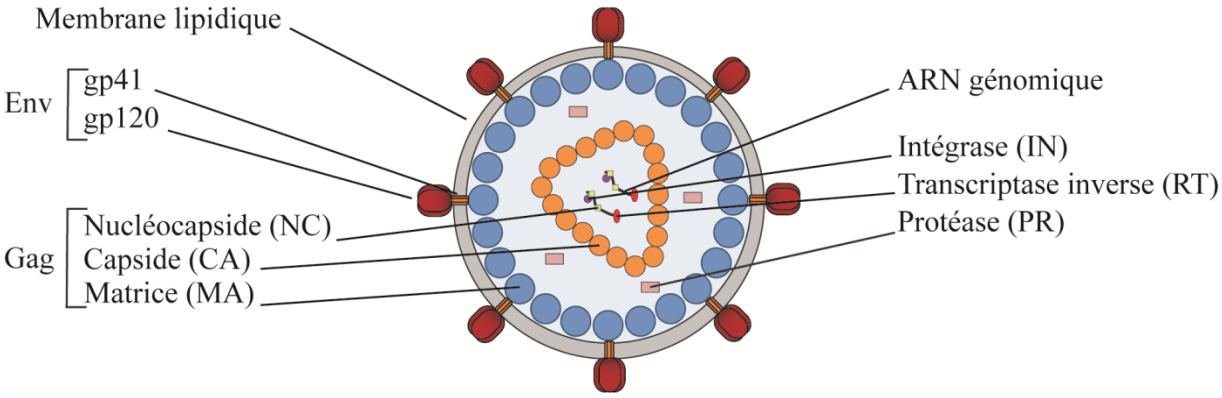


Figure 1.1 La particule virale mature du VIH-1
Schématisation d'une particule virale mature du VIH-1.

Le noyau de la particule virale (autrement nommé *viral core*) est formé de l'assemblage multimérique d'environ 1000-1500 sous-unités de la protéine de la capside virale (CA, p24) (32). Les monomères de CA s'organisent tout d'abord en sous-éléments hexamèriques, pour ensuite être reliés entre eux pour former la structure capsidique conique caractéristique des rétrovirus (33-35). L'obturation des extrémités sphériques du cœur viral nécessite toutefois l'incorporation d'assemblages de CA sous forme pentamérique, permettant ainsi le changement de l'angle de courbure de la lattice de la capside et donc sa fermeture (36, 37). Le cœur viral est incorporé dans une bicouche lipidique, tapissée de la matrice virale (MA), dans laquelle sont présents les trimères des glycoprotéines d'enveloppe (Env). Diverses protéines cellulaires ainsi que la protéase virale (PR) se retrouvent également dans le virion.

1.1.4 *Organisation génomique du VIH-1*

Démontrant une organisation génomique caractéristique des lentivirus, le VIH-1 possède un génome d'environ 9200 paires de bases présentant 9 cadres de lecture ouverts (*open reading frame*, ORF) codant, après maturation, pour 15 protéines virales distinctes (Figure 1.2) (38). Plus précisément, les gènes *gag*, *pol* et *env* sont d'abord synthétisés sous forme de précurseurs polyprotéiques avant d'être clivés en leurs différents constituants par PR ou des protéases cellulaires. Le précurseur Gag ($Pr55^{Gag}$) est ainsi clivé par PR pour générer les protéines structurelles et régulatrices MA, CA, NC, la protéine p6, SP1 et SP2 (39). Le gène *pol* code pour les protéines enzymatiques du virus, soit PR, RT et IN qui sont produites à

partir du clivage du précurseur $Pr160^{Gag\text{-}Pol}$ résultant d'un glissement ribosomal induisant un changement de cadre de lecture lors de la traduction de *gag (40, 41)*. La protéine d'enveloppe virale (Env) est quant à elle synthétisée sous forme d'un précurseur glycoprotéique (gp160) qui est clivé en ses deux constituants, les glycoprotéines de surface (gp120) et transmembranaire (gp41), par des protéases cellulaires de la famille des furines (42). Le génome du VIH-1 code aussi pour les protéines virales Vif (*virus infectivity factor*), Vpr (*viral protein R*), Vpu (*viral protein U*), Nef (*negative factor*), Tat et Rev.

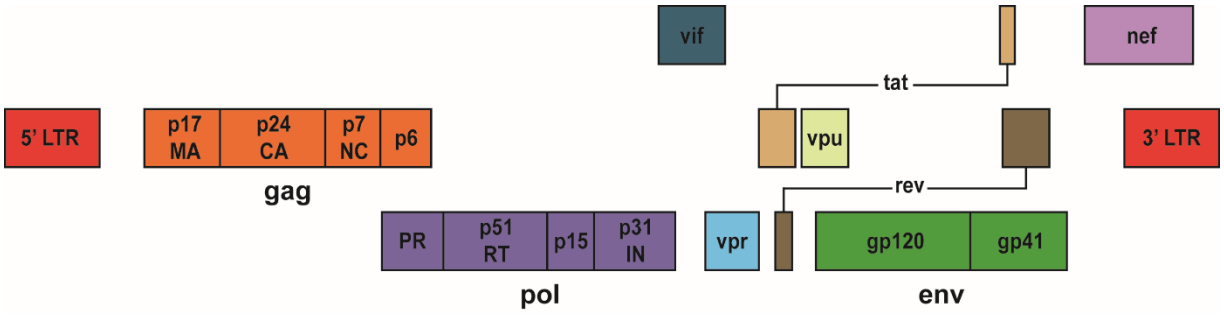


Figure 1.2 L'organisation génomique du VIH-1
Schéma démontrant l'organisation génomique du VIH-1. D'une longueur approximative de 9200 paires de bases, son génome comprend neuf gènes répartis sur trois cadres de lecture. Il est flanqué de longues répétitions terminales (LTR) identiques.

Les régions non-codantes du génome du VIH-1 sont aussi essentielles au cycle réplicatif viral. Tout d'abord, et c'est également un trait caractéristique des rétrovirus, son génome est flanqué à ses deux extrémités par de longues séquences terminales répétées identiques (*long terminal repeats*, LTR). Les LTRs ont des rôles majeurs dans l'intégration de l'ADN viral dans le génome cellulaire, dans le contrôle de la transcription des gènes viraux et dans l'encapsidation des ARN génomiques viraux (43, 44). D'autres motifs conservés dans le génome du VIH-1 sont également présents et exercent différents rôles tout au long du cycle de réplication viral : le motif de changement de cadre de lecture (*frameshifting*, FS) entre *gag* et *pol*, le motif d'instabilité ARN (INSs), le tract central polypurine (PPT_c), les accepteurs et donneurs d'épissage, l'élément de réponse à Rev (*rev-responsive element*, RRE), le tract 3' polypurine (PPT) et le signal de polyadénylation (PA) (38).

1.1.5 *Cycle réplicatif*

Le cycle de réplication du VIH-1 se divise en une phase *précoce*, allant de la fusion de la particule virale avec la membrane cellulaire jusqu'à l'intégration de l'ADN viral dans le génome cellulaire, et une phase *tardive* allant de l'expression des protéines du provirus jusqu'au bourgeonnement et à la maturation des nouveaux virions (Figure 1.3).

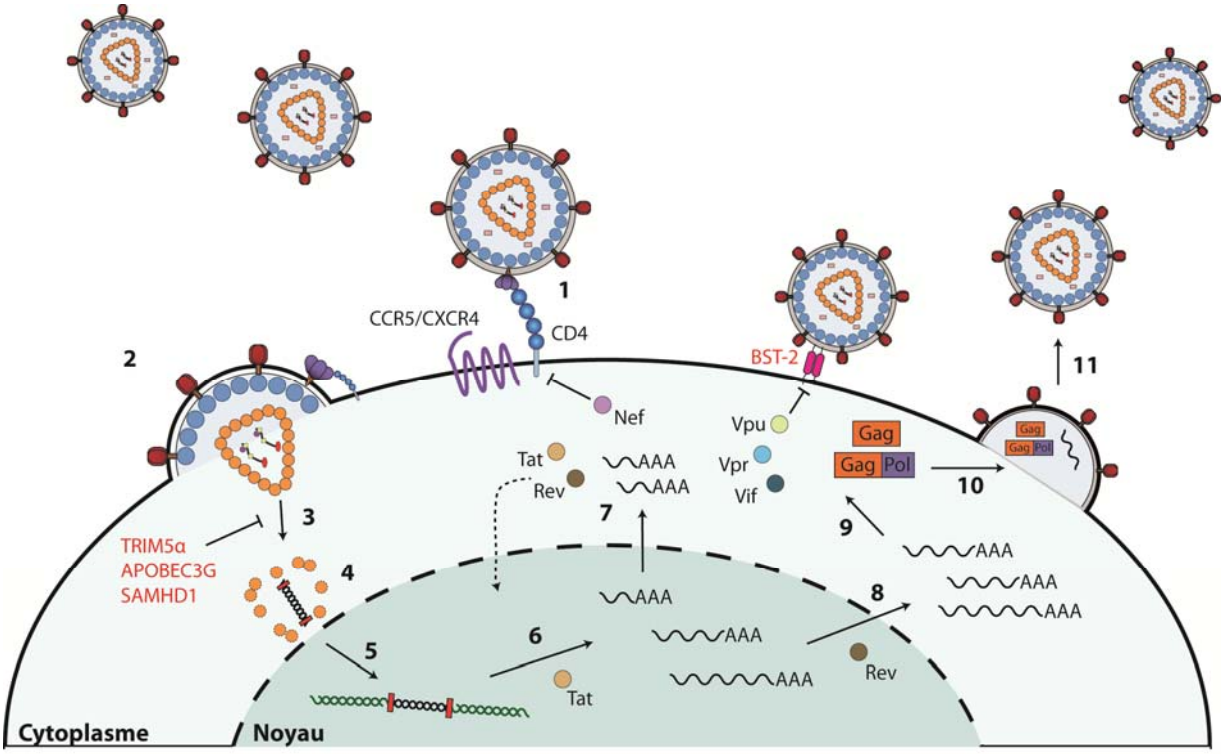


Figure 1.3 **Le cycle de réplication du VIH-1**
Représentation schématique des principales étapes du cycle de réplication du VIH-1 : 1) l'attachement, 2) l'entrée virale des suites de la fusion des membranes virale et cellulaire, 3) la décapsidation du cœur viral, la transcription inverse et la translocation vers le noyau, 4) la finalisation de la transcription inverse et de la décapsidation à la périphérie du noyau et l'importation nucléaire, 5) l'intégration, 6) la transcription des ARNm viraux, 7) l'export des ARNm et traduction précoces, 8) l'export des ARNm Rev-dépendants, 9) la traduction des protéines virales, 10) l'assemblage et 11) la relâche et maturation du virus.

1.1.5.1 Entrée virale

L'étape initiale de l'infection par le VIH-1 est l'interaction spécifique entre la glycoprotéine d'enveloppe gp120 et son récepteur cellulaire CD4 (45, 46). Ce récepteur est présent majoritairement à la surface des lymphocytes T CD4+ et des macrophages mais sa seule présence n'est pas suffisante pour rendre une cellule susceptible à l'infection au VIH-1 (47, 48). Ces observations ont par la suite été expliquées par la découverte que l'entrée du VIH-1 dans une cellule cible nécessite la présence d'un de ses deux corécepteurs, les récepteurs de chimiokines CCR5 ou CXCR4 (49-56).

Le processus de la fusion membranaire et de l'entrée virale comprend plusieurs étapes séquentielles. Tout d'abord, l'interaction initiale entre la gp120 et le récepteur cellulaire CD4 induit des changements de conformation dans la gp120 qui occasionne l'exposition de la boucle variable 3 (V_3), normalement enfouie dans la protéine (57, 58). C'est cette région nouvellement exposée qui est responsable de l'interaction avec le corécepteur (59, 60), déterminant ainsi le tropisme du virus : CCR5 (R5)-tropique, CXCR4 (X4)-tropique ou encore à double tropisme R5/X4-tropique (61). Après que le complexe tertiaire Env – CD4 – corécepteur soit formé, d'autres changements de conformation sont transmis par la gp120 vers gp41, engendrant ainsi l'exposition de la superhélice 1 de la gp41 (*helical heptad repeat 1*, HR1) et l'insertion du peptide de fusion dans la membrane plasmique de la cellule cible (62-65). L'homologie entre les hélices HR1 et HR2 de gp41 cause ensuite son repliement en une structure nommée le paquet de six hélices (*six-helix bundle*) rapprochant ainsi les membranes lipidiques virale et cellulaire et occasionnant leur fusion (66, 67). La fusion de la particule virale avec la cellule cible permet la relâche du cœur viral dans le cytoplasme cellulaire.

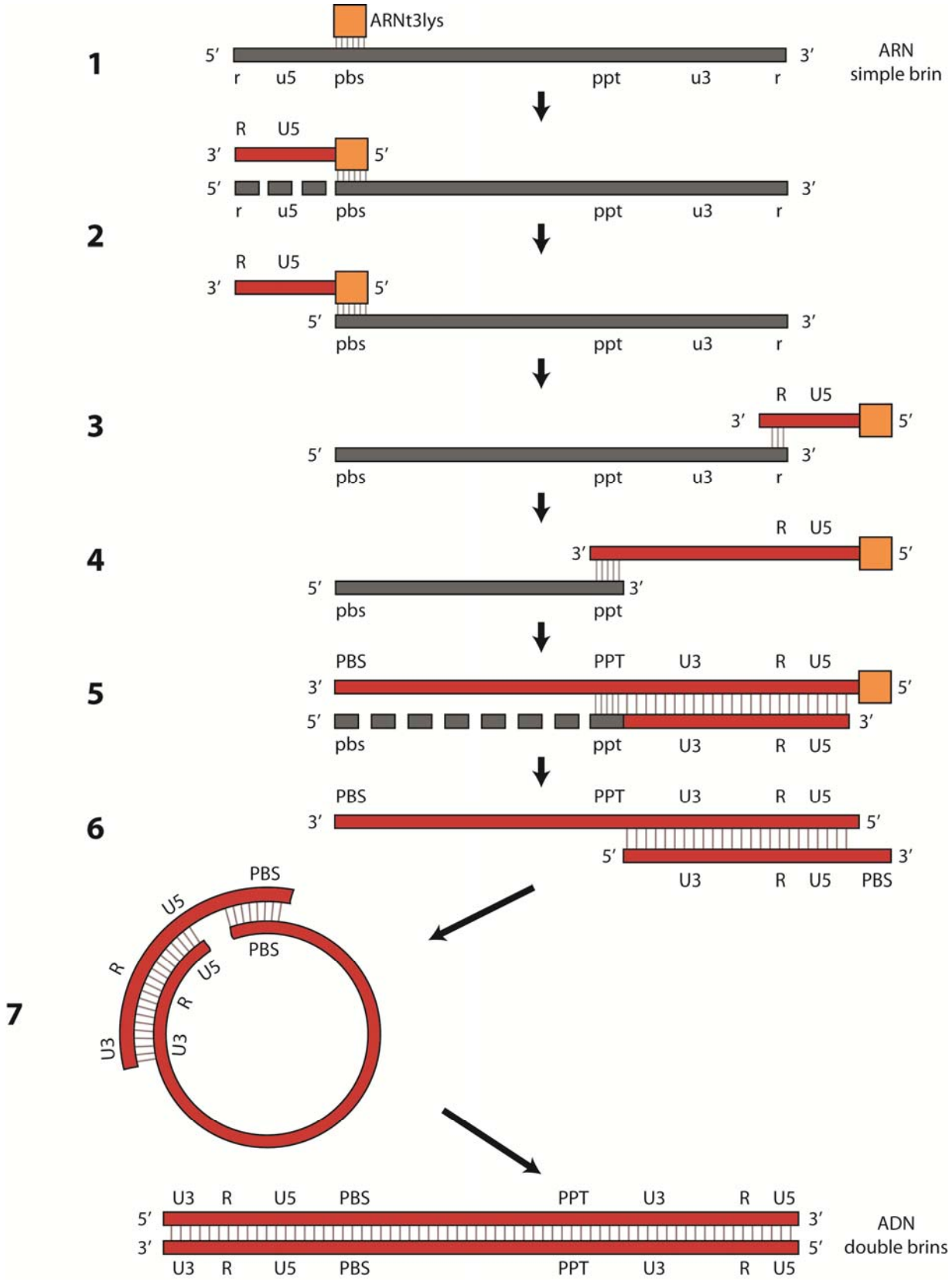
1.1.5.2 Les étapes précoces post-entrée

Les évènements de la phase post-entrée et pré-intégration du cycle de réplication viral du VIH-1 sont étroitement régulés par celui-ci mais la complexité d'étudier ces phénomènes limite une connaissance approfondie de ces mécanismes. Comme pour tous les rétrovirus, le VIH-1 doit invariablement transcrire, à l'aide de la RT, son génome en un double brin d'ADN

qui pourra ultimement être intégré dans le génome de la cellule cible. Ce processus est enclenché lors de la relâche du cœur viral dans le cytoplasme. Des études suggèrent que le processus de transcription inverse s'effectue en une heure ou plus rapidement après l'entrée du virus dans la cellule cible (68-71). L'étape de la transcription inverse nécessite toutefois que des changements dans l'organisation structurelle de l'assemblage de la capside virale s'effectuent de manière strictement contrôlée. En effet, l'assemblage multimérique de CA se désorganise (étape de décapsidation) rapidement après l'entrée du cœur viral dans le cytoplasme. Il est maintenant de mieux en mieux accepté que ces deux étapes sont extrêmement liées, et la dérégulation de la décapsidation du cœur viral engendre un retard voire même l'interruption des événements de transcription inverse et du transport au noyau de l'ADN virale (voir plus bas), occasionnant ainsi une perte d'infectivité pour le virus (68, 72-76). Néanmoins, plusieurs controverses concernant la décapsidation du VIH-1 demeurent dans le collectif scientifique. Certaines études suggèrent que cette étape aurait lieu dans le cytoplasme, alors que d'autres affirment qu'elle ne s'effectuerait qu'à l'arrivée du cœur viral aux pores nucléaires (68, 77, 78). Il est de plus en plus clair que la protéine CA régule les événements post-entrée virale en interagissant avec des facteurs cellulaires afin d'échapper à la reconnaissance et à l'activation des défenses antivirales de l'hôte (79-81).

La rétro-transcription de l'ARN viral en ADN est catalysée par la RT. Dans sa forme mature, elle est formée par l'hétérodimérisation de sous-unités de 66 (p66) et 51 (p51) kDa. La sous-unité p51 est dérivée du clivage de p66 par PR. La RT possède trois activités enzymatiques distinctes qui sont essentielles au processus de rétro-transcription : la polymérisation d'ADN à partir d'un brin d'ARN (polymérase ARN-dépendante), une activité ribonucléase (RNaseH) et la polymérisation d'ADN à partir d'un brin d'ADN (polymérase ADN-dépendante). Brièvement, la synthèse du brin d'ADN à polarité négative s'effectue d'abord à l'extrémité 3' de l'ARNt(Lys3) qui est encapsidé sélectivement dans la particule virale et agit en tant qu'amorce pour la réaction de rétro-transcription (27, 28) (voir Figure 1.4, étape 1). La transcription se poursuit jusqu'à l'extrémité 5' du génome alors que la RT digère de manière spécifique la portion ARN de l'hybride ARN/ADN ainsi formé (étape 2). Il y a alors relâche et formation d'un fragment d'ADN simple brin (*minus strand strong-stop DNA*). Ce fragment s'hybride grâce à l'homologie avec l'extrémité 3' du génome via la région

répétée R du LTR présente à chaque extrémité du génome viral (étape 3). La synthèse du brin d'ADN de polarité négative se poursuit alors que l'ARN est digéré par l'activité RNAseH de la RT (étape 4). Cependant, des régions du génome qui sont résistantes à la dégradation de l'ARN par la RT (notamment le PPT et PPT_c) servent d'amorce pour la synthèse du brin d'ADN de polarité positive (82) (étape 5). L'activité RNaseH de la RT digère l'ARNt(Lys3) ayant servi d'amorce pour la synthèse du brin d'ADN de polarité négative, exposant ainsi la région PBS sur le brin de polarité positive (étape 6). Ceci permet à ce dernier de s'hybrider avec sa région homologue à l'extrémité 3' du brin de polarité négative (étape 7, nommée *second-strand transfer*). L'élongation peut ainsi se poursuivre pour chacun des brins. À noter que l'élongation du brin de polarité positive se termine dans la région CTS (*central termination signal*) et nécessite le déplacement d'une centaine de nucléotides qui se superposent ainsi à l'ADN double brins (83). Nommée *DNA flap*, cette structure semble être importante pour le transport au noyau du complexe de préintégration (PIC, *pre-integration complex*) (84, 85).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ADN
double brins

Figure 1.4 La rétrotranscription de l'ARN viral en double brins d'ADN
Schématisation des principaux événements de la rétrotranscription de l'ARN viral simple brin en double brins d'ADN. Voir section 1.1.5.2 pour plus de détails sur les différentes étapes.

L'ADN viral double brin ainsi généré constitue l'élément central du PIC qui comprend également plusieurs protéines virales et cellulaires (86, 87). Puisque son diamètre est estimé à 56 nm et que la limite de diffusion passive par les pores nucléaires est d'environ 9 nm, on a supposé que le VIH-1 devait être capable d'induire sa translocation active par les pores nucléaires (88, 89). Plusieurs modèles différents, impliquant l'une ou plusieurs des protéines virales MA, Vpr et IN ou encore la structure nucléique *DNA flap*, ont été proposés mais demeurent controversés (84, 85, 90-95). Cependant, une étude a démontré qu'une construction chimérique du VIH-1 codant pour l'IN du virus de la leucémie murine (MLV, *murine leukemia virus*), un rétrovirus incapable d'infecter des cellules non-prolifératives, et ne présentant pas les autres déterminants proposés pour le transport au noyau (MA, Vpr, le *DNA flap*), présentait tout de même la capacité d'infecter des cellules non-prolifératives, suggérant ainsi qu'aucun de ces éléments n'étaient critiques à l'infection des cellules non-prolifératives par le VIH-1 (96). Il est maintenant mieux accepté que des quantités significatives de CA se retrouvent jointes au PIC (76-78) et des études suggérant un lien entre CA et l'import au noyau du PIC continuent de s'accumuler (75, 76). Principalement, la comparaison de chimères entre le VIH-1 et MLV dans leur capacité à infecter des cellules non-prolifératives a identifié la CA comme déterminant principal de l'import nucléaire (97). De plus, la CA est le déterminant viral de l'interaction avec des facteurs de l'hôte impliqués dans le transport nucléaire et essentiels à l'infectivité du VIH-1 (TNPO3, NUP153, NUP358/RanBP2) (80, 98, 99). Des mutations ponctuelles dans la protéine CA sont également capables d'induire des changements dans la nécessité du VIH-1 face à ces facteurs de l'hôte pour son transport au noyau (99-101).

Une fois l'ADN viral double brin présent au noyau, l'étape d'intégration de celui-ci dans le génome de la cellule cible s'effectue. Catalysée par IN et requérant la présence d'un cofacteur de l'hôte nommé LEDGF (*lens epithelium-derived growth factor*) (102), cette étape du cycle réplicatif s'initie par la reconnaissance et clivage des LTR aux deux extrémités du

génome viral, créant ainsi des extrémités 3'-hydroxyle. L'IN catalyse aussi le clivage de l'ADN génomique cellulaire et les extrémités3'-hydroxyle de l'ADN proviral sont liguées aux extrémités 5'-phosphate de l'ADN cellulaire. La machinerie cellulaire de l'hôte répare ensuite les dommages à l'ADN au niveau du site d'intégration (87), complétant ainsi l'intégration de l'ADN viral dans le génome cellulaire. Le VIH-1 s'intègre préférentiellement dans des sites actifs de transcription (103). Bien que le mécanisme par lequel le site d'intégration est déterminé fut longtemps incompris, il est maintenant plus clair que LEDGF et la voie d'entrée au noyau (dont CA semble être le déterminant) jouent des rôles majeurs dans ce processus (80, 104).

1.1.5.3 Les étapes tardives post-entrée

Une fois intégré dans le génome cellulaire, l'ADN viral se comporte essentiellement comme un gène cellulaire. La région LTR située en 5' du génome est le site de régulation de la transcription, possédant une séquence promotrice et des sites de liaison pour différents facteurs de transcription (SP1, NF-κB, AP1 et NFAT [*nuclear factor of activated T cells]*) (105-108). Ces régions, majoritairement celles liant le facteur de transcription SP1, sont responsables du recrutement de l'ARN polymérase II (ARN Pol-II) au niveau du site d'initiation de la transcription. Alors que les facteurs de transcription importants pour le VIH-1 autre que SP1, tels que NF-κB, NFAT et AP-1, sont induits lors de l'activation de la cellule infectée, SP1 joue quant à lui un rôle majeur dans l'initiation de niveaux basaux de transcription (109). Cependant, l'activité de l'ARN Pol-II est intrinsèquement instable et les niveaux basaux de transcription entraînent la formation de transcrits épissés non-polyadénylés qui donneront naissance aux protéines virales Tat, Rev et Nef (110).

La protéine Tat est le déterminant majeur de l'activation de la transcription de l'ARN viral, sa délétion ou mutagénèse empêchant la formation de particules virales (111, 112). Il s'agit d'une protéine de 101 acides aminés encodés par deux exons distincts qui est responsable de l'augmentation marquée (plusieurs centaines de fois) de la production d'ARNs viraux en accentuant la processivité de l'ARN Pol-II. Pour effectuer cette fonction, Tat lie une tige-boucle de l'ARN viral en cours de synthèse (nommée TAR, *trans-activating response*

element) et y recrute les protéines cellulaires cycline T1, protéine kinase-9 cycline-dépendante (Cdk9), et le facteur d'élongation de la transcription b (pTEF-b), catalysant ainsi l'hyperphosphorylation du domaine C terminal de l'ARN Pol-II et l'augmentation drastique de son activité (113-118).

La synthèse des ARN messagers (ARNm) du VIH-1 utilise la machinerie cellulaire et est donc sujette aux mêmes modifications que les ARNm des gènes cellulaires (coiffe en 5', polyadénylation en 3' et épissage). Les ARNm viraux multi-épissés peuvent être exportés du noyau par la machinerie traditionnelle de l'hôte mais ne codent que pour les protéines virales Tat, Rev et Nef. Le VIH-1 nécessite donc un mécanisme viral afin de forcer l'exportation d'ARNm mono ou non-épissés hors du noyau. Rev, une protéine de 19 kDa et comprenant 116 acides aminés, est responsable de l'export de ces ARNm en se liant à une région de l'ARNm hautement structurée, le RRE (119). Un signal de localisation nucléaire (NLS, *nuclear localisation signal*) dans Rev lui permet d'avoir une localisation nucléaire après sa synthèse cytoplasmique. C'est à cet endroit que Rev lie les ARNm viraux (mono- ou non-épissés) contenant le RRE et recrute les protéines cellulaires CRM1 (*chromosome maintenance gene 1*) et RanGTP grâce à sa région de signal d'export nucléaire (NES, *nuclear export signal*) (120, 121). Le complexe ainsi formé est capable d'interagir avec la machinerie des pores nucléaires et d'être relâché dans le cytoplasme où RanGTP sera convertie en RanGDP et induira la déstructuration du complexe Rev / ARNm / CRM1 / RanGTP/GDP (122-124). Les ARNm ainsi relâchés seront incorporés dans les particules virales naissantes (ARN génomique complet) ou traduits en diverses protéines virales accessoires, enzymatiques ou structurelles. À noter que le ratio optimal entre les quantités de protéines de structure codées par le gène *gag* et les protéines enzymatiques comprises dans le précurseur Gag-Pol est assuré par un décalage du cadre de lecture à la fin de la traduction du précurseur Gag. Ce décalage, nécessitant la présence du FS dans l'ARNm de Gag, s'effectue d'une manière sous-optimale, résultant en la production de 20 précurseurs Gag pour chaque précurseur Gag-Pol (40, 125). Ce ratio a été démontré comme étant critique à la production de particules virales infectieuses (126, 127).

L'assemblage des virions s'effectue principalement à la membrane plasmique de la cellule infectée (128-130). Le précurseur Gag est nécessaire et suffisant à la formation de

particules non-infectieuses ressemblant des virions (VLP, *virus-like particles*). Néanmoins, la formation de particules virales infectieuses requiert la présence du précurseur Gag-Pol et l'incorporation des protéines Env et de l'ARN génomique viral. Gag est donc responsable de plusieurs activités lors de l'assemblage et de la relâche des particules virales naissantes : il représente la base structurelle de la particule virale, est responsable de l'encapsidation du génome viral, recrute les facteurs cellulaires nécessaires au bourgeonnement des particules virales, et permet l'acquisition de la membrane lipidique et des protéines Env associées (131). Le sous-domaine MA de Gag (et de Gag-Pol) possède un domaine myristoylé qui est essentiel à la liaison et à la localisation de Gag à la membrane plasmique via une association avec le lipide phosphatidylinositol-(4,5)-bisphosphate(PI(4,5)P2) (132-135). Le sous-domaine NC de Gag initie ensuite la multimérisation de Gag par son interaction avec l'ARN génomique (136, 137), la présence d'ARN étant si critique à l'initiation de la multimérisation de Gag que de l'ARN cellulaire sera incorporé dans les VLP si l'ARN génomique n'est pas présent (138, 139). Les complexes NC-ARN sont critiques pour la dimérisation de Gag, étape cruciale dans l'assemblage de structures d'ordres supérieurs et la formation de VLPs (140-144). Le mécanisme responsable de l'incorporation d'Env dans la particule virale est toujours méconnu mais il est probable que diverses stratégies soient impliquées (145). Les mécanismes proposés varient de 1) la simple présence d'Env à la surface cellulaire pourrait faire en sorte qu'elle serait incorporée d'une manière passive dans la particule virale naissante; 2) Env et Gag pourraient être ciblés aux mêmes domaines de la membrane cellulaire et donc être concentrés dans la même région; 3) une interaction directe entre Gag et Env permettrait son incorporation dans les particules virales; ou encore 4) cette interaction pourrait être indirecte et s'effectuer via une protéine adaptatrice de l'hôte. Néanmoins, des études basées sur la mutagénèse d'Env et du sous-domaine MA de Gag soutiennent que le rôle de l'interaction de MA avec la queue cytoplasmique d'Env joue un rôle majeur dans son incorporation au sein de la particule virale (146, 147).

Le processus par lequel la particule virale assemblée peut bourgeonner hors de la cellule infectée est orchestré par le sous-domaine p6 du précurseur Gag. Pour ce faire, le domaine p6 comprend des motifs P(T/S)AP et $YP(X)_nL$ dans sa portion C-terminale permettant le recrutement de composantes des complexes ESCRT-I et –III (*endosomal sorting complex 1 –*

3 required for transport), respectivement Tsg101 (*tumour susceptibility gene 101*) et AIP1/Alix (*ASK-interacting protein 1*) (148-151). Les complexes ESCRT sont normalement impliqués dans la fission des vésicules endosomales et leur recrutement par le VIH-1 permet ainsi la fission des membranes cellulaire et virale et donc la relâche de particules virales hors de la cellule infectée. L'observation d'une certaine accumulation de protéines Gag et de particules virales dans les corps multivésiculaires (CMVs) est un phénomène encore mal compris mais qui est suggéré comme s'effectuant soit par le ciblage direct de Gag aux CMVs ou encore par une accumulation de Gag aux CMVs des suites de son internalisation de la membrane plasmique (152-158). Néanmoins, un processus de maturation doit être mis en branle par le domaine PR du précurseur Gag-Pol avant que la particule nouvellement relâchée devienne infectieuse (159, 160). Ce processus, s'effectuant durant et/ou immédiatement après la relâche virale, consiste en le clivage d'une manière séquentielle des précurseurs polyprotéiques Gag et Gag-Pol par PR pour ainsi permettre la formation des protéines virales MA, CA, NC, p6, PR, RT et IN. La première étape de ce processus est suggérée comme étant intramoléculaire, permettant ainsi la relâche de PR du précurseur Gag-Pol (161). La disponibilité stérique et la spécificité des sites de reconnaissance par PR dictent l'ordre dans lequel seront clivées les différentes sous-unités des précurseurs polyprotéiques (162, 163). La maturation de la particule virale mène à la formation du cœur viral, dont le composant majeur est la CA. Des études par microscopie électronique ont démontré que les virions immatures présentent un assemblage radial des précurseurs Gag et Gag-Pol et que cet assemblage était délaissé pour donner place à la forme conique caractéristique des rétrovirus dans les particules matures (164-166). Il est suggéré que le réarrangement de la lattice de CA nécessite sa désorganisation et sa restructuration autour des complexes NC-ARN (167), mais des études par imagerie moderne suggèrent plutôt une restructuration graduelle en forme de feuille couvrant le cœur viral (168, 169). Une fois le processus de maturation complété, la particule virale nouvellement synthétisée est infectieuse.

1.1.6 *La pathogénèse du VIH-1*

Le VIH-1 se transmet lors du contact avec des liquides biologiques d'un individu. Ces fluides comprennent le sang, le sperme, les fluides vaginaux, ou encore le lait maternel. La

transmission du VIH-1 à l'échelle mondiale est principalement causée par des rapports sexuels non protégés mais peut également être occasionnée par l'utilisation d'aiguilles contaminées (surtout chez les utilisateurs de drogues par injection), la transfusion de sang contaminé et via une transmission verticale de la mère à l'enfant lors de l'accouchement ou l'allaitement. Ces transmissions s'effectuent dans la majorité des cas (60 à 80 %) par un isolat viral nommé virus transmis / fondateur (170, 171).

Les cellules cibles pour le VIH-1 comprennent différents types cellulaires, incluant les lymphocytes T CD4+, les monocytes / macrophages, les cellules dendritiques ainsi que des cellules microgliales du système nerveux central (172). L'infection par le VIH-1 ciblant majoritairement les piliers du système immunitaire, elle est caractérisée cliniquement par une diminution graduelle mais marquée des lymphocytes T CD4 naïfs et mémoires ainsi qu'un état d'activation chronique du système immunitaire (173, 174). Prises ensemble, ces caractéristiques mènent à la détérioration et à la dysfonction des différents compartiments immuns ouvrant ainsi la porte à l'infection par des pathogènes opportunistes et aux complications cliniques incluant la mort des individus infectés.

1.1.6.1 Phases

L'infection au VIH-1 donne lieu à trois phases distinctes : la phase aigüe (primo-infection), la phase chronique (asymptomatique) et la phase SIDA.

La phase aigüe comprend les premiers jours ou semaines suivant l'exposition de l'hôte au virus. Avec des symptômes rappelant ceux d'infections communes telles qu'une grippe ou une mononucléose (175, 176), cette phase passe souvent inaperçue aux yeux de l'individu nouvellement infecté. Le site initial de l'infection se situe généralement au niveau des muqueuses mais le VIH est rapidement disséminé aux tissus lymphoïdes. La phase aigüe de l'infection au VIH-1 est associée par une charge virale extrêmement élevée (entre 10^6 et 10^7 copies d'ARN viral par ml de sang), une dissémination du virus dans l'organisme et une diminution marquée des niveaux de lymphocytes T CD4+ (177, 178). Cette déplétion se fait non seulement au niveau des T CD4+ périphériques mais essentiellement dans les tissus

lymphoïdes associés au tractus gastro-intestinal (GALT, *gut-associated lymphoid tissue*) (179, 180), où près de 30 à 60 % des cellules T CD4+ deviennent productivement infectées et sont éliminées en seulement quatre jours lors d'études *in vivo* dans un modèle d'infection au VIS chez les macaques (181, 182). L'analyse des marqueurs immunologiques des cellules permissives et déplétées par l'infection au VIS suggère que les cellules T CD4+ mémoires actives ou non (*resting*) constituent le compartiment le plus vulnérable lors de la phase aigüe de l'infection (182). La fin de cette phase initiale de l'infection au VIH-1 est marquée par un certain contrôle immunologique du virus (183), diminuant ainsi la charge virale dans le sang périphérique et rétablissant partiellement les niveaux de cellules T CD4+ circulantes. L'importance relative dans la réduction de la virémie initiale de l'efficacité de la réponse immunitaire montée contre le VIH-1 et la diminution de la quantité de cellules cibles permissives est cependant toujours sujette à débat (184, 185). C'est également durant cette phase qu'un réservoir latent de cellules infectées (majoritairement des cellules CD4 mémoires non-activées) est établi (186, 187). Ces cellules ne sont toutefois pas infectées de manière productive et ne sont ainsi pas susceptibles à l'action des traitements antirétroviraux standards et à l'action du système immunitaire. Ce réservoir est maintenu dans l'organisme par la persistance des cellules T et leur capacité à proliférer et maintenir un état d'homéostasie (188). Il est donc considéré comme l'un des plus grands obstacles à l'éradication du VIH-1 chez un individu infecté.

Si la phase aigüe de l'infection au VIH-1 peut être caractérisée par une réplication virale massive et une activation importante du système immunitaire, la phase chronique est plutôt asymptomatique et peut durer de 8 à 12 ans (189, 190). Une réplication virale persistante continue de fournir la stimulation antigénique constante nécessaire à la sur-activation et à l'épuisement du système immunitaire. Initialement, il est constaté que le rythme de déplétion des cellules T CD4+ et la réplication virale arrive à un état d'équilibre apparent (191). Néanmoins, la balance tourne peu à peu en faveur du virus, les niveaux de T CD4+ continuent de diminuer à un rythme annuel de 25 à 60 cellules / mm^3 et la population virale devient de plus en plus hétérogène des suites de l'évasion de la réponse immunitaire et de l'établissement de réservoirs cellulaires et anatomiques permettant une réplication virale constante (192-196).

Lorsque laissée sans traitement, l'infection au VIH-1 atteint finalement une phase critique où le dérèglement du système immunitaire des suites de sa sur-activation constante et de la déplétion des lymphocytes T CD4+ permet un retour d'une charge virale élevée. Lorsque les niveaux de CD4+ chutent en dessous des 200 cellules / mm^3, un individu infecté est alors dans la phase SIDA. Il existe une forte corrélation entre les niveaux de CD4+ en périphérie et le risque relatif d'infection par des pathogènes opportunistes, avec le risque de présenter les complications les plus néfastes (telles que l'infection disséminée à *Mycobacterium avium* ou une réactivation de l'infection au CMV) présent à des niveaux de moins de 50 cellules / mm^3 (197, 198). La dérégulation du système immunitaire chez les patients infectés au VIH-1 corrèle aussi avec le risque de développer certains cancers caractéristiques tels que le sarcome de Kaposi et le lymphome de Hodgkin (199).

1.1.6.2 Élimination des lymphocytes T CD4+ infectés et non infectés

L'élimination des lymphocytes T CD4+ naïfs et mémoires constitue l'un des piliers de la pathogénèse de l'infection au VIH-1. Étant impliqués dans l'orchestration des réponses immunes adaptatives humorale et cellulaire, la diminution de la quantité et de la qualité des lymphocytes T CD4+ mène à l'apparition des symptômes typiques du stade SIDA tels que l'infection aux pathogènes opportunistes et aux cancers caractéristiques. Plus particulièrement, la destruction des cellules T CD4+ dans le GALT est suggérée comme jouant un rôle important dans l'activation chronique du système immunitaire observée pendant la phase chronique de l'infection au VIH-1. Ceci s'explique entre autres par le fait que la majorité des lymphocytes T CD4+ CCR5+ mémoires activés résident dans ces tissus lymphoïdes et représentent des cellules permissives au virus qui sont majoritairement (environ 80%) éliminées en trois semaines après l'infection (179, 200, 201). De ces cellules, on peut identifier une population hautement permissive : les lymphocytes T auxiliaires Th17, cellules responsables de la production d'interleukine 17 et 22 (200, 202, 203). Une étude comparant les caractéristiques de l'infection pathogénique et non pathogénique au VIS chez le singe a entre autres révélé que cette élimination des Th17 s'effectue uniquement dans les cas de pathogénicité (200). Ces cellules ont pour rôle de participer au maintien de l'homéostasie intestinale par la production de cytokines et défensines anti-microbiennes, leur dysfonction

entraînant ainsi une perte graduelle de l'intégrité de la barrière gastro-intestinale (204-207). Cette perte du contrôle immunitaire au niveau de la barrière gastro-intestinale mène à la translocation de produits microbiens tels que le lipopolysacharride (LPS) vers la circulation sanguine, favorisant ainsi une activation systémique de l'immunité, un renouvellement des cellules immunitaires susceptibles à l'infection et donc à l'éventuelle réplication accrue du virus.

Les mécanismes moléculaires favorisant l'élimination des cellules T CD4+ au cours de l'infection au VIH-1 demeurent incertains et sources de débats dans la communauté scientifique (208-210). Il est connu que l'infection au VIH-1 induit d'une manière directe la mort des cellules T CD4+ infectées par divers mécanismes incluant une cytotoxicité induite par les glycoprotéines d'enveloppe, l'apoptose occasionnée par l'activation des caspases et des réponses aux dommages à l'ADN (211-214) ou encore par la réponse immunitaire des cellules T cytotoxiques CD8+ directe contre les cellules infectées (215). Néanmoins, il est suggéré qu'une forte proportion des cellules T CD4+ éliminées au cours de l'infection au VIH-1 n'est pas infectée (216). Les mécanismes proposés pour expliquer la mort des cellules non-infectées incluent notamment la surexpression de ligands de mort programmée (211) ou le dérèglement de l'homéostasie cellulaire dû à un état d'hyper-inflammation (217) causés par l'activation chronique du système immunitaire, l'effet cytotoxique direct de certaines protéines virales telles que Tat, Vpr, Nef et la gp120 (218-220), ou encore par la pyroptose caspase-1-dépendante induite lors d'infections abortives (211, 221, 222). Un nouveau mécanisme impliquant la relâche de gp120 par les cellules infectées et induisant la reconnaissance et élimination des cellules CD4+ non-infectées par la réponse cytotoxique dépendante des anticorps (ADCC, *antibody-dependent cell-mediated cytotoxicity*) est présenté dans le chapitre 4.

1.1.6.3 Traitements et approches cliniques

L'arrivée de combinaisons efficaces de molécules antirétrovirales en clinique a fait en sorte que le taux de mortalité associé à l'infection au VIH-1 a chuté de plus de 80 % dans les pays développés (223-225). À l'heure actuelle, il existe plus de 30 molécules antirétrovirales

approuvées pour leur utilisation clinique qui se répartissent dans six classes distinctes pouvant être combinées. La première molécule à être approuvée par la *Federal Drugs Administration* (FDA) américaine en 1987 est la zidovudine, également mieux connue sous le nom de azidothymidine (AZT) (226). Appartenant à la classe des inhibiteurs nucléotidiques de la transcriptase inverse (NRTI, *nucleoside and nucleotide RT inhibitors*), la zidovudine est un analogue de la thymidine ne comprenant pas de groupement –OH en 3', engendrant ainsi l'arrêt de la synthèse du brin d'ADN complémentaire lorsqu'elle est intégrée dans celui-ci par la RT (227). La RT est également ciblée par des inhibiteurs non-nucléotidiques (NNRTI, *non-nucleoside RT inhibitors*) qui s'insèrent dans un site potentiel de liaison de molécules de la RT et induisent des changements de conformation dans la RT qui inhibe son activité (228, 229). Les NNRTI constituent une classe de composés antirétroviraux de choix pour les traitements de première ligne et dans la prévention de la transmission de la mère à l'enfant.

Le processus d'entrée et de fusion viral est également une cible importante pour les composés antirétroviraux. Bien que le traitement par une forme soluble de CD4 (sCD4) n'a démontré qu'une efficacité limitée pour la neutralisation de particules virales (230, 231), de petits composés chimiques ont été développés et présentent un fort potentiel (232). Ces composés et leur implication dans les changements de conformation de l'Env virale sont davantage décrits dans le chapitre 4. Néanmoins, un anticorps monoclonal humanisé, l'Ibalizumab, liant le domaine 2 de la portion extracellulaire de CD4, agit comme un inhibiteur de l'entrée virale après l'attachement de la particule virale et a fait l'objet d'études cliniques (233, 234). Maraviroc, un composé antagoniste du corécepteur viral CCR5 empêchant l'interaction de la gp120 avec celui-ci, a été démontré comme pouvant inhiber de manière significative la réplication virale dans des études cliniques (235, 236). Probablement l'un des inhibiteurs de la fusion virale les plus connus, l'enfuvirtide (T-20) est un peptide synthétique de 36 acides aminés qui lie la boucle HR1 de gp41 et empêche la formation du paquet de six hélices (237, 238).

D'autres protéines virales telles que la PR et l'IN sont également les cibles de composés antirétroviraux très efficaces (239-241).

1.1.7 *Rôle des protéines accessoires dans la réplication et la pathogénèse virale*

1.1.7.1 Nef

Nef est une protéine myristoylée de 27 kDa qui est exprimée précocement dans le cycle de réplication viral (242). Elle joue de multiples rôles importants dans la pathologie de l'infection au VIH-1 et augmente la réplication et l'infectivité virales. Bien que facultative pour l'infectivité *in vitro* du VIH-1, des individus infectés par des virus codant pour une forme inactive de Nef ne présentent pas ou progressent très lentement vers les symptômes de la maladie qui sont normalement observés (243-246). De multiples fonctions ont été associées à Nef : 1) le contrôle de l'expression de surface de différents marqueurs tels que CD4, le complexe majeur d'histocompatibilité de classe I (CMH-I), CD8, le complexe du récepteur des cellules T CD3, et la molécule de co-stimulation CD28; 2) l'augmentation de l'infectivité virale; et 3) la modulation de l'activation cellulaire.

Nef, de manière conjointe avec Vpu, diminue la quantité de récepteur CD4 présente à la surface des cellules infectées en interagissant de manière précoce avec la queue cytoplasmique de CD4. Cette interaction mène au recrutement de la protéine adaptatrice-2 (AP-2), induisant ainsi l'endocytose clathrine-dépendante et la dégradation lysosomale de CD4 (247-250). La protéine β-COP (*coatomer protein complex subunit beta*) est également essentiel pour le transport vers et la dégradation de CD4 dans les lysosomes (251). On pense que le retrait de CD4 de la surface cellulaire limite la surinfection et les effets négatifs de la forte présence de CD4 sur l'incorporation d'Env dans les particules virales et leur relâche (252, 253).

Nef participe à la protection des cellules infectées contre la réponse immunitaire. Pour se faire, Nef limite l'expression de surface du CMH-1 en recrutant la protéine adaptatrice-1 (AP-1) et β-COP (251, 254, 255). Le retrait sélectif des protéines HLA-A et HLA-B, sans affecter les niveaux de HLA-C et HLA-E, permet de limiter la susceptibilité des cellule infectées à la réponse des lymphocytes T cytotoxiques tout en gardant l'interaction de HLA-C et HLA-E avec les récepteurs inhibiteurs des cellules NK (256, 257). Nef diminue également l'expression des ligands des récepteurs activateurs des cellules NK NKG2D, DNAM-1 et

NKp44 (258-260). De plus, Nef affecte négativement la réponse immunitaire adaptative en diminuant l'expression de surface des molécules de CMH-II, limitant ainsi la présentation antigénique par les cellules infectées (261).

Les cellules dendritiques (CDs) capturent des particules virales infectieuses via un récepteur spécifique (DC-SIGN) et peuvent les transmettre à des cellules cibles permissives (262, 263). Nef, en augmentant les niveaux de DC-SIGN à la surface des CDs, pourrait ainsi faciliter la transmission virale (264).

Le mécanisme par lequel Nef augmente l'infectivité *in vitro* du VIH-1 d'une manière indépendante de CD4 a récemment été élucidé. Plusieurs possibilités avaient été émises par la communauté scientifique, incluant l'implication de la dynamine et l'altération générale des endosomes précoces et de leur recyclage (265, 266). Deux études concomitantes ont révélé que Nef empêcherait l'incorporation des protéines cellulaires SERINC3 et SERINC5 (*serine incorporator 3 and 5*) dans la particule virale, prévenant ainsi un blocage précoce dans le cycle réplicatif au niveau post-entrée (267, 268).

Finalement, Nef influence les voies de transduction des signaux cellulaires et affecte l'état d'activation des cellules infectées. Le mécanisme par lequel Nef module l'activation cellulaire est controversé, différentes études exposant des observations contradictoires malgré une approche expérimentale similaire (269). Il est néanmoins de plus en plus accepté que Nef formerait un complexe de signalisation en interagissant, entre autres, avec la kinase Ser/Thr activée par la protéine 21 (*p21-activated kinase,* PAK2) au niveau des radeaux lipidiques (270-272). Ce complexe occasionnerait l'augmentation de l'expression de NFAT, induisant l'activation cellulaire et la transcription du génome viral (273).

1.1.7.2 Vif

Vif est une protéine virale de 23 kDa qui est exprimée tardivement dans le cycle de réplication viral. Elle joue un rôle critique dans la production de particules virales infectieuses par certains types cellulaires dont les lymphocytes T CD4+ ou macrophages primaires en plus

de quelques lignées de cellules T (274). La comparaison de cellules permissives ou non à la production de particules virales infectieuses en absence du gène *vif* a permis d'identifier le facteur cellulaire APOBEC3G (*apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G*) comme étant responsable de ce phénotype dominant (275-277).

APOBEC3G est une enzyme appartenant à la famille des cytidine désaminases qui catalyse le changement de bases cytidines en uraciles (mutation C à U) dans l'ADN simple brin. En absence de Vif, APOBEC3G est incorporée dans les virions et, suivant l'entrée de la particule virale dans une nouvelle cellule cible, s'attaque à l'ADN viral en cours de synthèse par la RT (278-280). Cette attaque induit l'accumulation de mutations C à U dans le brin de polarité négative de l'ADN viral, se traduisant ainsi par une signature typique d'hypermutations G à A sur le brin de polarité positive qui va par la suite coder pour des protéines virales défectueuses (281-283). De plus, APOBEC3G pourrait directement inhiber l'étape de transcription inverse en réduisant la capacité de translocation de la RT le long de l'ARN viral (284). D'autres membres de la famille APOBEC, dont APOBEC3F, sont également aptes à inhiber la réplication du VIH-1 en absence de Vif (285, 286). Enfin, pour empêcher APOBEC3G d'être incorporé dans la particule virale, Vif interagit directement avec le facteur de restriction et recrute la E3 ubiquitine ligase Cullin5 (287). Le complexe occasionne alors la poly-ubiquitination d'APOBEC3G et cible ce dernier pour être dégradé par la machinerie du protéasome (287, 288).

1.1.7.3 Vpr

Vpr est une protéine accessoire de 14 kDa qui est hautement conservée chez les lentivirus de primates (289). Une interaction avec la protéine p6 permet son incorporation dans les particules virales (290-293), suggérant qu'elle peut jouer un rôle important dans les étapes précoces du cycle de réplication avant la synthèse *de novo* des protéines virales.

L'une de ces fonctions les mieux connues est certainement la capacité de Vpr à induire un arrêt du cycle cellulaire en phase G2/M (294-296). Il a été démontré que les protéines Vpr incorporées dans les particules virales, et non celles produites *de novo*, sont responsables et

suffisantes pour induire l'arrêt de cycle (297). Le mécanisme sous-jacent a été partiellement élucidé, mais demeure controversé et impliquerait la voie de dommage à l'ADN via l'activation de la protéine ATR (*ataxia telangiectasia-mutated and Rad3-related*) (298, 299). Très récemment, une étude a démontré que Vpr, en liant VprBP (*Vpr binding protein*), permet de recruter le complexe E3 ubiquitine ligase Cul4/DDB1/VprBP et d'occasionner la poly-ubiquitination et la dégradation de la protéine MCM10 (300). Bien que le substrat de Vpr n'ait été que récemment identifié, plusieurs études avaient suggéré qu'un tel mécanisme serait impliqué dans l'activation de la voie ATR et dans l'induction de l'arrêt de cycle en G2/M (301-303). De plus, une étude récente a identifié Vpr comme étant responsable, d'une manière qui nécessite l'activation de la voie ATR, de l'augmentation d'expression des ligands du récepteur activateur des cellules NK, NKG2D, augmentant ainsi la susceptibilité des cellules infectées ou non à la reconnaissance et lyse par les cellules NK (220).

Les autres fonctions de Vpr impliquent notamment sa capacité à activer la transcription au niveau des promoteurs viraux (le LTR) ainsi que certains promoteurs de l'hôte (290, 304). Cette augmentation de la transcription virale serait plus particulièrement en lien avec l'ADN viral non intégré ou en voie de l'être (305, 306), bien que d'autres études aient démontré un lien entre Vpr et des facteurs de transcriptions canonicaux du LTR viral tels que SP1 (307, 308). De plus, Vpr pourrait induire l'apoptose des cellules infectées ou qui se retrouvent en présence de cette protéine (via son incorporation dans les particules virales défectueuses) (309-312). Cette activité semble néanmoins découler de la capacité de Vpr à induire l'arrêt de cycle en phase G2/M puisqu'elle semble être dépendante de l'activation de la voie d'ATR et de la phosphorylation de l'un de ses substrats, BRCA1 (310, 311). Il en découle une régulation positive de GADD45α et l'induction subséquente de l'apoptose via la perméabilisation de la membrane mitochondriale par la protéine proapoptotique Bax (310, 311).

Vpr serait par ailleurs impliquée dans l'augmentation de la fidélité de la RT via une interaction spécifique avec l'enzyme uracile glycosylase UNG2 (313). Ceci permettrait l'incorporation d'UNG2 dans la particule virale et, au moment de l'étape de transcription inverse, cette enzyme pourrait limiter l'incorporation d'uracile dans l'ADN viral et ainsi augmenter la fiabilité de la RT (314-316). Finalement, et tel que mentionné dans la section

1.1.5.2, Vpr pourrait faciliter la translocation du PIC vers le noyau cellulaire (317, 318). Ce phénomène serait facilité par l'interaction de Vpr avec l'importine alpha et serait essentiel au transport nucléaire du PIC dans les macrophages (319-321).

1.1.7.4 Vpu

Vpu est une protéine transmembranaire de type 1 de 16 kDa unique au VIH-1 et quelques VIS (20, 322, 323). Elle se caractérise par un domaine N-terminal transmembranaire hydrophobe et un domaine C-terminal cytoplasmique hydrophile (324, 325). Au sein de son domaine cytoplasmique, deux sérines phosphorylées (S52 et S56) semblent être des déterminants majeurs de l'activité biologique de Vpu (326). Ainsi, Vpu est responsable de la dégradation de CD4 (de concert avec Nef et Env) et de l'inhibition du facteur de restriction BST-2/tétherine (327, 328).

Tout d'abord, l'activité de Vpu, contrairement à celle de Nef, s'effectue sur les protéines CD4 nouvellement synthétisées lors de leur passage dans le réticulum endoplasmique (RE). Afin de favoriser la dégradation de CD4, Vpu interagit avec la queue cytoplasmique du récepteur puis recrute le complexe E3 ubiquitine ligase SCF (Skp1/Cullin 1/F-box) via une interaction avec β-TrCP (329-331). Ce complexe est alors responsable de la polyubiquitination de la queue cytoplasmique de CD4, favorisant ainsi sa reconnaissance et dégradation par le protéasome dans la voie ERAD (*ER-associated protein degradation*) (329, 332-335).

Ensuite, Vpu est un élément clé favorisant la relâche des particules virales. En effet, en absence de Vpu, il a été démontré que le facteur de restriction BST-2/tétherine occasionne une accumulation drastique de particules virales bourgeonnées à la surface cellulaire (327, 328). Pour se faire, BST-2 possède un domaine transmembranaire et une ancre GPI (glycosylphosphatidylinositol) lui permettant de s'ancrer à la fois dans les membranes cellulaires et virales (327, 328). Le mécanisme par lequel Vpu empêche cette activité antivirale est toujours mal compris, mais il est suggéré que Vpu pourrait déplacer BST-2 hors du site d'assemblage viral en occasionnant sa séquestration ou sa dégradation endosomale ou lysosomale suite à l'interaction entre leurs domaines transmembranaires (336-341). Certaines

études suggèrent que cette séquestration pourrait s'effectuer sur le BST-2 nouvellement synthétisé (342, 343), alors que d'autres suggèrent qu'elle s'effectuerait au niveau des endosomes de recyclage après l'internalisation de BST-2 (344). De plus, Vpu semble également induire l'ubiquitination de BST-2 via son interaction avec β-TrCP (345). Cependant, deux isoformes distincts de BST-2 ont été identifiés et semblent montrer une susceptibilité différente à l'inhibition par Vpu (346, 347).

Une autre fonction mal connue de Vpu est sa capacité à diminuer la réponse immunitaire des cellules NK contre les cellules infectées. En effet, il a été démontré que Vpu engendre la diminution de l'expression de surface de CD1d (348) et de ligands de NTB-A (349), contribuant ainsi à diminuer la reconnaissance des cellules infectées par les cellules NK. Vpu, par son activité sur BST-2, aiderait également à limiter l'activation des réponses immunitaires innées via la voie NF-κB (350).

1.1.8 *Les glycoprotéines d'enveloppe virales*

Les glycoprotéines d'enveloppe sont un déterminant majeur de l'infectivité et de la pathogénicité du VIH-1. En ce sens, elles sont responsables de l'interaction initiale de la particule virale avec la cellule cible et représentent le seul antigène viral présent à la surface des cellules infectées (351).

Les glycoprotéines gp120 et gp41 sont le fruit du clivage de gp160 (voir Figure 1.5), synthétisé à partir d'un ARNm mono-épissé codant aussi pour la protéine Vpu. Cet ARNm contient un peptide signal qui sera clivé post-traduction et sert à cibler le transport de la protéine en voie d'être traduite au RE (352). Sa traduction est caractérisée par un complexe processus de glycosylation et d'association à des chaperonnes cellulaires telles que BiP/GRP78, la calréticuline et la calnexine (353-355). Ces chaperonnes sont essentielles à l'adoption d'une structure tertiaire adéquate et à l'oligomérisation de gp160 en trimères. Ces derniers sont alors transportés jusqu'à l'appareil de Golgi où des protéases cellulaires de la famille des furines clivent le précurseur en ses deux formes distinctives gp120 et gp41 (42, 356, 357) au niveau d'un site consensus RX(K/R)R de la gp120 (358, 359). La gp120 demeure

ensuite reliée à la portion transmembranaire gp41 via des interactions non-covalentes (360-363).

La détermination de l'organisation structurelle du trimère d'Env natif constitue une épreuve qui demeure à être surmontée. En effet, bien que plusieurs études aient tiré profit d'un éventail de différentes techniques pour résoudre des portions de cette organisation, l'information disponible demeure à ce jour incomplète. De multiples défis se présentent aux scientifiques s'attaquant à la caractérisation de l'organisation structurelle d'Env : sa forte glycosylation, sa haute flexibilité, la propension de la gp120 de se détacher de la sous-unité gp41 et, finalement, le fait que la gp41 soit attachée à la membrane cellulaire ou virale. Les premières structures à avoir été résolues étaient basées sur des monomères de gp120 mutés souvent liés à des anticorps monoclonaux ou à CD4 afin de limiter leur flexibilité (364, 365). Celles-ci ont permis de mieux comprendre comment la gp120 interagit avec CD4, cache ses sites conservés d'interaction avec le récepteur et le corécepteur par la présence de boucles variables glycosylées au pourtour de ces sites conservés, créant ainsi un bouclier de glycans. Il est donc appréciable que l'épitope reconnu par plusieurs anticorps neutralisants ayant un large spectre d'activité comprend en partie certains glycans (366).

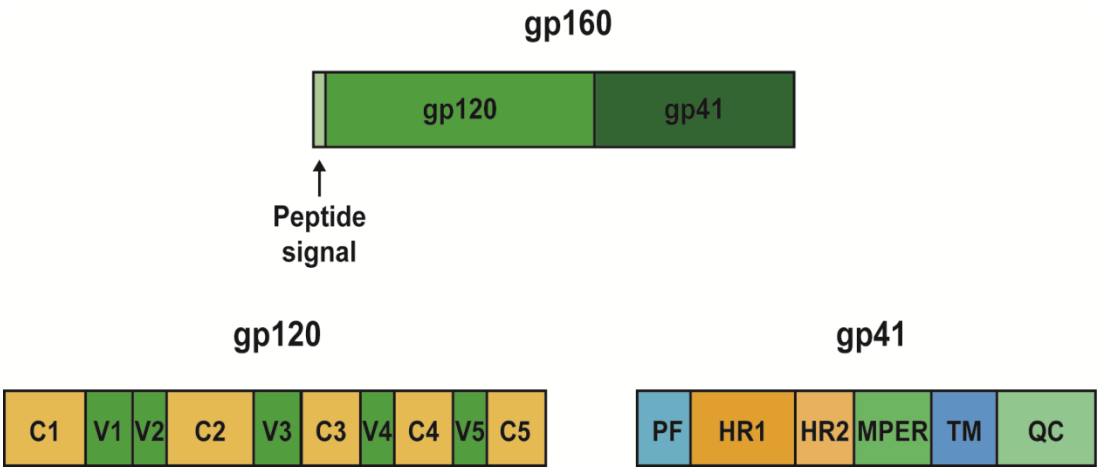

Figure 1.5 **La protéine d'enveloppe du VIH-1 : la gp160 et ses dérivées gp120 et gp41**
Schéma illustrant l'organisation de la gp160 et de ses sous-produits gp120 et gp41. PF : peptide de fusion, HR1 : *heptad repeat 1*, HR2 : *heptad repeat 2*, MPER : *membrane-proximal external region*, TM : domaine transmembranaire, QC : queue cytoplasmique.

Néanmoins, ces constructions ne constituent pas l'organisation structurelle native du trimère d'Env et donc des efforts continuent d'être mis de l'avant afin d'obtenir cette structure. À cette fin, de récentes études ont publié une structure d'une construction trimèrique soluble (367, 368). Celle-ci présente une gp41 clivée au résidu 664 lui permettant ainsi de ne pas être ancrée à la membrane cellulaire. De plus, deux cystéines sont introduites en positions 501 (gp120) et 605 (gp41), ce qui permet la création d'un pont disulfure entre les deux résidus attachant par le fait même les deux sous-unités. Finalement, afin de favoriser l'association trimèrique de cette construction, une mutation dans HR1 gp41 (I559P) est utilisée (SOSIP). Bien que cette construction et les études structurelles l'employant donne un aperçu de l'organisation d'Env sous forme trimèrique et représentent un avancement majeur pour le domaine de recherche, il est possible que l'introduction d'une proline dans une hélice alpha de HR1 (I559P) affecte la conformation de la protéine, rendant ainsi questionnable l'importance biologique des structures résolues (369). Néanmoins, ces études permettent d'apprécier comment les régions variables V_1, V_2 et V_3 sont situées à l'apex du trimère d'Env et semblent participer au maintien du trimère. Plus de détails sur la structure du domaine interne de la gp120 sont donnés dans la deuxième section de ce chapitre.

1.1.8.1 Gp120

Au sein du trimère mature d'Env, la gp120 se retrouve attachée par des interactions non-covalente à la glycoprotéine transmembranaire gp41. Cette protéine de surface est caractérisée par une forte glycosylation facilitant le masquage des régions à potentiel antigénique (370, 371). La gp120 possède cinq régions variables (V_1 à V_5) intercalées au sein de cinq régions conservées (C_1 à C_5) (voir Figure 1.5) (372). C'est dans ces régions conservées qu'on retrouve des structures nommées « couches topologiques », qui sont essentielles à la transition de conformations d'Env engendrée par la liaison avec CD4 (362, 373, 374).

Le domaine interne, dont font partie les couches topologiques, est responsable de l'interaction et de l'association avec la gp41 (361, 362, 365, 375, 376). Les études structurelles de la gp120 en liaison avec CD4 ou divers anticorps ont démontré que ses N- et C-terminaux forment un domaine rapproché qui émane du domaine interne de la gp120 vers la membrane virale et la gp41 (376). Aussi trouvés dans le domaine interne sont deux domaines : 1) l'assemblage de feuillets β nommé le β-sandwich, qui semble également contribuer à l'association avec gp41 (361, 363); et 2) les trois boucles formant les couches topologiques mobiles (362). D'un point de vue antigénique, le domaine interne de la gp120 offre des épitopes hautement conservés (377). Néanmoins, la majorité des anticorps ciblant ce domaine sont caractérisés comme étant non-neutralisants, et il est suggéré que ces anticorps ne reconnaissent pas le trimère d'Env natif non lié à son récepteur car leur épitope se retrouve au sein du complexe trimèrique (voir chapitre 2). Les épitopes du domaine interne sont exposés lors de l'interaction avec le récepteur, mais le processus d'entrée virale semble être trop avancée pour permettre la neutralisation du virus par ce type d'anticorps (378). Ces anticorps, qui sont dits « induits par CD4 » (CD4i), reconnaissent aussi des épitopes précocement exposés après l'interaction avec CD4 mais avant le contact avec le corécepteur. Puisque ces anticorps ciblent une région formée de la région conservée C_4 et des boucles variables V_1 et V_2, responsables de la liaison avec le corécepteur, ils sont nommés *co-receptor binding site antibodies* (58, 365). Quelques sites de vulnérabilité ciblés par des anticorps neutralisant un large spectre d'isolats viraux ont été identifiés sur le trimère d'Env natif, notamment le site de liaison à CD4 (379), le domaine V1/V2, les glycans de la boucle V3, ainsi que l'interface entre

gp120 et gp41 (380). Le Tableau 1.1 donne un exemple de quelques anticorps neutralisants à large spectre pour chacune des catégories des sites de vulnérabilité d'Env.

Tableau 1.1
Exemples d'anticorps neutralisant à large spectre et le site de vulnaribilité qu'ils reconnaissent

Anticorps	Spectre de neutralisation	CI_{80} (µg / ml)
Site de liaison à CD4 (gp120)		
b12	33 %	2,70
VRC01	87 %	0,98
8ANC131	57 %	4,02
V1/V2 (gp120)		
PG9	70 %	0,31
PGT145	60 %	0,31
Glycans V3 (gp120)		
2G12	18 %	4,85
PGT121	53 %	0,08
PGT135	56 %	0,11
MPER (gp41)		
2F5	48 %	9,42
4E10	88 %	8,98
10E8	97 %	2,05

CI_{80} : Concentration inhibitrice 80 %

Adapté de Kwong, Mascola, Nabel. Nat Rev Immunol. Septembre 2013 (381)

1.1.8.2 Gp41

La gp41, portion transmembranaire de la protéine Env, est caractérisée par trois domaines : l'ectodomaine en N-terminal, le domaine transmembranaire et le domaine cytoplasmique.

Tout d'abord, l'ectodomaine de gp41 contient les éléments constitutifs de la machinerie de fusion membranaire. Ceux-ci semblent être similaires à la protéine HA du virus influenza (66, 364, 382, 383) et il a été démontré que l'exposition du peptide de fusion ne peut s'effectuer en absence de la transmission d'un signal via gp120 de la liaison du récepteur CD4 (62). Tel que discuté dans la section 1.1.6.3 sur les traitements et approches cliniques, l'ectodomaine de gp41 est la cible du peptide antiviral T20 (384). De plus, l'ectodomaine de gp41 forme une région, nommée la région proximale à la membrane (MPER), qui est la cible d'anticorps neutralisants à large spectre (385).

Pour sa part, le domaine transmembranaire de gp41 comprend 21 résidus qui fixent Env à la membrane cellulaire (386). Bien qu'étant controversé suite à des études contradictoires (387), ce domaine pourrait avoir des implications dans le processus de fusion d'Env (388).

Finalement, le domaine cytoplasmique de gp41 est un long domaine de 150 résidus se retrouvant près de la membrane cytoplasmique interne. Tel que discuté dans la section 1.1.5.3, cette région, aussi appelée queue cytoplasmique, semble importante pour l'incorporation d'Env dans la particule virale. Fait intéressant, la longue queue cytoplasmique est un élément fréquent chez les lentivirus mais très peu répandu chez les rétrovirus, suggérant que celle-ci peut jouer un important rôle dans la réplication et le contrôle de la réponse immunitaire contre le virus (389). De plus, il est maintenant mieux apprécié comment la queue cytoplasmique permet à Env d'être internalisée de la surface cellulaire afin de limiter son exposition et sa reconnaissance par le système immunitaire (390, 391).

1.1.9 *Stratégies vaccinales contre le VIH-1*

Très tôt après la reconnaissance que le SIDA était causé par un agent pathogène, les agences gouvernementales ont déclaré que des vaccins seraient prêts pour études préliminaires dans les années suivantes l'isolation du VIH-1. Ce vaccin se fait toujours attendre 30 ans plus tard. Néanmoins, plusieurs essais cliniques de vaccins candidats ont été réalisés depuis ce temps.

Les stratégies vaccinales contre le VIH-1 peuvent jusqu'à ce jour se diviser en trois catégories : 1) la mise en place et le maintien d'une réponse humorale protectrice (basée sur la génération d'anticorps neutralisant ou induisant une réponse effectrice) ou 2) d'une réponse cellulaire cytotoxique CD8+ et 3) la combinaison des deux stratégies précédentes (392). Ces approches sont basées sur l'utilisation d'une panoplie (au moins 12 différentes) de stratégies vaccinales et vecteurs viraux pour l'expression antigénique de diverses portions du VIH-1, incluant Env (gp120 recombinante) et les gènes *gag*, *pol,* et *nef* (393). Cependant, à ce jour seulement quatre approches vaccinales différentes ont été testées dans six essais d'efficacité clinique (393, 394). Ces études passées ont certainement démontré à la communauté scientifique et médicale que le VIH-1 se veut plus complexe à combattre que d'autres pathogènes. Un des exemples les plus appropriés pour illustrer ce fait est clairement l'essai clinique HVTN 502, aussi connu sous le nom d'essais vaccinal STEP (*STEP trial*). Exécuté par Merck & Co, cette étude tentait de démontrer que l'utilisation d'un vecteur viral adénovirus de type 5 encodant les gènes *gag, pol* et *nef* similaires à la séquence consensus du clade B pourrait provoquer une réponse immunitaire à médiation cellulaire suffisante pour protéger contre l'acquisition du VIH-1 (395, 396). Cependant, il a été révélé que certains groupes d'individus vaccinés, notamment les hommes non-circoncis et ayant une immunité préexistante à l'adénovirus de type 5, ont semblé être, d'une manière passagère après la vaccination, plus susceptibles à l'infection au VIH-1 que les personnes ayant reçu le placebo (392, 396).

Néanmoins, un récent essai vaccinal contre le VIH-1 en Thaïlande, le RV144, a su démontrer une efficacité, quoique limitée, contre l'acquisition du VIH-1 (397). L'approche utilisée consistait d'un régime de vaccination avec deux vaccins différents. Tout d'abord, un vaccin basé sur un vecteur viral canarypox, l'ALVAC-HIV, fut administré aux mois 0, 1, 3 et 6 en plus d'un vaccin AIDSVAX B/E aux mois 3 et 6. L'ALVAC-HIV encode le gène *gag-pol* et le domaine transmembranaire de gp41 de la souche virale de clade B LAI ainsi que la portion gp120 du gène *env* de 92TH023, un isolat du clade CRF01-AE. Pour sa part, l'AIDSVAX B/E consiste de gp120 recombinante monomérique des isolats CM244 (du clade CRF01-AE) ainsi que de MN (clade B). L'efficacité vaccinale calculée à 42 mois post-vaccination était de 31.2%, alors qu'elle semblait être de 60% à 12 mois post-vaccination avec

un déclin en lien avec la diminution de la réponse immunitaire avec le temps (397-399). Malgré cette apparente efficacité dans la prévention de l'infection, le vaccin n'a pas eu d'effet sur la progression de la maladie chez les personnes vaccinées qui ont tout de même été infectées (400). La comparaison de la réponse immune observée deux semaines après la dernière vaccination chez 41 individus vaccinés ayant été infectés versus 205 vaccinés non-infectés ont permis d'identifier deux corrélats de protections principaux : 1) la présence d'IgA reconnaissant Env corrélait avec le risque d'infection; et 2) la protection corrélait avec la présence d'IgG reconnaissant une molécule présentant les épitopes des boucles variables V_1 et V_2 (401, 402). Plus particulièrement, la présence d'IgA semblait faciliter l'infection en diminuant la réponse immunitaire protectrice, notamment la réponse cytotoxique dépendante des anticorps (ADCC) et la capacité des anticorps à neutraliser le VIH-1 (403-406).

1.2 Les fonctions effectrices des anticorps

1.2.1 *Les généralités*

Autre leur capacité à neutraliser des pathogènes, les anticorps produits par le système immunitaire sont responsables de l'initiation d'une multitude de réponses immunes alliant les axes immunitaires humorale et cellulaire. Puisque cet ouvrage se centre sur une réponse effectrice des anticorps, l'ADCC, cette prochaine section introduira les différentes classes d'anticorps ainsi que leur structure, les mécanismes effecteurs que peuvent avoir les anticorps ainsi que les cellules responsables d'induire ces réponses immunes.

Les différents types d'anticorps présentent tous une structure similaire avec une variabilité accrue aux régions de reconnaissance de leur antigène. Les fonctions effectrices sont associées aux régions plus conservées. Un anticorps est constitué symétriquement de deux chaînes lourdes et légères reliées ensemble par des ponts disulfures (Figure 1.6). Chacune de ces chaînes présente des régions avec des unités homologues répétées d'environ 110 acides aminés, nommées domaines d'immunoglobuline (Ig). La chaîne lourde comporte une région variable (V) constituée d'un domaine Ig, et d'un domaine conservé (C) formé de trois ou quatre domaines Ig. Lors de la digestion de l'anticorps ainsi formé par la papaïne, il y

a formation de trois résidus : 2 fragments Fab formés des domaines variables ainsi qu'un fragment Fc formé des domaines conservés. C'est par ailleurs ce dernier qui est responsable des fonctions effectrices des anticorps. Pour sa part, la chaîne légère ne comporte qu'un domaine Ig dans son domaine V et C. Les domaines V forment le site de reconnaissance antigénique grâce à leur variabilité, mais elle est surtout présente au niveau de trois sous-régions hypervariables nommées *complementarity determining regions* (CDR).

De par les différences dans la région C des chaînes lourdes des différents anticorps, on peut reconnaître cinq classes d'anticorps : IgA, IgD, IgE, IgG et IgM. Les IgG sont de loin les plus abondants dans la circulation (70-75%), suivi des IgA et IgM (10-10%). Les IgD et IgE sont beaucoup moins fréquent avec moins de 1% du total de la fraction des immunoglobulines. Puisque la région C est responsable de l'interaction avec les cellules (phagocytes, cellules NK, etc.) ou protéines solubles effectrices (le complément), elle détermine ainsi la réponse effectrice possible pour chacune des cinq classes d'anticorps. En ce qui concerne cet ouvrage, un intérêt particulier est porté à la réponse ADCC, laquelle nécessite la région C des anticorps de type IgG.

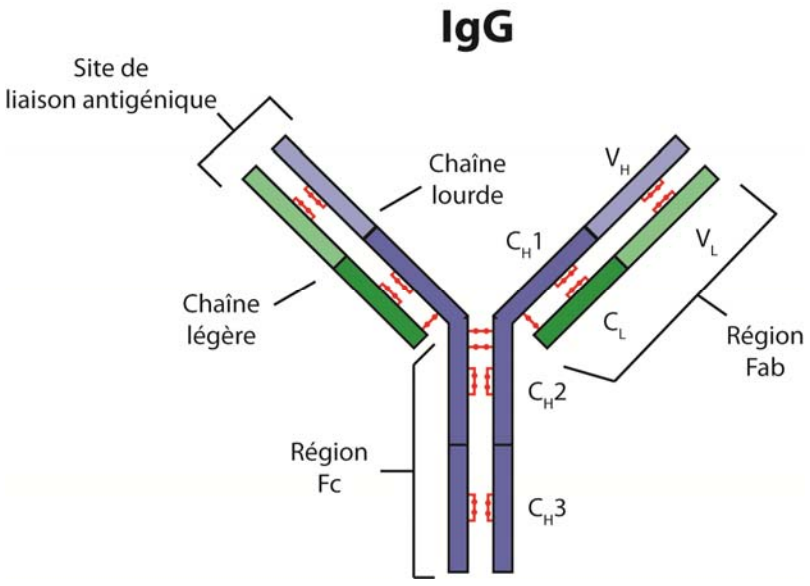

Figure 1.6 L'anticorps IgG et ses composantes
Schéma illustrant la composition structurale d'un anticorps de classe IgG. On dénote ses chaînes lourges et légères, reliées par des ponts dissulfures (rouge).

La région Fab, Fc etle site de laision antigénique y sont illustrés. Voir section 1.2.1 pour plus de détails.

La fonction la mieux connue des anticorps est certainement la neutralisation directe des pathogènes. Néanmoins, l'opsonisation de pathogènes permet d'engendrer un éventail de réponses immunes, notamment la phagocytose, la lyse du pathogène ou d'une cellule infectée des suites de la réponse ADCC, ou encore l'activation du complément. Ce dernier peut à son tour orchestrer la phagocytose des pathogènes opsonisés par des fragments du complément, inciter une réponse inflammatoire ou encore favoriser la lyse directe des pathogènes.

La fonction effectrice des anticorps à médiation cellulaire nécessite la reconnaissance de la région C des anticorps par un récepteur spécifique à la surface des cellules (les FcR). Ces derniers forment une vaste famille avec différentes implications dans les réponses immunes et sont exprimés à la surface d'une grande variété de cellules. À titre d'exemple, la phagocytose des pathogènes couverts d'anticorps s'effectue majoritairement par les macrophages et les neutrophiles grâce aux récepteurs FcγRI (CD64), FcγRIIA et FcγRIIC (CD32) présents à leur surface et qui interagissent avec la portion Fc des IgG (407). Pour sa part, la réponse ADCC est principalement induite lors de la reconnaissance d'IgG par le récepteur FcγRIIIA à la surface de cellules NK (*natural killers*), avec une plus faible contribution des macrophages et neutrophiles (407). Cette interaction entre la région Fc de l'anticorps et le récepteur cellulaire mène à l'activation des sous-unités CD3ζ et/ou FcεRIγ qui contiennent des motifs ITAM (*immunoreceptor tyrosine-based activation motif*) favorisant l'activation et la dégranulation de la cellule effectrice (408, 409). Un déterminant de l'activité effectrice des anticorps est le profil de glycosylation de leur région Fc (410). Plus particulièrement, la glycosylation de l'asparagine 297 affecte la conformation de l'anticorps et module son affinité pour les différents récepteurs Fc (411-413).

1.2.2 *Leurs implications dans l'infection au VIH-1 et la nécessité d'élargir les connaissances scientifiques*

Il est bien connu que l'infection au VIH-1 engendre l'activation de réponses effectrices des anticorps dépendantes d'une part du complément et d'une autre part des cellules

effectrices. D'un côté, il est suggéré que les anticorps liés aux particules virales contribuent à activer le système du complément jusqu'à l'induction du complexe d'attaque membranaire et la lyse de la particule virale ou cellule infectée (414, 415). Cependant, bien que des études aient démontré que des virions puissent être lysés *in vitro* (416, 417), l'importance biologique de l'activation du complément *in vivo* est toujours controversée. Ceci est dû principalement au fait que plusieurs virus, dont notamment le VIH-1, ont évolué des mécanismes afin de contrer ce type de réponse immunitaire (418). Dans le cas du VIH-1, les isolats primaires sont capables d'incorporer des molécules régulatrices du complément (CD46, CD55, CD59) lors du bourgeonnement hors de la cellule infectée et une protéine régulatrice soluble présente dans le sérum (le facteur H) peut lier directement la gp120 et gp41 (414, 419, 420), participant ainsi à protéger les virions et cellules infectées de la réponse du complément. De plus, la liaison d'anticorps et du complément à la surface des particules virales semblent, sous certaines conditions, augmenter l'infectivité du virus (421, 422).

La littérature concernant le rôle des fonctions effectrices des anticorps à médiation cellulaire dans le contrôle de l'infection au VIH-1 demeure à ce jour controversée. D'une part, des études suggèrent que la capacité de la portion Fc des anticorps à lier le récepteur FcR est essentielle pour leur rôle protecteur *in vivo*. Ceci a été illustré entre autres avec l'anticorps monoclonal b12, présentant ou non une capacité à lier le récepteur FcR, en comparant son efficacité à protéger des macaques contre l'infection (423). Ceci contredit cependant d'autres études réalisées chez les macaques et qui n'ont pas identifié de rôle de la réponse ADCC dans la protection des animaux contre l'infection au VIS (424-426). De plus, plusieurs études, mais pas toutes (427-429), ont démontré que les fonctions effectrices des anticorps semblent être liées à une charge virale réduite et à une progression de la maladie plus lente, ainsi qu'à une diminution du risque de transmission verticale de l'infection (430-436). Plus récemment, il a également été suggéré que chez le macaque, les anticorps non-neutralisants ne peuvent conférer une protection complète contre l'acquisition du virus mais exercent plutôt une pression sélective contre les souches virales utilisées pour infecter les animaux (437). De plus, de récents travaux ont suggéré que le profil de glycosylation des anticorps anti-VIH-1 influencerait leur capacité à induire une réponse effectrice mais les implications de ces travaux dans le design d'approches vaccinales sont toujours méconnues (438-440).

Cette controverse dans la littérature sur le rôle et les déterminants des réponses effectrices des anticorps au cours de l'infection au VIH-1 ouvre la porte aux travaux présentés dans cet ouvrage. Ils se concentrent donc sur **1)** la mise en place de techniques *in vitro* permettant de mesurer les changements de conformation d'Env et de déterminer la réponse ADCC d'anticorps reconnaissant des conformations précises d'Env, **2)** l'identification des déterminants viraux permettant la reconnaissance de ces antigènes d'Env à la surface des cellules infectées, **3)** déterminer comment il est possible de tirer avantage de ces stratégies virales d'évasion de la réponse ADCC et mieux comprendre leur rôle potentiel dans la pathogénèse virale. La prochaine section de ce chapitre continue d'introduire les notions essentielles à cet ouvrage, notamment concernant les différentes techniques *in vitro* de mesure de l'ADCC, les changements de conformation des glycoprotéines d'Env, la structure du domaine interne de la gp120 qui est la cible de plusieurs anticorps capables d'induire une réponse ADCC, l'hypothèse que le virus pourrait être transmis par des cellules provenant d'individus infectés, ainsi qu'un bref aperçu des travaux qui sont présentés dans les prochains chapitres.

ROLE OF ENV CONFORMATION AND ACCESSORY PROTEINS ON ADCC RESPONSES

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Cette section est une revue de la littérure pertinente aux travaux abordés dans les prochains chapitres de cet ouvrage et qui en résume les principaux points. Notre revue présente les plus récentes études, incluant certaines de celles couvertes par les prochains chapitres de cet ouvrage, concernant le rôle de la conformation de l'Env du VIH-1 et comment elle est modulée par les protéines accessoires Nef et Vpu. Un aperçu de différentes techniques pour mesurer l'ADCC y est également présenté. De plus, l'article explore l'hypothèse que des cellules infectées dites « chevaux de Troie » pourraient être à la base de la transmission du virus et comment des anticorps et les fonctions effectrices associées pourraient être impliqués dans la limitation de la transmission mucosale du virus.

Marzena Pazgier et George K. Lewis ont écrit la section sur l'information structurale des épitopes dits du groupe A et ont réalisé la modélisation présentée en Figure 1 de l'article. Matthew S. Parsons a écrit la section sur l'hypothèse des cellules chevaux de Troie et du rôle des anticorps et de leurs fonctions effectrices dans la transmission muquosale du virus. Jonathan Richard a écrit la section résumant les techniques *in vitro* de mesure de la réponse ADCC. Maxime Veillette a écrit les sections sur les changements de conformation d'Env et

sur le rôle des protéines accessoires Nef et Vpu dans la réponse ADCC. Andrés Finzi a contribué à la rédaction et préparation du manuscrit dans son ensemble.

Résumé

Le rôle des fonctions effectrices des anticorps dans le contrôle et la prévention de l'infection aux virus du VIH-1 et du VIS a récemment été souligné dans plusieurs études. L'une de ces fonctions, la réponse ADCC, corrèle avec un risque diminué à l'infection par le VIH-1 dans l'essai vaccinal anti-VIH-1 RV144. Des anticorps induits par le vaccin RV144 et qui ont une forte capacité à induire une réponse ADCC reconnaissent des épitopes d'Env exposés lors de l'interaction Env-CD4. Cependant, le VIH-1 limite d'une manière très stringente l'exposition de ces épitopes en retirant CD4 de la surface cellulaire grâce à sa protéine Nef, tout en empêchant l'accumulation d'Env à la surface des cellules infectées via la neutralisation du facteur de restriction tétherine par la protéine virale Vpu. Ces protéines accessoires ont donc été proposées comme jouant un rôle majeur dans le contrôle de la susceptibilité des cellules infectées à la réponse ADCC. Dans cette revue, nous résumons ces récents travaux et discutons le rôle critique que les changements conformationels des glycoprotéines du VIH-1 jouent dans la réponse ADCC et comment ces connaissances peuvent guider la conception de nouvelles stratégies visant à cibler les cellules infectées par le VIH-1.

Abstract

The role of antibody Fc-mediated effector functions in controlling or preventing infections by human immunodeficiency type 1 (HIV-1) and simian immunodeficiency (SIV) viruses has been recently highlighted in multiple studies. One of those effector functions, antibody-dependent cellular cytotoxicity (ADCC) was suggested as correlating with decreased HIV-1 acquisition risk in the recent Thai RV144 vaccine trial. RV144-elicited antibodies with potent ADCC activity were recently found to recognize HIV envelope (Env) epitopes exposed upon Env-CD4 interaction. However, HIV-1 efficiently limits the exposure of those epitopes by strongly downregulating CD4 by both Nef and Vpu accessory proteins, as well as indirectly preventing the accumulation of Env at the cell surface by Vpu-mediated BST-2 antagonism.

These accessory proteins were thus proposed to play a critical role in decreasing the susceptibility of HIV-infected cells to elimination by ADCC. In this review we will summarize these recent findings and discuss the critical role that HIV-1 envelope glycoproteins conformation plays on ADCC responses, how these responses can be measured in the laboratory, the role of HIV-1-transmission on ADCC responses and how this knowledge can be used to develop new strategies aimed at targeting HIV-1-infected cells.

Introduction

The human immunodeficiency virus type 1 (HIV-1) continues to infect more than 2 million individuals annually for an estimated total of 35 million people living with this virus. Enormous efforts have been placed forward by research teams all over the world, which led to great improvements in the clinical management of HIV/AIDS through highly-active antiretroviral drugs. Accordingly, HIV infection is now well controlled by these drugs and, in most cases, allows for a significant increase in the life expectancy of infected individuals. Despite this, efforts to design efficient preventive or curative strategies have yet to produce results in the clinic. Choosing an adequate viral target as well as characterizing the immune functions needed to establish a protective immunity and how the virus responds and protects itself from these immune functions represent a highly complex, multi-faceted problem.

As of the writing of this review, only one anti-HIV-1 vaccine trial, the RV144 Thai trial, presented a modest (31.2%) efficacy in preventing infection by the virus[1]. Interestingly, correlates of protection in this trial trial suggest that an increased antibody-dependent cellular cytotoxicity activity (ADCC) could be linked with decreased HIV-1 acquisition [2]. Moreover, researchers were able to isolate Abs with potent ADCC activity from some RV144 vaccinees [3]. ADCC is thought to represent an important immune effector function in the protection and control of different viral infections [4-6]. Decreased viral load, rate of disease progression and decreased mother to child transmission correlated with Fc-mediated effector functions in HIV-1 and SIV infections in some [7-17], but not in all studies [18-20]. While studies failed to show that passively administered ADCC antibodies could confer protection against SIV or

SHIV challenges in macaque models [21-23], a recent study suggests effector functions of antibodies could limit the number of transmitter founder viruses [24]. As the only viral protein exposed at the cell surface, HIV-1 Env represents the main target of ADCC-mediating Abs [25]. Recent studies, including ours [26-30], reported that the viral accessory proteins Nef and Vpu decrease the susceptibility of HIV-1-infected cells to anti-HIV-1 envelope (Env)-mediated ADCC responses [30-32]. We described that Env – CD4 interaction at the surface of the same infected cell exposes CD4i epitopes in an angle that favors the recognition and activity of ADCC-mediating monoclonal Abs [30, 33] as well as Abs present in sera [28] or in cervico vaginal lavages (CVLs) from HIV-1-infected individuals [26]. It is increasingly clear that Env CD4-bound conformation is a major target of ADCC-mediating Abs from HIV-1-infected individuals [27-30], which represent a significant proportion of anti-Env Abs elicited during natural HIV infection[28, 34]. Moreover, multiple mAbs with potent ADCC activity, including those isolated from RV144 vaccinees (CH54, CH91, CH92, etc) and the recently-described anti-Cluster A Abs (N5-i5, L9-i1, etc.) [35], were found to recognize highly-conserved Env inner domain CD4i epitopes similar to the well-described A32 Ab [30, 33, 36, 37]. It is noteworthy that the ability of Env to transit from the unbound to the CD4-bound conformation is required for Env recognition at the cell surface by CD4i antibodies [29, 30] and this is conserved among Env isolated from several primary HIV-1 isolates but also within some SIV and HIV-2 strains [30]. In addition, efficient internalization of Env which translates into decreased amount of Env at the cell surface also helps to avoid ADCC responses [38]. However, HIV has evolved a series of mechanisms to prevent exposure of epitopes that could be detrimental to the infected cell survival or to the infectivity of viral particles by evading Env recognition by the highly prevalent, easily-elicited, so called non-neutralizing antibodies [34, 39, 40].

Env Glycoproteins Conformation

The trimeric viral Env complex mediates HIV-1 entry in the target cell, the first of many essential steps in the viral replication cycle. Env also represents the only virus-specific antigen present at the surface of viral particles and this exposure results in generation of both neutralizing and non-neutralizing antibodies. The mature HIV-1 Env trimer, comprising the

exterior gp120 and transmembrane gp41 subunits [37, 41, 42], results from the processing of a trimeric gp160 precursor by furin family proteases [43, 44]. The first step in the viral fusion process consists of the interaction of the gp120 glycoprotein with the main cellular receptor, CD4 [45, 46], which triggers conformational changes in gp120 that promote its interaction with one of HIV-1 co-receptors, CCR5 or CXCR4 [47-54]. The binding of both the receptor and co-receptor by gp120 also leads to the exposure of gp41 helical heptad repeat (HR1) segment and insertion of the viral fusion peptide in the target membrane [55-58]. Interaction between the HR1 and HR2 gp41 segments causes the collapse of the extended HR1-HR2 arm into a six-helix bundle, bringing cellular and viral membranes in close proximity thus allowing their fusion [59-61].

In the course of the viral fusion process, each step (CD4 binding, HR1 exposure, co-receptor binding, etc) represents distinct conformational 'checkpoints' in which new epitopes are transiently exposed while Env transitions to lower energy states. One of the first sets of conformational transitions to occur in Env upon binding its cellular receptor CD4 exposes the co-receptor binding site, followed by a quaternary rearrangement of the Env spike to a more open conformation [62, 63]. This results in the exposure of highly-conserved gp120 inner domain epitopes [30, 33, 37]. Multiple reports attribute a lack of neutralizing activity to Abs targeting the gp120 inner domain [34, 54, 64], which is in part explained by the fact that those epitopes are only exposed after Env conformational transitions towards the CD4-bound conformation. The fact that the gp120 inner domain represents a highly-conserved region critical for gp120-gp41 interaction [37, 41, 42, 65] as well as some conformational transitions critical for the fusion process [37, 65] might explain why this region is not exposed. However, we recently demonstrated that Env interaction with CD4 at the surface of infected cells results in Env conformational changes and exposure of CD4-induced epitopes critical for the recognition of infected cells by monoclonal antibodies (mAbs) [30] and sera from HIV-1-infected individuals [28] able to mediate potent ADCC responses. In our view, the biological relevance of the ability of CD4-dependent antibodies to mediate ADCC has been underscored. The virus developed a sophisticated strategy to prevent premature display of CD4-induced epitopes in order to prevent the recognition and subsequent elimination of infected cells by the immune system [40].

The HIV-1 Env tightly controls its native closed conformation by complex mechanisms that involve numerous parts of the glycoproteins. The topological layers in the gp120 inner domain play an important role in controlling and orchestrating the conformational changes upon CD4 binding [37, 65, 66]. As such, mutations of certain highly-conserved residues in those layers can diminish the propensity of the unliganded Env to spontaneously sample the CD4-bound conformation [37, 65]. The arrangement of the variable regions in the native Env conformation most likely impedes the spontaneous transition to the CD4-bound state; thus resulting in decreased CD4i epitopes exposure [67-70]. Finally, it is possible that CD4-dependent ADCC could play an important role in limiting co-receptor switch in vivo as studies showed that the 'opening' of Env (i.e., more accessible for CD4i ADCC Abs) precedes co-receptor switch [71, 72].

CD4 induced epitopes in the C1-C2 region of Env as potent ADCC targets: epitope cluster A

Our recent studies identified a cluster of epitopes in the gp41-interactive region of gp120 that are targets of non-neutralizing antibodies that mediate potent ADCC on target cells sensitized with entering virions [35]. This region, we termed Epitope Cluster A, is comprised of two epitope sub-regions that are defined by the first mAbs shown to recognize these structure. The A32 epitope-subregion of Epitope Cluster A is defined by reactivity with the A32 monoclonal antibody that was isolated by James Robinson's group [73] and shown by mutagenesis to recognize a determinant associated with the C1 region of gp120 [37, 74, 75]. More recent studies showed that the A32 epitope sub-region is a significant ADCC target of circulating antibodies in most HIV-1 infected individuals [30, 36] as well as in RV144 vaccinees [2, 3, 76]. The C11 epitope subregion of Epitope Cluster A is defined by another monoclonal antibody isolated by James Robinson's group that recognizes an epitope associated with the 7-stranded α-sandwich of gp120 [37, 75, 77]. Based on these studies we initiated a program to resolve Epitope Cluster A at atomic resolution.

We have successfully solved the co-crystal structures of CD4-triggered-gp120 cores with two A32-like mAbs, N5-5 and 2.2c, that map to the A32 epitope sub-region [33]. These antibodies recognize overlapping but distinct structures and differ dramatically in their relative abilities to mediate ADCC, with N5-i5 being almost two logs more potent than 2.2c. Further, N5-i5 has a larger epitope footprint involving the α0 and α1 helices in the C1 region of gp120 as well as residues from the C2 region (Figure 1). By contrast, 2.2c binds the α0 helix but it does not contact the α1 helix, resulting in a distinct gp120-binding angle compared with N5-i5 [33]. We have solved co-crystal structures for three additional mAbs, including A32 itself, and it appears that the epitope recognized by 2.2c is the outlier as the other structures involved the α0 and α1 helices as well as the C2 region [78]. The unusual binding angle of 2.2c is a major contribution to its lower ADCC potency as compared with the other mAbs. N5-i5 (and the three other mAbs) binds gp120 at an angle that should orient its CH2 domain more toward the effector cell favoring FcR binding and potent ADCC. By contrast, the 2.2c binding angle is predicted to orient its CH2 domain more toward the target cell surface, disfavoring FcR binding and ADCC. This hypothesis was confirmed by VH/VL domain swaps, which had no effect on N5-i5 but improved the potency of 2.2c [33]. Collectively, these studies provide an increasingly clear view of the A32 epitope subregion and how it becomes available during viral entry and viral budding as a major target of non-neutralizing antibodies that mediate potent ADCC.

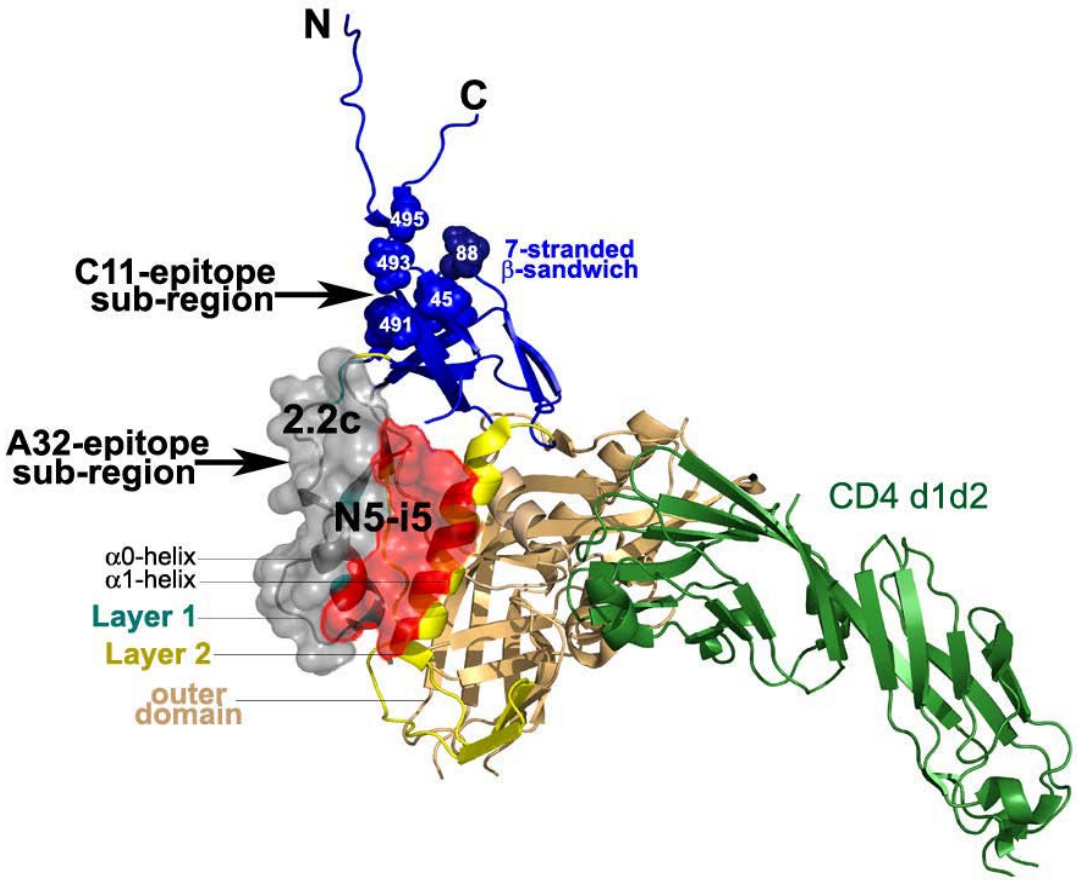

Figure 1.7 **(Article 1 Figure 1) C11 and A32-epitope sub-regions of Cluster A region.** Residues shown by mutagenesis studies [37, 75, 77] to affect binding of mAb C11 to gp120 Env are shown as spheres in blue. The mAb 2.2c and N5-i5 epitope footprint (from crystal structures, PDB code: 4H8W and 4R4F, [33]) is highlighted in grey and grey-red, respectively. The 7-stranded α-sandwich of inner domain is shown in blue. Env layer 1 and 2 are shown in cyan and yellow, respectively. The figure was assembled by a superimposition of structure of N5-i5 Fab- $gp120_{93TH057}$ $core_e$-CD4 d1d2 onto the HIV-1 gp120 with gp41-interactive region [75].

In contrast to the A32 epitope subregion, we have no structural information on the C11 epitope subregion, save for a structure of unliganded C11[78]. As pointed out above, mutagenesis data suggests that C11 recognizes a structure associated with the 7-stranded α-sandwich [37, 75, 77] (Figure 1). We have used this information to carry out docking simulations of the C11 Fab structure and have developed a low energy model that places the C11 epitope subregion adjacent to the A32 subregion but without significant overlap. It should

be noted that mAb competition studies using ELISA suggested that A32 can non-reciprocally block the binding of C11 to gp120 at high concentrations [35, 54]. Blocking studies using 7S antibodies (immunoglobulin G characterized by sedimentation coefficient 7s, in Svedberg units, which corresponds to antibody fraction with a molecular weight of 150 kDa) cannot distinguish between the possibility that the two mAbs recognize the same epitope from the possibility that there is no epitope overlap but that the binding of one full-length antibody occludes access of the other full-length antibody by steric hindrance. Our most recent studies using Fab fragments and a combination of surface plasmon resonance and fluorescence correlation spectroscopy confirm that A32 and C11 recognize completely distinct structures and that any blocking using 7S antibodies is due to steric hindrance [33].

Collectively, our studies provide an increasingly clear picture of Epitope Cluster A structure that inform not only ADCC potency but the exposure of this region during viral entry and viral budding. Epitope cluster A is not exposed on cell free virus [79] but becomes exposed shortly after binding to cell surface CD4 where it remains exposed for at least two hours [80], which we've shown sufficient for target cell killing by ADCC [35]. This information also informs the temporal exposure of Epitope Cluster A as discussed in other parts of this review.

Role of HIV-1 accessory proteins on Env conformational state

Env-CD4 interaction is modulated by the HIV-1 accessory proteins Nef and Vpu, which decrease CD4 cell-surface levels [81, 82]. Moreover, Vpu facilitates viral release by antagonizing the restriction factor Tetherin/BST-2 [83, 84]. Production of viral particles in cells infected with viruses deficient for Vpu leads to a dramatic accumulation of viral particles at the cell surface, thus greatly increasing Env exposure [28, 30-32]. Hence, it is thought that cellular activities of both Nef and Vpu help to indirectly control Env–CD4 interaction at the surface of infected cells [28, 30] (Figure 2A). Cells infected with viruses defective for Nef or expressing attenuated forms, the latter have been described in a cohort of elite controllers [85, 86], show an incomplete downregulation of CD4 surface levels compared to cells infected with a wt virus. Those CD4 levels are sufficient to allow Env-CD4 interaction to occur at the

cell surface, leading to increased CD4i epitopes exposure and an increased susceptibility of the infected cell to CD4i ADCC-mediating epitopes (Figure 2B). However, when vpu-deleted viruses are used, Env surface accumulation, most-likely resulting from BST-2/tetherin trapped viruses, only slightly increase the overall exposure of CD4i (Figure 2C). The consequences of Nef and Vpu deletions are synergistic, since their deletion results in an increase in both CD4 and Env levels at the cell surface. CD4 and Env interaction results in the exposure of CD4i Env epitopes recognized by ADCC-mediating Abs and HIV-1+ sera [28, 30] (Figure 2D).

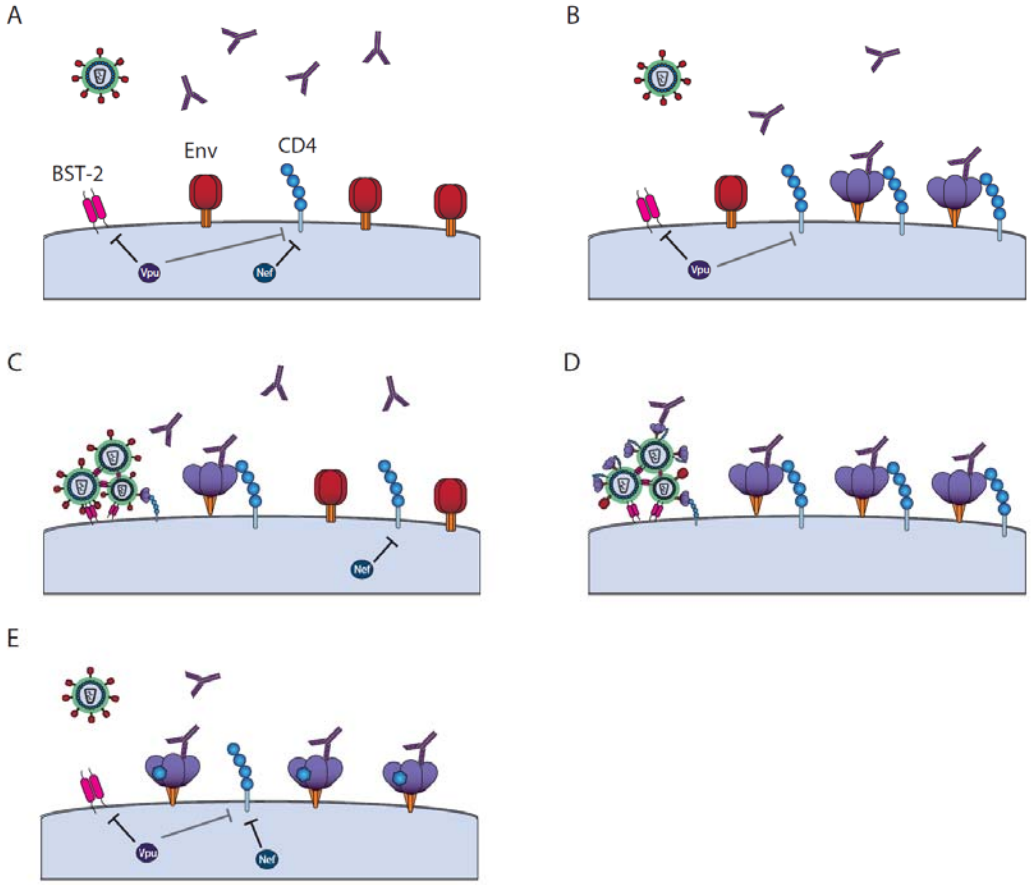

Figure 1.8 **(Article 1 Figure 2) Role of Env conformation, CD4 downregulation by Nef and Vpu, as well as Vpu-mediated BST-2 antagonism on ADCC responses.** (**A**) HIV-1 evolved a highly-conserved and sophisticated mechanism to avoid exposure of Env CD4-induced (CD4i) epitopes at the cell surface. In cells infected with a wild-type (wt) virus, Vpu counteracts the restriction factor BST-2 limiting the amount of total Env present at the cell-surface and, in conjunction with Nef, they downregulate CD4, thus limiting the possibility of Env-CD4 interaction at the cell surface. As a consequence, there is no exposure of Env CD4i epitopes. (**B**) In the absence of Nef (Nef-), Vpu is still able to counteract

BST-2 thus limiting the overall amount of Env at the cell. However, due to the absence of Nef, there is an accumulation of CD4 at the cell surface free to engage with Env resulting in exposure of Env CD4i epitopes (recognized by ADCC-mediating Abs). (**C**) In the absence of Vpu, there is an accumulation of Env at the cell surface due to the trapping of outgoing viral particles by BST-2. Also, Vpu is known to downregulate CD4 and in its absence there is a small increase in cell surface levels of CD4. Therefore, in absence of Vpu there is a significant increase in Env and some CD4 at the cell surface. This allows a limited engagement of Env with CD4 which results in a slight increase of Env recognition by CD4i ADCC-mediating Abs. (**D**) In absence of Nef and Vpu, there is no CD4 downregulation (i.e., high levels of CD4 at the cell surface) and a significant accumulation of Env at the cell surface (mostly due to BST-2-trapped virions). Therefore, a large quantity of Env engages with CD4, inducing Env conformational changes that result in exposure of CD4i epitopes. Thus explaining enhanced sensitivity of nef-vpu- infected cells to ADCC. (**E**) Finally, CD4 mimetics (small molecules NBD-556 derivatives and mini-proteins mimicking CD4) can engage with Env present at the surface of wt-infected cells (coding for functional Nef and Vpu) and force Env to sample the CD4-bound conformation, thus exposing CD4i epitopes which are readily recognized by ADCC-mediating Abs. These Abs are generated early during HIV-1 infection, present in sera, cervico-vaginal fluids and breast milk and are present in large quantities in HIV-1-infected individuals. Thus, effectively sensitizing HIV-1 wt –infected cells to ADCC-mediated killing.

Despite the cooperation of these two accessory proteins to decrease the overall exposure of CD4i epitopes at the cell surface, it is yet unclear which of the two accessory proteins have a stronger impact on Env conformation [28, 30-32]. We hypothesize that viruses encoding Env variants prone to spontaneously sample the CD4-bound conformation by presenting a more open quaternary structure (such as those from some lab-adapted and X4-tropic HIV-1 strains [70, 87, 88]) would be more dependent on the anti-tetherin Vpu activity to protect infected cells from ADCC responses by preventing the accumulation of highly-targeted Env. Cells exposing even moderate to high amounts of Env sampling highly-compact ‘closed’ conformations with low intrinsic reactivity [89, 90] would not be more susceptible to ADCC responses in absence of surface CD4 and therefore would find themselves more dependent on a strong CD4-downregulation activity by both Nef and Vpu.

Role of easy-to-elicit non-neutralizing antibodies on ADCC responses

The vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, likely limiting the exposure of CD4i Env epitopes at the surface of infected cells and thus preventing ADCC responses. Therefore, strategies to prevent Nef and Vpu-mediated CD4 / BST2 downregulation or to directly alter Env conformation could potentially increase the susceptibility of HIV-1-infected cells to ADCC. In this sense, agents promoting the CD4-bound Env conformation should expose CD4i epitopes that are readily recognized by ADCC-mediating Abs present in sera and CVLs from vaccinated and infected individuals [26-30]. Importantly, modulating Env conformation at the surface of HIV-1-infected cells is now an attractive strategy after the discovery and design of small CD4-mimetic compounds. Members of the first generation of those compounds, NBD-556 and NBD-557, were the results from a screen for inhibitors of gp120-CD4 interaction [91]. These small-molecule ~337-dalton compounds and recent derivatives (DMJ-I-228) bind in the Phe 43 cavity [92-94], a highly conserved ~150-Å3 pocket in the gp120 glycoprotein formed by the interface of the inner domain, outer domain, the bridging sheet and the CD4 receptor [95]. CD4-mimetics block gp120-CD4 interaction and induce thermodynamic changes in gp120 similar to those observed upon soluble CD4 (sCD4) binding [96]. Accordingly, these small molecules as well as sCD4 can promote the transition of Env to the CD4-bound conformation, thus sensitizing HIV-1 particles to neutralization by otherwise non-neutralizing CD4i Abs [34, 64]. Additional strategies using scaffolded miniproteins targeting critical gp120 elements required for CD4 interaction allowed the identification of CD4-mimetics with nanomolar affinity for gp120 [97]. One of these variants, M48U1, displayed remarkably potent neutralization of three HIV-1 isolates[98]; its crystal structure in complex with HIV-1 gp120 was recently solved, showing that M48U1 engages the Phe 43 cavity in a manner similar to that of CD4 [99]. Thus, CD4-mimetics might induce gp120 to adopt the CD4-bound conformation, expose CD4i epitopes at the surface of infected cells and thus sensitize them to ADCC-mediated killing (Figure 2E). We recently demonstrated that this strategy works as CD4-mimetics were able to potently sensitize HIV-1-infected cells to ADCC mediated by antibodies present in sera, CVLs and breast milk (Figure 1.8E) [100].

While CD4-mimetic compounds and miniproteins offer an attractive way of forcing exposure of CD4i epitopes at the surface of infected cells, it is of interest to note that

alternative approaches to directly target Nef accessory proteins are starting to emerge in the literature. Recently, Bouchet and collegues reported the construction of a single-domain antibody (sdAb) that presents high affinity for HIV-1 Nef, named sdAb19 or Neffin [101]. This sdAb was isolated from llama immunized with a recombinant Nef protein and, when expressed intracellularly, showed anti-Nef activity that resulted in the inhibition of the Nef-mediated surface CD4 downregulation. We hypothesize that expression of this sdAb in HIV-1 infected cells would result in increased Env-CD4 interaction and thus exposure of CD4i epitopes at the cell-surface, potentially increasing recognition and elimination of infected cells via the ADCC-effector functions of CD4i-targeting Abs. However, while gene therapy technologies have come a long way in the past few years and could have a promising future in HIV treatment and prevention, targeting potentially infected cells with a safe and efficient vector to express recombinant protein such as sdAb19 remains a challenge.

Methods to measure ADCC:

Traditionally, the first procedures to measure ADCC responses against HIV-1 used the 51Chromium release assay (CRA) [10, 102-104], a technique originally established to measure killing by cytotoxic T cells. In this assay, targets cells are labeled with the radioactive 51Chromium and antibody-mediated target cell killing is measured by the release of the radioactive isotope into the cell culture supernatant [104]. However, this assay is cumbersome and presents several disadvantages including lack of sensitivity, weak labelling or high spontaneous isotope release for certain cell types. In recent years a number of alternative flow-cytometry-based assays were subsequently developed. The majority of these assays use the CEM.NKr.CCR5 cell-line as target cells. These CD4+ cells are resistant to elimination by the innate killing ability of NK cell [105], although primary CD4+ T cells can also be used. Target cells can either be pulsed with HIV-1 Env (recombinant gp120 or gp140) or infected with different HIV-1 strains. These assays commonly evaluate NK cell activation, granzyme B delivery into target cells or ultimately the elimination of the targets cells; Tableau 1.2 summarizes these methods.

NK cell activation assays

Simple whole-blood assays assessing cytokine expression in responses to overlapping peptides were already used to study of HIV-specific T-cell immunity. A similar assay was next adapted by Kent's group [106, 107] to study HIV-1 specific ADCC response. Rather than using the previously standard process of assaying the elimination of immortalized cell lines presenting HIV antigens, this assay is based on primary blood cells and measures several functions of NK cells in response to ADCC triggering. Antibody-dependent activation of NK cells identified as CD3-CD56+ cells, are assessed by detection of intracellular IFNy and cell-surface expression of CD107a, a surrogate marker of NK cell degranulation [108], or by measuring the loss of intracellular granzyme B in NK cells. Notably, this activation of NK cells can be evaluated in responses to antigens comprising both Env glycoproteins and HIV peptides from all nine HIV proteins. In that context, this approach was successfully employed to map epitopes that are recognized by potentially ADCC-mediating antibodies [109-111]. Alternatively, NK activation can also be measured using whole blood sample or PBMC as source of effector cells in this assay using target cells either coated with HIV-1 Env or infected with HIV-1 virus [112].

Fluorometric granzyme cytotoxicity assay

Ferrari and colleagues engineered a high-throughput platform to ascertain the presence of ADCC-mediating antibody, using an assay based on hydrolysis of a cell-permeable fluorogenic peptide substrate that generates a fluorescent signal when it gets hydrolyzed by granzyme B [113]. This assay allows identification of target cells that received granzyme B from effector cells as a consequence of target cells recognition by specific antibodies. Target cells used for this assay included primary CD4+ T cells or the CEM.NKr cell line either infected or coated with Env proteins, while freshly isolated or cryopreserved PBMC or NK cells can be used as effector cells. Target cells are incubated for a short period of time with effector cells in the presence of the granzyme B substrate and purified antibodies or sera. The level of target cells positive for granzyme B is then determined by flow-cytometry. Notably, this method was the secondary ADCC assay used to measure the ADCC response reported for the RV144 trial (2) and subsequently used to characterize the ADCC activity of plasma and monoclonal antibodies isolated from vaccinees [3, 76]. Alternatively, measurement of

granzyme B delivery into target cells and infected cell elimination can be assessed together by staining intracellularly for p24 expression [18]. Using the latter method, Small-Mantey and colleagues determined that anti-HIV-1 ADCC activity was mainly directed against HIV-1 Env and strongly correlated with total and IgG1 binding to infected cells [18].

The rapid and fluorometric ADCC (RFADCC)

The rapid and fluorometric ADCC (RFADCC) assay was developed based on the FATAL assay, a flow-cytometry-based assay originally designed to test T cell responses [114]. In this method, target cells are stained with a membrane dye (PKH-26) and a viability dye (CFSE) prior to incubation with unstained effector cells in presence of antibodies [115]. The ADCC-mediated killing of target cells is calculated by the emergence of a cell population positively labeled by the membrane dye but that loses the viability dye during cytolysis (CFSE-PKH-26+). This assay was found to provide a higher cell killing readout compared to CRA [115] and consequently was widely used in the field, including correlating vaccine-induced ADCC with protection in macaques [116-119]. However, it has been demonstrated that RFADCC mainly measure the IgG-mediated monocytes functions rather than NK cell effector function [120]. Indeed, the CFSE-PKH-26+ cell population normally defined as the killed target cells in this assay were reported to mainly contain monocytes (CD3-CD14-) that have internalized PKH-26 labeled membrane fragment rather than killed target cells. Accordingly, a meagre correlation between the RFADCC and assays looking at NK cell activation was previously observed [106, 107]. Nevertheless, further analysis confirmed that focussing the analysis on PKH26+ monocytes rather than CFSE-PKH-26+ target cells was a better way to assay ADCC using this technique [120].

Infected-cells or gp120-coated cells elimination assay (ICE and CCE).

During viral infection, the specific recognition of infected cells by antibodies and, most importantly, the subsequent elimination of the opsonized cells by ADCC contribute to the disease outcome. Most of the assays described above do not directly measure elimination of target cells. Nevertheless, the specific measurement of HIV-1-infected cell elimination by

ADCC (ICE) was reported using different methods including LTR-Luciferase engineered target cells [9]. In this assay, NK cells expressing human or macaque CD16 are used as effector cells, while a CEM.NKr cell lines expressing luciferase under a Tat-inducible promoter is used as target cells. After incubating effector cells and virally infected target cells with plasma dilutions, the extent of the loss of luciferase activity is correlated to the ADCC-mediated killing in a dose-dependent manner. Titers of ADCC-mediating antibody in the plasma of both HIV-infected individuals and SIV-infected macaques were successfully measured using this assay. Accordingly, this method was the primary assay used to assess the ADCC response reported for the RV144 trial [2] and was also used to characterize the ADCC activity mediated by plasma and monoclonal antibodies isolated from vaccines [3]. Alternatively, measurement of HIV-1-infected cell elimination by ADCC was also reported using GFP-expressing viruses [28, 30, 121] or with intracellular anti-p24 staining [18, 100].

We recently developed a simple and rapid flow-cytometry based assays that allows the accurate calculation of gp120-coated cells [26, 27, 112] (CCE) or HIV-1-infected cells elimination by ADCC (ICE)[28, 30, 100]. In the gp120-coated cell assay, CEM.NKr cells coated with HIV-1 gp120 are used as target cells, while human PBMC are used as effector cells. Target and effector cells are stained with two different cellular dyes before being incubated with mAbs or human sera. This approach allows the specific gating of target cells, while the exact number of surviving target cells is calculated trough normalization with flow-cytometry particles. Importantly, this double staining allows the precise calculation of target cell number; excluding effector cells that could have internalized target cells membrane fragments, as reported for the RFADCC. We adapted our assay to measure the elimination of HIV-1-infected cells either by following the elimination of GFP-expressing cells (when using GFP-encoding proviruses) [28, 30] or p24-positive cells [100]. Accordingly, in contrast to RFADCC, our assays likely evaluate NK cell effector functions since similar ADCC responses were obtained using purified NK cells as effector cells [27]. This method was successfully applied to screen human or macaque sera for ADCC activity directed against HIV-1 gp120 Env. Alternatively, using primary CD4+ T cells coated with HIV-1 gp120 as target cells, this method was also used to compare anti-HIV-1 ADCC function of autologous and allogeneic effector cells [112]. Notably, by using small concentrations of recombinant gp120 Env,

suitable target cells that recapitulate the ADCC response mediated against cells infected with Nef-Vpu- were generated for this method [27]. This is likely due to the fact that gp120 Env coats target cells through CD4 interaction, thus forcing gp120 Env to sample the CD4-bound conformation, which results in the exposure of CD4-induced ADCC-mediating epitopes. In that context, we found that introduction of a mutation in the HIV-1 gp120 CD4-binding site (D368R), known to impair gp120-CD4 interaction, completely abrogated binding of gp120 to target cells and consequently their ADCC-mediated elimination. These results highlight the fact that gp120-coated cells, generally used for most of the ADCC assays described above, are presenting CD4-induced epitopes, in contrast to HIV-1 infected cells in which such epitope exposure is avoided through CD4 downregulation. Consequently, ADCC assays using gp120-coated cells measure the ADCC response directed to CD4-induced HIV-1 Env epitopes. Interestingly, utilization of target cells infected with lab-adapted virus defective for Vpu and/or Nef could explain why strong ADCC responses directed against HIV-1 Env were previously reported in infected cell lines [36].

In brief, there is no clear consensus on the best ADCC assay to use to assess the ADCC response mediated against HIV-1, since all these methods evaluate different overlapping function of ADCC, using different target cells and diverse read-out. However, the type of targets cells (i.e., gp120-coated cells or HIV-1 infected cells) should be carefully chosen, as this will definitively dictate the type of Env epitopes (CD4-induced or unbound) driving the ADCC response.

Tableau 1.2
(Article 1 Table 1) Description of commonly used ADCC assays

Type of assay	Description	References
Chromium release assay (CRA)	Targets cells are labeled with the radioactive ^{51}Chromium and ADCC-mediated killing is measured by the release of the radioactive isotope into the cell culture supernatant from killed target cells.	(10,102-104)
NK cell activation assays	Antibody-dependent activation of NK cells are assessed by detection of intracellular IFN-γ, cell-surface expression of CD107a (a surrogate marker of NK cell degranulation) or by measuring the loss of intracellular granzyme B by NK cells.	(106,107,109-112)
Fluorometric granzyme cytotoxicity assay	Target cells are incubated with a cell-permeable fluorogenic peptide substrate that generates a fluorescent signal when it gets hydrolyzed by granzyme B. The level of target cells that receive granzyme B from effector cells as a result of an ADCC response is then determined by flow-cytometry.	(2,3,18,76,113)
The rapid and fluorometric ADCC (RFADCC)	Target cells are stained with a membrane dye (PKH-26) and a viability dye (CFSE). ADCC-mediated killing of target cells is calculated by the emergence of a cell population positively labeled by the membrane dye that loses the viability dye (PKH-26+ CFSE-).	(115-120)
Infected-cells elimination assay (ICE) (Luciferase assay)	CEM.NKr cells expressing luciferase from a Tat-inducible promoter upon HIV-1 or SIV infection are used as target cells. The level of ADCC-mediated killing is measured as the dose-dependent loss of luciferase activity.	(2,3,9)
Infected-cells elimination assay (ICE) (% of infected cells)	Measurement of infected cell elimination by ADCC is assessed by calculating the loss of the percentage of infected cells using GFP-expressing viruses or with intracellular anti-p24 staining.	(18,28,30,100)
Infected or gp120-coated cells elimination assay (ICE, CCE) (with flow-cytometry particles)	Target and effector cells are stained with different cellular dyes. The percentage of ADCC-mediated killing is evaluated by calculating the exact number of surviving target cells through normalization with flow-cytometry particles.	(26-28,100, 112)

Defining the enemy: the "Trojan Horse" hypothesis of cell-to-cell HIV transmission

Just prior to the confirmation of HIV as the causative agent of acquired immunodeficiency over 30 years ago, it was proposed that the etiological agent of AIDS was transmitted by leukocytes within infectious bodily fluids. The "Trojan horse" hypothesis, originally proposed by Anderson and Yunis (1983) and updated in a more recent iteration by Anderson et al. (2010), predicts that infectious HIV enters the uninfected donor in the form of virus-infected leukocytes that transmit HIV in a cell-to-cell fashion, while being protected from antibodies within the infected bodily fluid that can neutralize free virions [122, 123] (Figure 1.9). Since its original publication, several independent investigators have championed the "Trojan horse" hypothesis, and in vitro experiments, animal studies and clinical data now

support the plausibility of cell-to-cell transmission being responsible for at least a proportion of newly acquired HIV infections [124, 125].

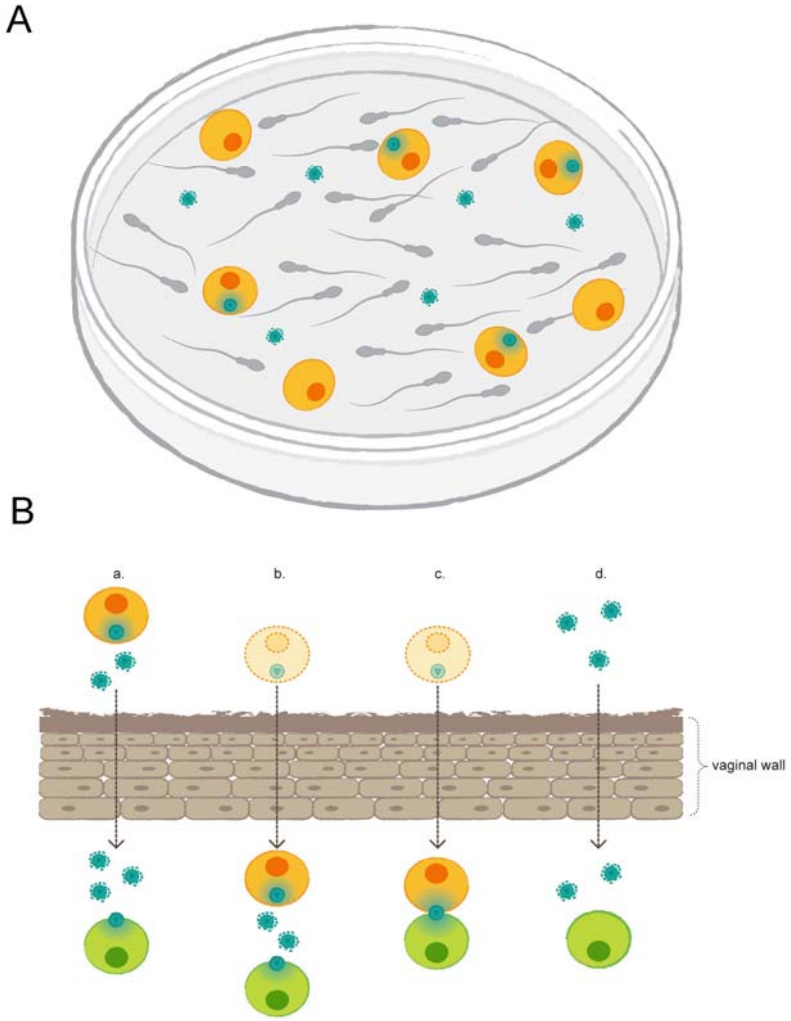

Figure 1.9 **(Article 1 Figure 3) The viral composition of seminal fluid and avenues for HIV transmission.**
(**A**) Seminal fluid, reflective of other bodily fluids capable of transmitting HIV, contains virus as both free virions and infected leukocytes. (**B**) Both cell-associated HIV and free virions have the potential to migrate across mucosal epithelia, such as the vaginal wall, and establish foci of infection. This might be accomplished by (a) HIV-infected leukocytes releasing virions towards the vaginal wall, transepithelial migration of virions and infection of susceptible cells; (b) Transepithelial migration of HIV-infected leukocytes, release of virions and infection of susceptible cells; (c) Transepithelial migration of HIV-infected leukocytes and cell-cell transmission of HIV to susceptible target cells; (d) Transepithelial migration of free virions and infection of susceptible cells.

The notion that cell-to-cell transmission of HIV establishes new infections is supported by the detection of cell-associated virus within infectious bodily fluids. Indeed, both cell-free and cell-associated HIV are present within infectious bodily fluids, such as blood, breast milk, semen and vaginal fluids, from HIV-infected donors [126-129]. Given that semen is a potent vehicle of both heterosexual and homosexual (men who have sex with men) HIV transmission, which account for the majority of new global HIV infections, many studies have focused on the infectiousness of viruses derived from semen. A recently published meta-analysis of the recovery rate of infectious viruses from semen revealed infectious virus to be significantly more likely to be recovered from seminal cells than the cell-free seminal plasma [122]. Although the detection of cell-associated HIV and demonstration of its superior in vitro infectiousness suggests cell-to-cell HIV infection could account for viral transmission, it should be noted that other barriers, such as the mucosal epithelial, still need to be overcome.

Given that most new HIV infections occur via mucosal exposures, the most essential requirement for the plausibility of the "Trojan horse" hypothesis of HIV transmission is that virus-infected allogeneic cells from the infected donor can traverse the mucosal epithelium and establish foci of infection within the recipient. Evidence of transepithelial migration has been demonstrated in humanized SCID mice following vaginal administration of HIV-infected allogeneic cells [130]. Similarly, vaginal challenge of macaques with CFSE-labeled SIV-infected allogeneic cells resulted in transepithelial migration of the infected cells, with CFSE-labeled cells being detected within both proximal and distal lymphoid tissues at one and two days post challenge [131]. Perhaps most relevant to the question of whether cell-associated HIV transmission is plausible is the evidence in humans that allogeneic cells traverse the mucosal epithelium. Indirect evidence of trans-epithelial migration in humans is provided by the detection of cellular immune responses against the allogeneic leukocytes of sexual partners in heterosexual and homosexuals practicing unprotected sex [132].

Corroborating the evidence that allogeneic cells can traverse the mucosal epithelial, studies in animal models have demonstrated cell-to-cell transmission to be a more effective means to establish infection with immunodeficiency viruses than free virions. Indeed, vaginal challenge of humanized SCID mice with HIV-infected cells resulted in establishment of

infection [130]. Challenges of cats with cell-associated and cell-free FIV establish infection through both the vaginal and rectal routes, but cell-associated virus is most infectious [133]. Likewise, cell-associated SIV and HIV can establish infections in macaques and chimpanzees, respectively [131, 134-138]. Intravenous challenges were utilized in the early studies assessing cell-associated viral transmission in non-human primates, with one study reporting that as few as two infected cells were required to establish systemic SIV infection in macaques [134, 136, 138]. More recent studies have revealed that vaginal challenge with cell-associated SIV or HIV establishes infections in macaques or chimpanzees with both ulcerative and intact vaginas [131, 135, 137]. Furthermore, comparisons of the relative abilities of cell-associated and cell-free SIV to establish infections upon vaginal challenges in macaques have revealed cell-associated virus to be a more robust means to establish infection [135]. That the viral copy number of cell-associated virus required for establishing infection more closely approximates the amount of cell-associated virus found in bodily fluids, such as semen, than does the amount of cell-free virus utilized in in vivo challenges has been put forth as a strength of the "Trojan horse" hypothesis [122].

In addition to evidence of cell-associated virus establishing infection in animal models, several lines of clinical evidence suggest cell-to-cell HIV transmission can account for at least a proportion of newly acquired HIV infections. Indeed, cell-associated HIV appears to be important in the transmission of HIV to infants through breastfeeding, as the amount of cell-associated HIV within breast milk is a more important factor in determining the risk of transmission within the first six weeks postpartum than the amount of cell-free virus present [126]. Evidence of sexual transmission of cell-associated virus has also been observed in a small cohort of transmission pairs. Utilizing their observation that cell-associated and cell-free viruses within semen are genetically distinct, Zhu et al. demonstrated that the viruses present in newly infected individuals were more similar to the seminal cell-associated virus than the cell-free virus of the originally infected partner in three out of five cases [129].

Cumulatively, evidence collected through in vitro experiments, animal models and clinical data suggest that cell-associated HIV might be responsible for a proportion of new HIV infections. Importantly, as several additional lines of evidence indicate that cell-

associated HIV is better equipped to evade humoral and cellular immune responses, cell-associated HIV transmission should be an essential component of conversations regarding the design of HIV vaccines and other prophylactic interventions.

ADCC: an immune response with potential to slay "Trojan horse" HIV

One of the central debates regarding HIV vaccine design over the past two decades has been whether induction of cytotoxic T lymphocytes (CTL) or neutralizing antibodies provides a more effective means of preventing systemic HIV infection. A major caveat of this debate, however, is that it often assumes that cell-free virus is responsible for new HIV transmissions. In the case of exposure to cell-associated virus, there is now ample evidence that CTL and neutralizing antibodies might be inefficient to protect against HIV infection, impractical and/or represent currently unachievable vaccination targets. As such, novel vaccine-inducible immune responses should be entered into the conversation regarding HIV vaccine design.

Broadly neutralizing antibodies (BnAbs) are thought to be ideal targets for induction via vaccination. These antibodies, which are observed in approximately 20% of HIV infected individuals, are capable of neutralizing in vitro infections by wide arrays of HIV isolates [139]. Furthermore, passive administration of BnAbs to macaques is sufficient to prevent infection by cell-free SHIV challenge [140]. Despite these attractive qualities of BnAbs, the utility of these antibodies in preventing infection by cell-associated virus have been called into doubt. Indeed, in vitro studies have demonstrated that BnAbs of certain specificities are either unable to prevent cell-to-cell HIV spread or require higher amounts of antibody to prevent cell-to-cell spread than are require to block cell-free HIV transmission [141, 142]. Most recently it has been demonstrated that certain BnAbs, such as the PGT121 antibody that targets an envelope epitope consisting of viral protein and glycan, can efficiently neutralize cell-to-cell transmission of HIV [142]. Notwithstanding this promising observation, strategies to induce BnAbs by immunization are currently unavailable. Several issues, including requirements for large amounts of somatic hypermutation and negative selection of B-cells with the capacity to become BnAbs, are thought to contribute to the current difficulties surrounding BnAb induction by vaccination [143, 144]. Given these drawbacks, recent studies

have begun to focus on the potential of utilizing non-neutralizing antibody functions to block HIV transmission.

Similar to BnAbs, CTL have traditionally been thought of as a potentially valuable anti-HIV immune response to induce by immunization. CTL-mediated immunity plays critical roles in slowing progression towards AIDS. Indeed, the slow disease progression of elite controllers and long-term non-progressors are linked to the function of their CTL [145]. This has been further corroborated by antibody-mediated depletion of CD8+ CTL in SIV-infected macaques, which is followed by a dramatic increase in viral load [146]. The potential of anti-viral CTL to serve as prophylactics, however, has not been as well established. Human clinical trials of vaccines designed to elicit anti-HIV CTL have twice failed to provide protection against HIV infection [147, 148]. With regards to preventing cell-associated HIV infections, vaccine-induced CTL have shown promise in non-human primates. Indeed, macaques carrying vaccine-induced CTL are protected against infection upon challenge with SIV-infected allogeneic cells, but only if the immunized macaque shares MHC-I alleles with the donor of the infected leukocytes [134]. Given that it would be extremely difficult to identify the MHC-I alleles of potential sexual partners, a vaccination approach based upon this observation seems impractical. The observation that cellular immunity might be sufficient to protect HIV infection by cell-associated virus, however, may be instructive for designing a successful prophylactic vaccine.

The mediation of ADCC by cells of the innate immune system, such as NK cells and monocytes, represents a mechanism outside of CTL for achieving cellular-mediated immunity. Furthermore, ADCC represents an immune response that overcomes some of the problems presented by BnAbs and CTL. Antibodies capable of mediating ADCC do not require the dramatic levels of somatic hypermutation that are required by BnAbs [3]. Further, ADCC effector cells do not exhibit the MHC-I restriction that is characteristic of CTL. In addition to these beneficial qualities of ADCC, the immune response appears to have utility against cell-associated HIV. Early evidence of the usefulness of ADCC against cell-associated HIV was provided by experiments in which the NK cell resistant CEM.NKr T-cell line was infected with HIV, haptenated and co-cultured with non-activated PBMC in the presence of anti-hapten

antibodies or Fab2 fragments [149]. Indicative of a role for ADCC in protecting T-cells from cell-to-cell transmission of HIV, viral production was observed under co-culture conditions containing anti-hapten Fab2, but not when whole antibodies were employed. In more recent research, we have demonstrated that allogeneic T-cells, which are coated with similar levels of gp120 as are observed on T-cells infected with Vpu and Nef deficient HIV, are susceptible to A32-mediated ADCC and capable of activating NK cells in the presence of polyclonal anti-HIV antibodies [112]. Interestingly, NK cells expressing the inhibitory KIR3DL1 receptor exhibited antibody-dependent NK cell activation against target cells carrying the receptor's HLA-Bw4 ligand, suggesting antibody-dependent activation of NK cells can overcome inhibitory signals through this inhibitory receptor/ligand combination. This observation implies that ADCC might reflect an immunological mechanism via which "Trojan horse" cells can be targeted without concern for matches or mismatches between the inhibitory KIR expressed by the NK cells of the recipients of "Trojan horse" cells and the HLA-I of the donors of these leukocytes.

Although both our data demonstrating anti-HIV ADCC against allogeneic cells and the previous data demonstrating that anti-hapten ADCC can prevent cell-associated HIV transmission in vitro clearly indicate that "Trojan horse" cells can be targeted via ADCC, many questions remain regarding the antibody specificities required for efficient targeting of "Trojan horse" cells. Indeed, the gp120 coated to allogeneic targets in our manuscript was able to obtain the CD4-induced conformation that we have previously demonstrated to be important for mediation of anti-HIV ADCC [30]. As wild-type viruses carry competent Vpu and Nef, envelope on the surface of naturally occurring "Trojan horse" cells is less likely to be able to obtain this CD4-induced conformation. As such, it is possible that trimer-binding antibodies might be required to efficiently target "Trojan horse" cells. It should be noted, however, that the partially effective RV144 vaccine elicited A32-like antibodies, which bind the CD4-induced epitopes of HIV envelope, and these antibodies might have contributed to the observed protection [3]. Albeit speculative, this data might reflect incomplete CD4 downregulation on "Trojan horse" cells infected with transmitted/founder viruses or indirect effects of A32-like antibodies.

Identifying antibodies capable of eliminating HIV-infected "Trojan horse" cells by ADCC

Future research is urgently required to identify antibody specificities sufficient for ADCC-mediated elimination of "Trojan horse" lymphocytes infected with wild type viruses. Identification of these antibodies will involve sifting through numerous biological samples from vaccinees and HIV-infected donors. Perhaps interesting sources of such antibodies are mucosal samples from individuals that have been exposed to HIV but remain uninfected [150]. Other conceivable sources of potentially useful antibody specificities are mucosal fluids involved in HIV transmission, such as semen and cervico-vaginal fluid. Indeed, it is plausible that antibodies within these fluids, which have been shown to be capable of mediating ADCC [26], could be utilized by effector cells within the exposed individual to eliminate HIV-infected allogeneic cells and/or early infected autologous cells.

Recently, we have attempted to identify antibodies capable of mediating ADCC within cervico-vaginal lavages from HIV-infected and exposed-uninfected women and semen samples from HIV-infected men (Parsons et al. Unpublished data) [26]. Although no anti-HIV ADCC antibodies were observed in CVL from exposed-uninfected donors [26] (and unpublished observations), CVL from HIV-infected women contained anti-HIV ADCC competent antibodies. Similar to our previous data regarding the specificities of ADCC antibodies with blood plasma, CVL ADCC antibodies targeted envelope in the CD4-bound conformation [26, 30]. Likewise, our analysis of semen samples for anti-HIV ADCC antibodies revealed antibodies capable of triggering ADCC and activating NK cells to produce cytokines. It should be noted, however, that the ability of anti-HIV antibodies within seminal plasma to activate NK cells was dramatically improved when IgG were enriched and the other components of seminal plasma were removed, suggesting the presence of factors within seminal plasma that can inhibit antibody-dependent NK cell activation. As of the writing of this review, the specificities of these seminal plasma antibodies have not been determined.

The identification of anti-HIV antibodies capable of mediating ADCC within CVL and semen samples from HIV-infected donors could represent a fruitful avenue for future research into protective ADCC responses. We hypothesize that such antibodies within the mucosal

fluids of infected individuals could be transferred to uninfected partners along with viral particles and HIV-infected cells. Additionally, effector cells within the uninfected donor might then utilize these antibodies to eliminate "Trojan horse" cells and/or infected autologous cells early post-exposure. The observation that some seminal plasma samples trigger anti-HIV antibody-dependent NK cell activation is supportive of this notion. However, the observation that seminal plasma heavily inhibits these antibody-dependent responses raises the possibility that semen could play a role in shielding HIV-infected target cells from antibody-dependent effector functions. Future research should be designed to identify serodiscordant couples, measure ADCC antibodies and viral load within the semen or CVL of the infected partner, follow the couples for HIV transmission (take into account the number of exposure events) and correlate the rate of transmission with the potency of ADCC antibodies.

Conclusion

It is now understood that antibodies without potent neutralization activity but presenting strong capabilities to induce Fc-mediated effector functions are highly prevalent during HIV-1 infection. Those antibodies mostly target epitopes present in the gp120 core, which can be exposed upon CD4 binding. There is now strong evidence supporting the hypothesis that HIV-1 and other lentiviruses evolved cooperative, highly-efficient mechanisms to prevent exposure of Env epitopes at the surface of infected cells that would otherwise be targeted by ADCC-mediating antibodies and lead to the premature elimination of the infected cell. Agents promoting the CD4-bound Env conformation could thus represent an interesting approach to expose CD4i epitopes that are readily recognized by ADCC-mediating Abs present in sera and CVLs from vaccinated and infected individuals. However, the impact and role of CD4i epitopes exposure at the mucosal sites in the context of viral transmission remains to be further characterized.

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CHAPITRE II

TECHNIQUES POUR MESURER LES CHANGEMENTS DE CONFORMATION D'ENV

Introduction

Ce chapitre présentera les techniques développées au cours des études présentées dans cet ouvrage et qui permettent de mieux étudier et d'évaluer les changements conformationels d'Env à la fois dans un système mécanistique et fonctionnel. Tout d'abord, une technique d'ÉLISA sur base cellulaire a été développée au laboratoire et permet d'évaluer l'exposition d'épitopes d'Env en réponse à diverses conditions (co-transfection de son récepteur CD4, traitements avec des molécules) tout en utilisant de petites quantités de réactifs. Ainsi, cette méthodologie est également applicable pour le criblage de banques de spécimens biologiques (*i.e.*, sérum de patients infectés ou vaccinés) qui sont parfois disponibles qu'en quantités très limitées. Ensuite, la deuxième section de ce chapitre est dédiée à une approche expérimentale permettant d'étudier la réponse ADCC contre l'Env du VIH-1. En ce sens, cette technique est à la fois utilisable non seulement pour quantifier le potentiel d'anticorps monoclonaux ou de spécimens biologiques à induire une réponse ADCC contre des cibles standardisées exposant des épitopes CD4-induits d'Env mais également pour déterminer les facteurs viraux qui modulent la susceptibilité de cellules infectées à la réponse ADCC. Les deux techniques introduites dans ce chapitre forment la pierre angulaire des chapitres suivants.

Pour la technique d'ÉLISA sur base cellulaire, publiée sous format vidéo dans le journal *The Journal of Visualized Experiments*, Mathieu Coutu, Jonathan Richard et Laurie-Anne Batraville ont réalisé les expériences démontrant les utilités de la technique pour le manuscrit. Maxime Veillette a réalisé le montage des figures, la rédaction de l'article et la préparation du script de la vidéo. Anik Désormeaux a participé à la mise sur pied de la technique dans le

laboratoire. Michel Roger a fourni des conseils et des réactifs. Andrés Finzi a participé à l'optimisation de la technique et à la préparation du manuscrit.

La technique permettant d'étudier la réponse ADCC d'anticorps ou de spécimens biologiques a été publiée en 2014 dans le journal *The Journal of Virological Methods*. Maxime Veillette a mis sur pied et optimisé la technique de mesure de l'ADCC contre des cellules infectées au VIH-1 et contribué des données pour les figures 1, 4 et 5. Jonathan Richard a adapté et optimisé la technique de mesure de l'ADCC pour être utilisable sur des cellules recouvertes de gp120 recombinante, réalisé la majorité des expériences du manuscrit et participer à la rédaction de celui-ci avec Andrés Finzi. Laurie-Anne Batraville et Jean-Philippe Chapleau ont contribué à la réalisation des expériences. Mathieu Coutu, Mattia Bonsignori, Nicole Bernard, Cécile Tremblay, Michel Roger et Daniel E. Kaufmann ont fourni des réactifs.

CONFORMATIONAL EVALUATION OF HIV-1 TRIMERIC ENVELOPE GLYCOPROTEINS USING A CELL-BASED ELISA ASSAY

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Résumé

Les glycoprotéines d'enveloppe du VIH-1 (Env) sont responsables de l'entrée virale dans les cellules cibles et sont donc critiques pour le cycle de réplication viral. Une compréhension accrue de comment Env est capable de fournir l'énergie nécessaire à la fusion virale via ses changements conformationels pourrait permettre le design de stratégies vaccinales plus appropriées. Dans cet article, nous décrivons une technique d'ÉLISA sur base cellulaire qui permet d'étudier la reconnaissance d'Env trimèrique du VIH-1 par des anticorps monoclonaux. Après l'expression d'Env trimèrique à la surface de cellules transfectées, des anticorps reconnaissant spécifiquement certaines conformations d'Env sont incubés avec les cellules. Un anticorps secondaire couplé à la peroxydase du raifort et une réaction de chimiluminescence sont alors utilisés pour détecter la liaison des anticorps aux cellules. Le système décrit est hautement flexible et permet de détecter les changements conformationels d'Env induits par du CD4 soluble ou des protéines cellulaires. Il demande de très faible quantité de matériel, aucun équipement hautement spécialisé ou de savoir-faire spécifique : il est donc approprié pour utilisation en criblage à moyen ou haut débit.

Abstract

HIV-1 envelope glycoproteins (Env) mediate viral entry into target cells and are essential to the infectious cycle. Understanding how those glycoproteins are able to fuel the fusion process through their conformational changes could lead to the design of better, more effective immunogens for vaccine strategies. Here we describe a cell-based ELISA assay that allows studying the recognition of trimeric HIV-1 Env by monoclonal antibodies. Following expression of HIV-1 trimeric Env at the surface of transfected cells, conformation specific anti-Env antibodies are incubated with the cells. A horseradish peroxidase-conjugated secondary antibody and a simple chemiluminescence reaction are then used to detect bound antibodies. This system is highly flexible and can detect Env conformational changes induced by soluble CD4 or cellular proteins. It requires minimal amount of material and no highly-specialized equipment or know-how. Thus, this technique can be established for medium to high throughput screening of antigens and antibodies, such as newly-isolated antibodies.

Introduction

Human immunodeficiency virus type 1 (HIV-1) entry, mediated by the trimeric viral envelope glycoproteins (Env) is the first step of the infectious cycle. Being the only exposed viral antigen presented at the surface of virions, the Env trimer elicits neutralizing and non-neutralizing antibodies. As such, it represents an interesting candidate for vaccine immunogen design. However, vaccination trials with Env in soluble or recombinant forms elicited responses with only minimal effectiveness against most primary HIV-1 isolates[1-3]. Nonetheless, partial efficacy observed in the RV144 vaccine trial[4] renewed interest in HIV-1 Env as an immunogen candidate. This was corroborated by a recent study describing that vaccine-elicited anti-Env antibodies were sufficient to generate a certain degree of protection against SIV and HIV challenges[5].

After being synthesized in the endoplasmic reticulum, the Env glycoprotein precursor, gp160, undergoes various post-translational modifications that are critical for its ability to fuel the viral fusion process. The Env precursor must fold properly and associate in trimers before

being cleaved into its extra-cytoplasmic gp120 and transmembrane gp41 subunits[6-10], with non-covalent interactions maintaining the gp120-gp41 liaison. The infected cell machinery is also responsible for heavily glycosylating Env, comprising about 50% of its total mass[11,12]. The resulting complex structure allows Env to be conformationally flexible[13,14], while providing a metastability that is thought to allow Env to adapt and hide certain highly immunogenic epitopes that would otherwise be exposed[15-19], highlighting the importance to better understand the different conformations sampled by the native Env trimer.

To date, several techniques have been developed and successfully used to study Env conformational changes. However, they vary in their limitations, being often restricted to specific Env contexts. For example, surface plasmon resonance or immunoprecipitation assays using conformation specific monoclonal antibodies (mAbs), rely either on monomeric soluble or solubilized Env molecules which are known to be immunogenetically different from their trimeric forms[20,21]. Recent studies also suggest that cleavage affects Env conformations resulting in the exposure of epitopes mainly recognized by non-neutralizing antibodies[14,22,23].

Here we describe in detail a method that allows for fast and easy determination of the conformation of cellularly-expressed Env trimers[18,24-26]. Following transient transfection of Env in a human adherent cell line the binding of Env-specific antibodies is detected using a simple chemiluminescence reaction. This technique can also be used to characterize the conformational preference of conformation-dependent antibodies. Thus, this assay provides a robust and highly flexible detection method.

Protocol

1. Day 1 – Cell Culture

1.1 Plate $2x10^4$ human osteosarcoma (HOS) cells per well in an opaque, 96-well cell-culture plate suitable for luminescence reading. Use Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. Incubate until next day at 37 °C, 5% CO_2.

2. Day 2 – Polyethylenimine (PEI) Transfection

2.1 Prepare transfection mix according to subsequent steps. Adjust reagents and DNA quantities according to the number of wells that are to be transfected with the same Env.
2.1.1 Tube A: Add 10 ng Tat-encoding plasmid (such as pTat-III[27]) and 150 ng Env-encoding plasmid to 5 µl DMEM supplemented with 25 mM HEPES.

Note: The Tat-encoding plasmid is only required when using Tat-dependent Env-encoding plasmids such as pSVIII.

2.1.2 Tube B: Add 450 ng PEI (from a 1 µg/µl solution) to 5 µl DMEM.

2.2 Add content of tube B to tube A. Mix thoroughly by vortexing for 10 sec and incubate transfection mix 10 min at room temperature (RT, 22 °C)

2.3 Add 10 µl of the transfection mix per well of the 96-well plate. Incubate for 48 h at 37 °C, 5% CO_2.

3. Day 4 – ELISA
All experiments must be performed at room temperature to minimize possible endocytosis of Env/antibodies complexes.

3.1 Prepare 250 ml of Washing Buffer per plate being used at the same time. Washing Buffer is Tris-buffered saline (TBS) 1X pH 7.5 (50 mM Tris-Cl, pH 7.5; 150 mM NaCl), supplemented with 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$.

3.2 Prepare 125 ml Blocking Buffer per plate by adding 1% non-fat dry milk and 5 mM Tris pH 8.0 to Washing Buffer.

3.3 Remove cell culture media and transfection mix (supernatant) from 96-well plate.

3.4 Add 100 µl of Blocking Buffer per well and incubate 20 min at RT.

3.5 Remove supernatant and add 50 µl of antibody (or serum) per well, diluted to appropriate concentration in Blocking Buffer. A concentration of 1 µg/ml is usually a good starting point. Incubate 1 h at RT.

3.6 Wash 3 times with 100 µl Blocking Buffer and then repeat washing process 3 times with 100 µl Washing Buffer.

3.7 Remove supernatant, add 100 µl Blocking Buffer and incubate 5 min at RT.

3.8 Remove supernatant and add 50 µl of secondary antibody, diluted 1/3000 in Blocking Buffer. Vary optimal antibody dilution according to manufacturer differences. Incubate 40 min at RT.

3.9 Wash 3 times with 100 µl Blocking Buffer and then repeat washing process 3 times with 100 µl Washing Buffer.

4. Data Acquisition

4.1 Remove supernatant from the plate and add 30 µl 1X enhanced chemiluminescence (ECL) substrate per well.

4.2 Acquire chemiluminescence signal for 1 sec per well on a suitable plate-reader according to manufacturer instructions. Reading time may differ according to hardware differences.

Representative results

Using the general procedure described above, we adapted the protocol to assay the impact of soluble CD4 (sCD4) and co-expressed cellular CD4 on the exposure of CD4i epitopes on either wild-type (wt) or mutated Env, as described previously[18,24,25,28]. Figure 1 schematically represents the general procedure and the exposure of CD4i epitopes following treatment with sCD4 or by co-expression of cellular CD4[18]. In Figure 2, we used sCD4 to induce Env conformational changes that expose CD4i mAbs 17b and 48d epitopes which overlap the coreceptor binding site[24,29], whereas the outer-domain recognizing mAb 2G12 is not affected by this treatment, as expected[18,24].

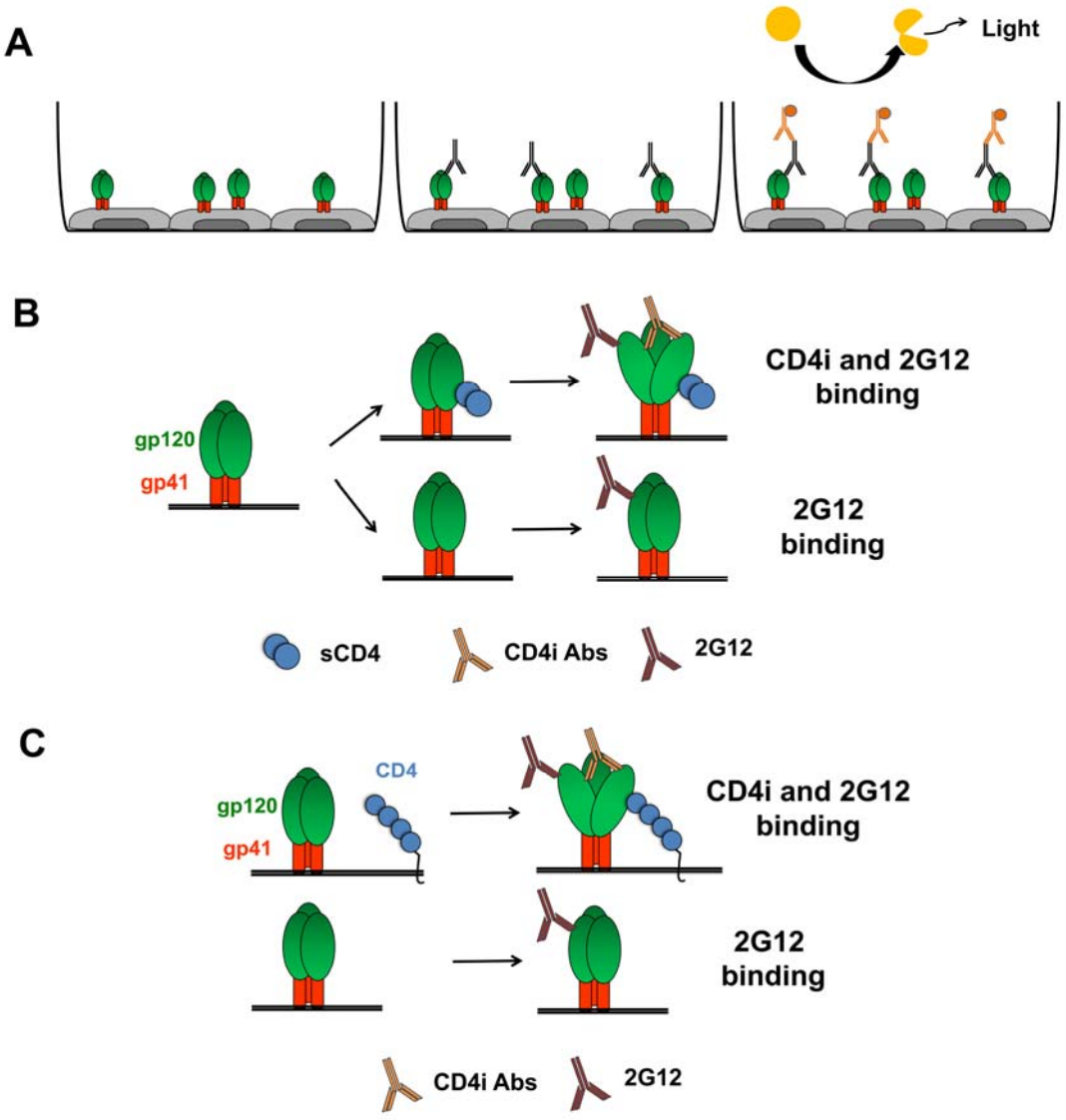

Figure 2.1 **(Article 2 Figure 1) Schematic representation of the anti-Env cell-based ELISA.**

(A) General scheme of the procedure in which HOS cells are transfected to express trimeric Env at the cell surface. Env conformation can then be sampled by using different antibodies recognizing specific conformations (such as CD4i mAbs). Signals are detected by chemiluminescence after staining with HRP-conjugated anti-human mAbs. sCD4 **(B)** or co-expression of cellular CD4 **(C)** can be used to induce Env conformational changes that lead to exposure of CD4i epitopes.

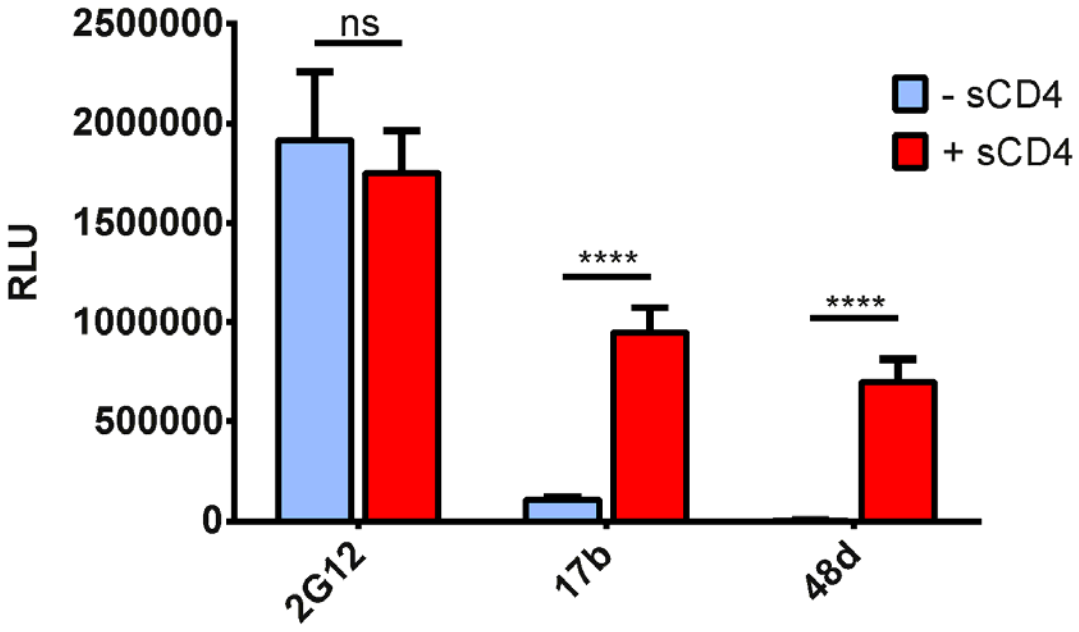

Figure 2.2 **(Article Figure 2) sCD4 induces Env conformational changes leading to exposure of CD4i epitopes.**

Interaction of sCD4 with HIV-1$_{CO\text{-}JRFL\Delta CT}$ Env enhances recognition by antibodies targeting CD4i epitopes (17b, 48d), but not by the gp120 outer-domain recognizing antibody 2G12. A plasmid encoding HIV-1$_{CO\text{-}JRFL\Delta CT}$ Env was transfected in each well and 48h later the cells were washed and incubated in presence or absence of 4 µg/ml sCD4 for 30 min at RT before continuing with the standard protocol (Day 4 – ELISA). Env conformation was then probed by incubating with 0.25 µg/ml 2G12, 1 µg/ml 17b or 48d anti-Env mAbs for 1h at RT. Signals were detected by chemiluminescence after incubation with an HRP-conjugated anti-human antibody for 45 min at RT. Shown are the mean RLU values ± SD of six replicates with signal obtained from wells transfected with an irrelevant plasmid (no Env) subtracted. Data is representative of results obtained in three independent experiments, with significance tested by two-way ANOVA (ns, not significant; ****, $p < 0.0001$).

The impact of point mutations in Env conformation can also be assessed using this assay, as presented in Figure 3. Here we used either the layer 1 Env mutant H66A, known to have a decreased propensity to spontaneously sample the CD4-bound conformation[18,24,30,31] or a mutant (S375W) which predisposes Env to the CD4-bound state[32] and obtained concordant results (Figure 3A). In cases where different Env expressors are used, it is often necessary to normalize the raw data expressed as relative light units (RLU) according to expression levels. In this case, we used PGT121, a mAb recognizing part of the Env glycan shield[33-35], as the normalizing antibody (Figure 3B).

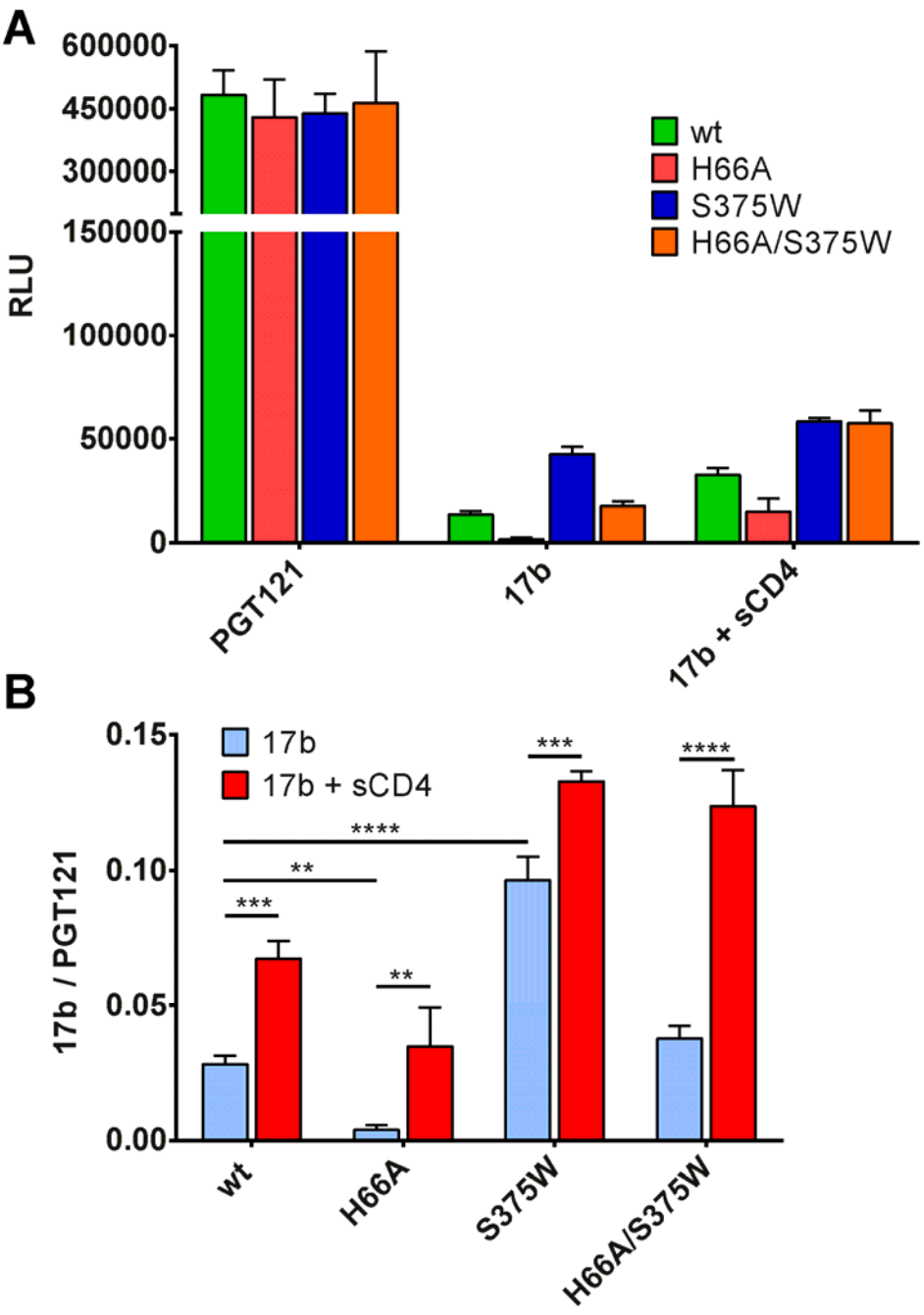

Figure 2.3 **(Article 2 Figure 3) Modulation of Env conformation.**
HIV-1 $_{YU2\Delta CT}$ layer 1 Env mutant (H66A) diminishes CD4i 17b recognition whereas the S375W variant exhibits increased 17b signal and is sufficient to restore the phenotype of the layer 1 mutant. **(A)** RLU values of the signals obtained using anti-Env PGT121 and 17b mAbs. **(B)** PGT121-normalized signals of CD4i mAb 17b following treatment with or without sCD4. Shown are the mean values ± SD of triplicates with signal obtained from wells transfected with an irrelevant plasmid (no Env) subtracted. Data is

representative of results obtained in three independent experiments, with significance tested by two-way ANOVA (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

As we recently described[18], interaction of Env and CD4 in the same cell leads to Env conformational changes that expose CD4i epitopes. In Figure 4, we co-transfected increasing amounts of a CD4 expressor together with Env in the cell-based ELISA assay and obtained increasing signals for CD4i mAbs A32 and C11[18,36-38], which recognize discontinuous epitopes in the inner domain of gp120, whereas Env recognition by the conformational-independent 2G12 antibody was not affected (Figure 4A). In order to control for transfection efficiency between conditions, raw data was normalized to 2G12 (Figure 4B). Increased signals obtained for A32 and C11 antibodies depended on Env-CD4 interaction as indicated by the absence of A32 and C11 modulation when Env was co-transfected with a CD4 mutant (F43H) with decreased ability to interact with Env[39].

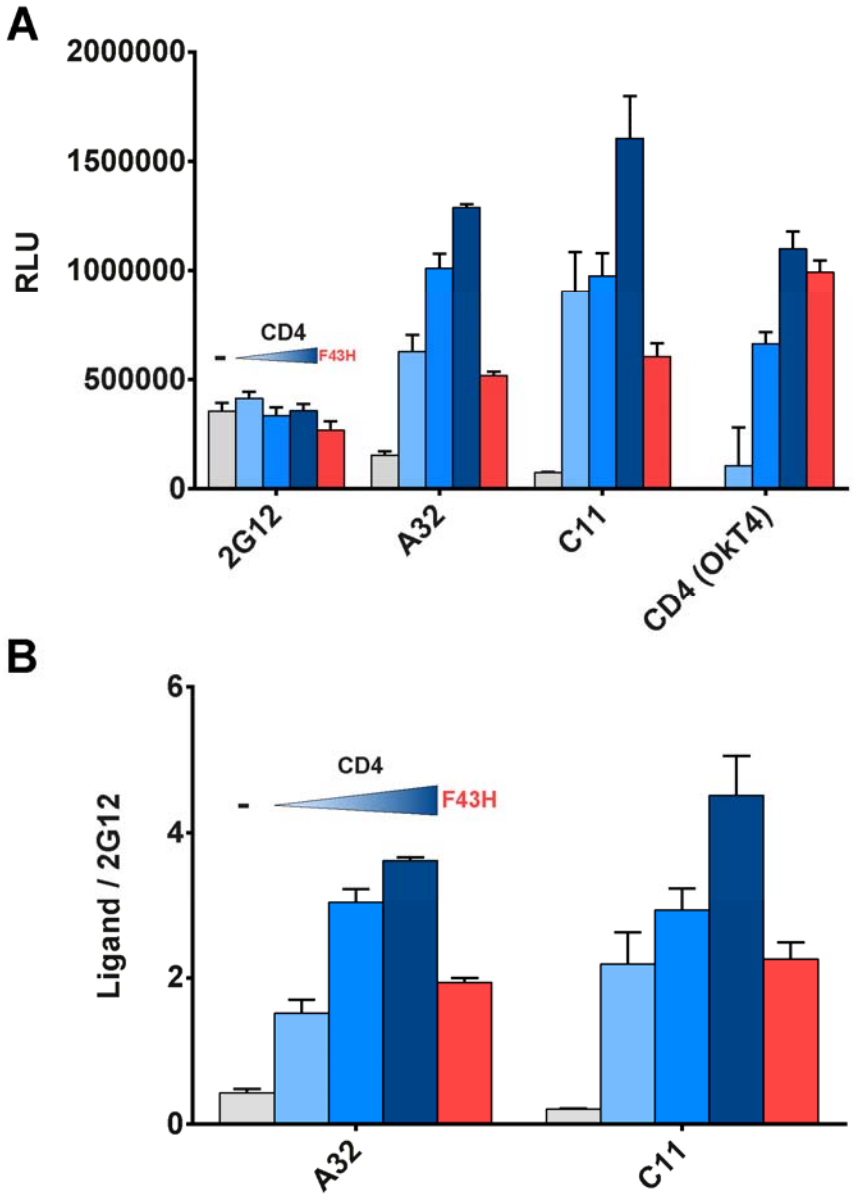

Figure 2.4 **(Article 2 Figure 4) Co-expression of cellular CD4 enhances recognition by CD4i antibodies.**

A CD4-encoding plasmid was co-transfected with HIV-1$_{YU2\Delta CT}$ Env in order to favor the CD4-bound conformation[18]. **(A)** RLU values of the signals obtained using anti-Env 2G12, A32 or C11 mAbs and the anti-CD4 OkT4 mAb. **(B)** 2G12-normalized signals of CD4i mAbs A32 and C11. Shown are the mean values ± SD of triplicates with signal obtained from wells transfected with an irrelevant plasmid (no Env) subtracted. Data is representative of results obtained in three independent experiments. Grey bar indicates in absence of CD4, whereas the increasing blue bar indicates a step-wise increase in the amount of CD4 expressor being transfected (1.7, 3.5 and 7 ng) and red bar

indicates the transfection of a CD4 mutant (F43H, 7 ng) with decreased capacity to interact with gp120.

Discussion

This assay is optimized to detect the interaction of specific mAbs with HIV-1 trimeric Env expressed at the cell surface. Once the protocol has been established, it can be used at medium to high throughputs with low overall material costs and little amounts of antibodies. Since this assay is transfection-based, it can easily be adapted for co-expression of cellular proteins such as CD4 in order to study their effects on Env conformation.

However, the transfection base of this protocol also implies that it is one of its most important pitfalls. First off, antigens to be studied with this technique are required to be available in an independent expression vector. As such, Env genes from various clinical sources or proviral constructs would need to be sub-cloned into mammalian expression vectors. While full-length proviral constructs can also be used in this technique (see reference 18), this also implies active viral particles production thus requiring work in appropriate bio-containment facilities.

Moreover, success of this technique is intimately linked with transfection efficiency. Low signals obtained are often due to poor expression of transfected antigens. Typical sources of problems are plasmid DNA quality, transfection reagents and cells viability. If required, optimization of transfection conditions could also be performed using other techniques, such as flow cytometry or western blotting. Of note, it is also important to be aware that expression of some Env constructs could be sub-optimal and could therefore affect the technique's outcome.

Here we focused on probing HIV-1 Env conformation using previously described CD4i mAbs. This setting allows for a broad range of analysis such as probing the effect of Env point mutations or the conformational consequences of co-expressed proteins. Moreover, the technique described here can also be used with well-characterized Env mutants with different conformational propensity in order to probe the specificity of different mAbs for various Env

conformations. This allows an easy and rapid characterization of newly isolated mAbs while not requiring highly-specialized equipment or know-how.

Although we only used this method against Env from various HIV-1 clades and other close relative lineages (HIV-2, SIV/Mac) [18], we believe this assay could be adapted for additional surface antigens, such as ones from other virus families.

Acknowledgments

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FLOW CYTOMETRY-BASED ASSAY TO STUDY HIV-1 GP120 SPECIFIC ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY RESPONSES

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Résumé

Une attention accrue sur le rôle des fonctions effectrices des anticorps dans le contrôle du VIH-1 a mené à un intérêt renouvelé envers le rôle que pourrait jouer la réponse ADCC dans le contrôle de la transmission virale ou dans la vitesse de progression de la maladie. Alors que des techniques basées sur la relâche de chrome[51] ont été fréquemment utilisées pour étudier la réponse ADCC contre le VIH-1, un bon nombre de techniques alternatives ont récemment été développées. Dans cet article, nous décrivons la mise en place d'une de ces techniques, qui permet de mesurer de manière non-radioactive via la cytométrie en flux l'élimination par la réponse ADCC de cellules couvertes par de la gp120 recombinante. Cette technique repose sur le marquage des cellules cibles et effectrices par des fluorochromes différents, ce qui permet de détecter adéquatement les cellules cibles et ainsi de quantifier la présence de cellules cibles qui ont survécu à la réponse ADCC en comparant à un nombre fixe de particules synthétiques qui ont été ajoutées à chacun des échantillons. En utilisant de faibles concentrations de gp120 recombinante, des cellules cibles se rapprochant de l'exposition standard d'épitopes à la surface de cellules infectées ont été générées. Finalement, cette

méthode a été utilisée avec succès pour cribler du sérum de patients infectés au VIH-1 pour déterminer leur activité ADCC contre la gp120.

Abstract

Increased attention on the role of Fc-mediated effector functions against HIV-1 has led to renewed interest into the role that antibody-dependent cellular cytotoxicity (ADCC) could play in controlling viral transmission and/or the rate of disease progression. While ^{51}Chromium release assays have traditionally been used to study ADCC responses against HIV-1, a number of alternative flow-cytometry-based assays were recently developed. In this study, an alternative flow-cytometry-based assay was established to allow non-radioactive measurement of ADCC-mediated elimination of HIV-1 gp120 envelope glycoprotein (Env)-coated target cells. This assay relies on staining target and effector cells with different dyes, which allows precise gating and permits the calculation of the number of surviving target cells by normalization to flow-cytometry particles. By using small concentrations of recombinant gp120 Env, suitable targets cells that recapitulate the ADCC response mediated against HIV-1-infected cells were generated. Finally, this method was applied successfully to screen human sera for ADCC activity directed against HIV-1 gp120 Env.

Text

Antibody-dependent cellular cytotoxicity (ADCC) response is an antibody-driven immune mechanism that recruits effector cells such as Natural Killer (NK) cells, monocytes and neutrophils and activates them against infected cells expressing surface antigens, resulting in their elimination. Besides antibody-mediated viral neutralization, ADCC was one of the earliest antibody functions observed in HIV-1-infected individuals (Lyerly et al., 1987). The HIV-1 envelope glycoprotein (Env) is the main target antigen at the surface of infected cells for antibodies that mediate these functions (Lyerly et al., 1987; Koup et al., 1989). The importance of ADCC in controlling HIV-1 and SIV was subsequently reported in several studies (reviewed in (Lewis, 2013)). The immune-correlates analysis of the RV144 vaccine trial indicated that ADCC activity in association with low levels of serum IgA was linked with

decreased HIV-1 acquisition (Bonsignori et al., 2012; Haynes et al., 2012). Accordingly, potent ADCC-mediating monoclonal antibodies (mAbs) were isolated from a subset of RV144 vaccinees (Bonsignori et al., 2012). It has been recently suggested that ADCC responses apply significant immune pressure on individuals infected with HIV-1 during the course of natural infection, further supporting the potential impact of ADCC on HIV-1 progression (Chung et al., 2011).

While ADCC responses to HIV-1 were first assayed using the ^{51}Chromium release assay (Tyler et al., 1990; Ahmad et al., 1994; Baum et al., 1996), a number of alternative flow-cytometry-based assays were subsequently developed. The majority of these assays use T cells coated with HIV-1 Env (gp120 or gp140) as target cells, although HIV-1-infected cell lines have also been used (reviewed in (Kramski et al., 2013)). These assays commonly evaluate NK cell activation (IFN-y, granzyme B and CD107a)(Stratov, Chung, and Kent, 2008; Chung et al., 2009; Madhavi et al., 2013), the delivery of granzyme B to target cells (Pollara et al., 2011; Smalls-Mantey et al., 2012) or the percentage of apoptotic target cells (Kantakamalakul et al., 2006). However, these techniques do not directly measure the elimination of target cells. To date, ADCC-mediated loss of HIV-1 infected cells has been measured using LTR-Luciferase engineered target cells (Alpert et al., 2012), GFP-expressing viruses (Pham et al., 2014; Veillette et al., 2014) and intracellular anti-p24 staining (Smalls-Mantey et al., 2012). The rapid and fluorometric ADCC (RFADCC) which relies on the release of intracellular fluorescent dye (Gomez-Roman et al., 2006), is a flow cytometry-based assay that allows assessment of gp120-coated target cells killing. However, a recent report suggested that this assay primarily reflects Ab-mediated monocyte phagocytosis (Kramski et al., 2012). Here, an alternative flow-cytometry-based assay was developed to permit accurate calculation of ADCC-mediated elimination of gp120-coated targets without the use of radioactivity.

The interaction of HIV-1 Env with the CD4 receptor was recently reported to be required for efficient exposure of ADCC-mediating Env epitopes at the surface of HIV-1 infected cells (Veillette et al., 2014). Accordingly, the ability of Env to reach the CD4-bound conformation was found to be critical for exposure of Env ADCC-mediating epitopes (Veillette et al., 2014). Thus, cell-surface CD4 down-regulation by Nef and Vpu accessory

proteins, as well as Vpu-mediated Tetherin/BST-2 antagonism reduced Env ADCC-mediating epitope exposure, negatively impacting the susceptibility of infected cells to this effector function (Veillette et al., 2014). Based on these data, cells infected with virus lacking both Vpu and Nef (i.e. presenting high levels of CD4 and Env at the cell surface) were shown to be suitable target cells to evaluate anti-HIV-1 Env ADCC responses. Since working with HIV-1-infected cells requires complex manipulations and a biosafety level containment laboratory to ensure safety, a simpler assay that could be carried out under standard level 2 biocontainment was developed in this study. Since HIV-1 gp120 Env represents one of the main targets for ADCC-mediating Abs (Lyerly et al., 1987), cells coated with recombinant HIV-1_{YU2} gp120 proteins were used as target cells. In order to specifically study ADCC responses, CEM.NKr, cells, a CD4+ T cell line resistant to direct NK cell killing mediated by natural cytotoxicity receptors, were used as target cells (Howell et al., 1985; Alpert et al., 2012).

Recombinant HIV-1_{YU2} gp120 proteins were produced and purified as previously described (Finzi et al., 2010; Desormeaux et al., 2013). First, the minimal amount of recombinant HIV-1 gp120 required to coat target cells was determined by flow cytometry, by evaluating the capacity of different specific anti-gp-120 mAbs to recognize gp120-coated cells and cells infected with HIV-1 lacking both Vpu and Nef. Briefly, CEM.NKr cells were coated with increasing concentrations of recombinant HIV-1_{YU2} gp120 (10, 50, 100 or 250 ng/ml) for 30 min at 37°C ($7.5x10^5$ cells/ml) or were infected with VSV-G-pseudotyped HIV-1 NL4.3.ADA.GFP.IRES.Nef- Vpu- virus, as previously described (Veillette et al., 2014). To evaluate the level of HIV-1 gp120 Env present at cell-surface, cells were stained with the conformation-independent gp120 outer domain-recognizing 2G12 antibody and then fluorescently labeled with an Alexa-fluor 647-coupled goat anti-human IgG secondary antibody (Invitrogen, San Diego, CA, USA). As expected, recognition of gp120-coated target cells by 2G12 was dependent on the concentration of recombinant HIV-1 gp120 used to coat the cells (Fig.1A-B). Interestingly, coating target cells with 50 ng/ml of recombinant HIV-1 gp120 was sufficient to reach 2G12 binding levels similar to those observed in HIV-1-infected cells (Fig.1A-B), thus indicating that, in this method, a concentration of 50 ng/ml gp120 Env per $7.5x10^5$ CEM-NKr/ml is sufficient to reach levels of Env similar to those observed at the surface of infected cells. Higher gp120 concentrations (100 ng/ml and 250 ng/ml) exceeded

Env levels present at the surface of HIV-1-infected cells. To specifically evaluate the exposure of ADCC-mediating gp120 epitopes, cells were also stained with different CD4-induced ADCC-mediating Abs, including the A32 mAb (Moore et al., 1994; Veillette et al., 2014), the A32-blockable CH54 mAb isolated from an RV144 trial vaccine recipient (Bonsignori et al., 2012) and the anti-cluster A L9-i1 mAb (Guan et al., 2013). Similarly, coating CEM.NKr cells with 50 ng/ml of recombinant gp120 was sufficient to reach the level of gp120 Env ADCC-mediating epitope recognized by these antibodies at the surface of HIV-1-infected cells (Fig.3C).

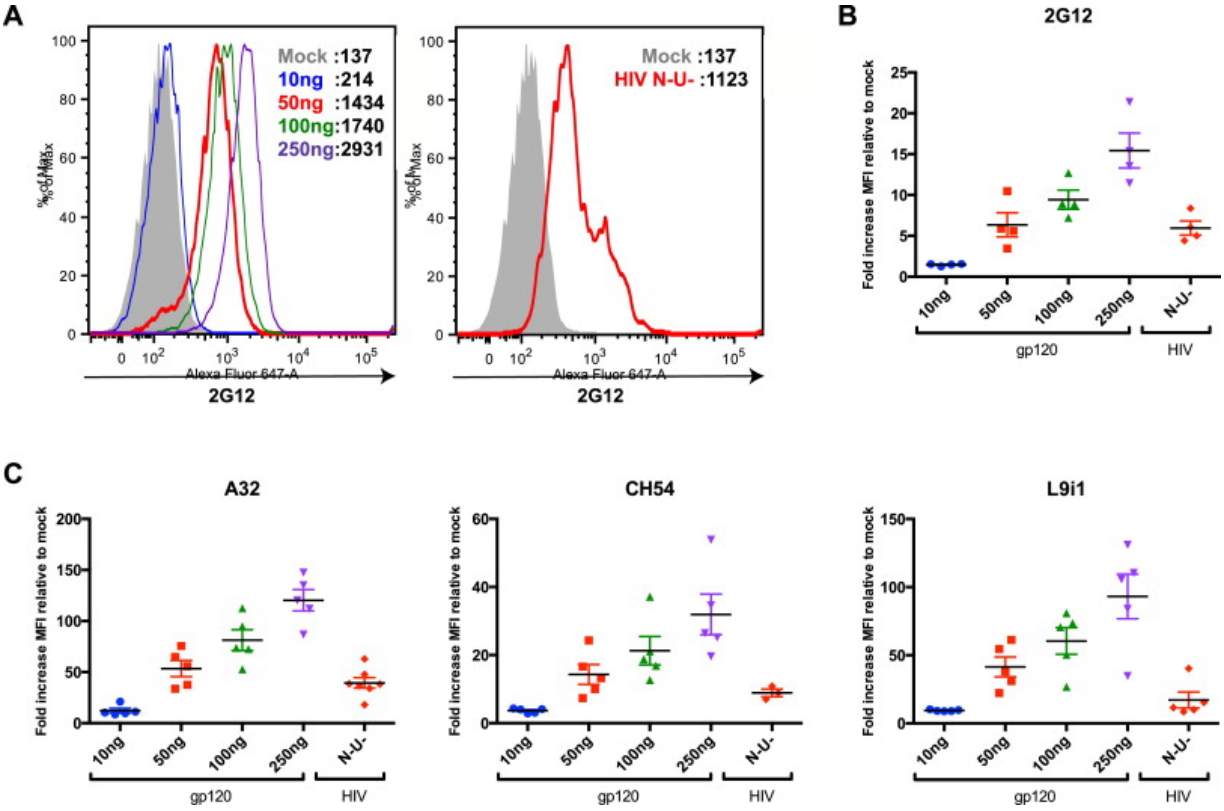

Figure 2.5 **(Article 3 Figure 1) Recognition of recombinant HIV-1 gp120-coated and HIV-1 infected target cells by specific anti-gp120 antibodies.**
CEM.NKr cells were either coated with increasing concentrations of recombinant HIV-1 gp120 (10, 50, 100 or 250 ng/ml) for 30 min or were infected with VSV-G-pseudotyped HIV-1 lacking Nef and Vpu (N-U-) for 48h. The ability of the anti-gp120 2G12 (**A-B**) or the CD4-induced ADCC-mediated mAbs A32, CH54 or L9i1 (**C**) to recognize gp120-coated or HIV-1 infected cells was evaluated by flow cytometry using appropriate secondary Abs. (**A**) The numbers shown in the graph represents mean fluorescence intensity (MFI) values. (**B-C**) Signals were normalized to the MFI value of the mock control

(fold-change). Data shown are the results of at least 3 independent experiments and error bars depict the standard error of the mean (SEM).

A recently-described assay that allowed to calculate elimination of HIV-1-infected cells (Veillette et al., 2014) was next adapted to obtain a simple and rapid FACS-based assay measuring elimination of gp120-coated target cells by ADCC. The new assay was performed as outlined in Figure 2. Briefly, target cells were labelled with the viability dye AquaVivid (Invitrogen, San Diego, CA, USA) and the cellular marker eFluor670 (eBiosciences, San Diego, CA, USA), whereas effector cells were labelled with the cellular marker eFluor450 (eBiosciences, San Diego, CA, USA). Cell labeling was performed according to manufacturer's directions. Cryopreserved peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were used as the source of effector cells. After washing with culture medium (RPMI, 10% FBS), $1x10^5$ target cells were mixed with $1x10^6$ effector cells in 96-wells V-bottom plates (Corning, Corning, NY, USA) in the presence or absence of a 1:1000 dilution of human serum or 5 µg/ml of ADCC-mediating mAbs for 4h to 6h at 37°C, in a humidified 5% CO_2 incubator. Cells were then fixed with a PBS-formaldehyde solution (2% formaldehyde final concentration) containing a constant number of flow cytometry particles ($5x10^4$/ml) (AccuCount Blank Particles, 5.3 µm; Spherotech, Lake Forest, IL, USA). These particles are designed to be used as reference particles since their concentration is known. Thus allowing to count the absolute cell number by flow cytometry. A constant number of particles (~$1x10^3$) were counted during cytometry acquisition in order to normalize the number of viable targets cells. Each sample was acquired with a LSRII (BD Bioscience, Mississauga, ON, Canada) and data analysis was performed using FlowJo vX.0.6 (Tree Star, Ashland, OR, USA).

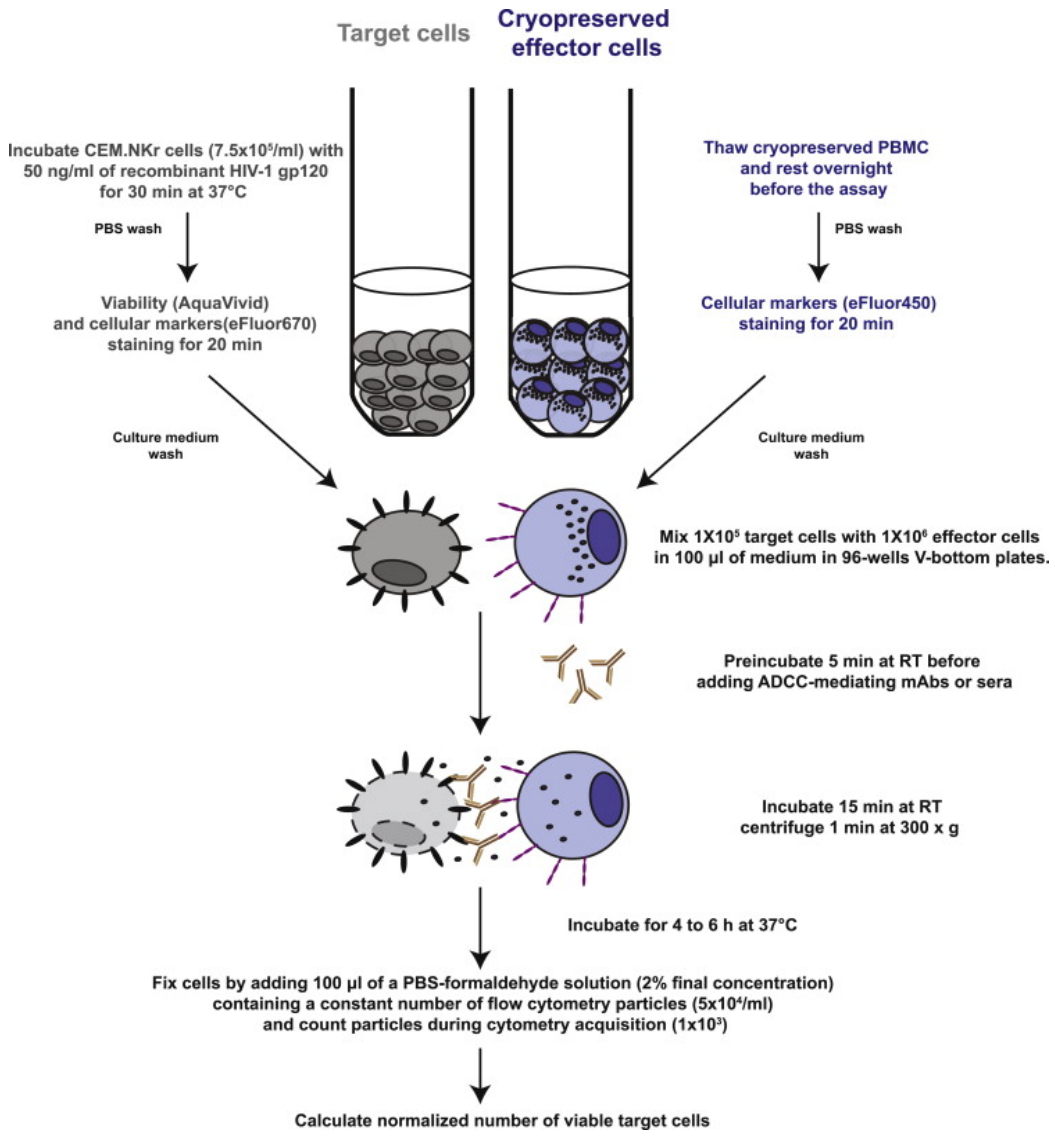

Figure 2.6 (Article 3 Figure 2) Assay overview.
A schematic representation of the assay providing the outline of the protocol.

To calculate the number of normalized single viable target cells that survived the incubation period in presence of mAbs and effector cells, the gating strategy shown in Figure 3A was developed. The first gate was set to include the target cells according to cell morphology by light-scatter parameters using target cells alone (first row). After gating on single cells (data not shown), target cells were identified based on the eFluor670 marker; thus excluding effector cells labeled with the eFluor450 cellular marker (second row). Finally, viable target cells were identified by exclusion of non-viable target cell population labeled

with Aquavivid (Third row). In parallel, flow cytometry particles were identified according to their morphology (first row) and served to normalize the number of viable target cells (Fig.3A). The normalized number of viable target cells was used to calculate the percentage (%) of cytolysis by using the formula presented in Figure 3C. As shown in Figure 3A, in the presence of effector and target cells alone (second column), a small decrease in the number of normalized viable target cells was observed compared to target cells alone (first column), which most likely represents ADCC-independent target cell killing, as previously reported with CEM.NKr cells (Richard et al., 2010). However, upon addition of the ADCC-mediating A32 mAb (third column), a significant decrease in the number of live target cells (6126 *vs* 10652, representing 35,9% of ADCC-mediated killing) was observed. Of note, this ADCC-mediated killing was specific to A32 since it was completely blocked by pre-incubating target cells with the A32 Fab fragment (5µg/ml, 30 min) (fourth column). Although an optimal ADCC response was obtained using 5µg/ml of A32 mAb, it is worth mentioning that ADCC-mediating killing was also detected using lower concentrations of ADCC-mediating Abs using this assay (Fig.S1 A).

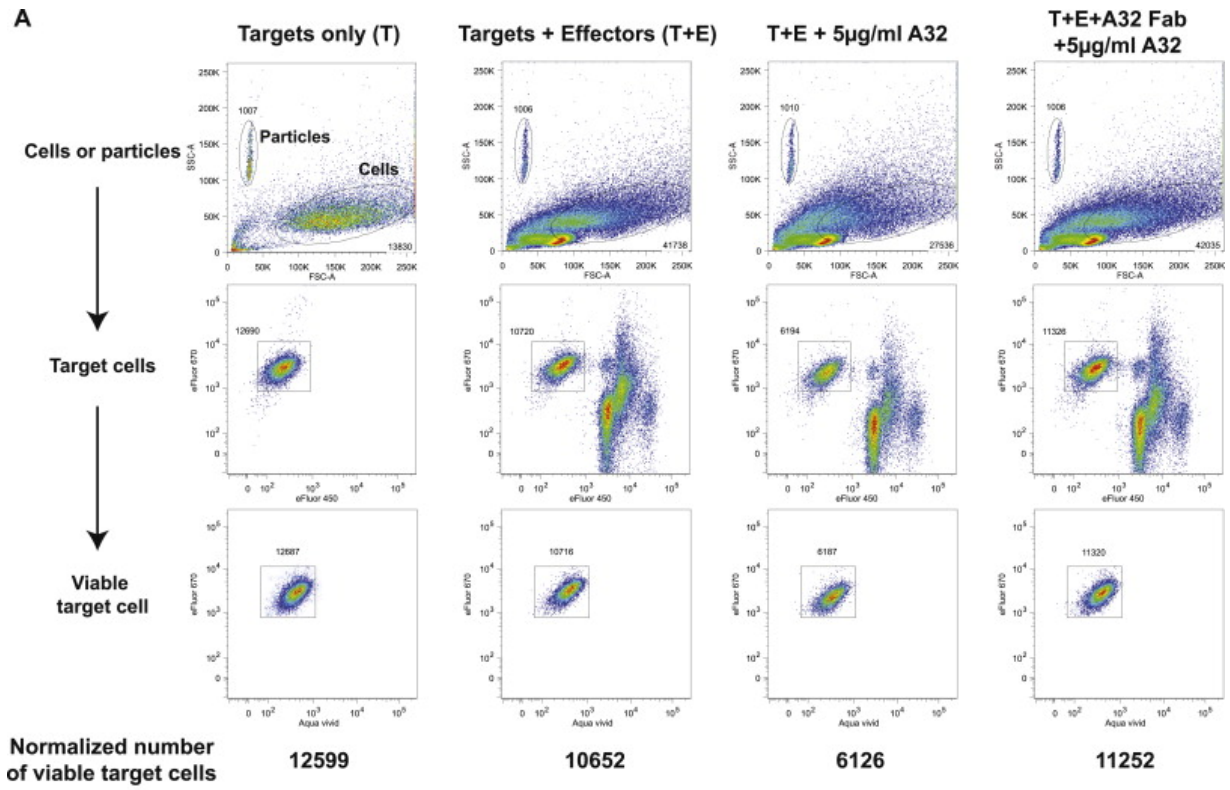

Figure 2.7 **(Article 3 Figure 3) Gating strategy and calculation to evaluate the percentage of ADCC-mediated killing.**

(A) Target cells were identified according to cell morphology by light-scatter parameters (first row), based on their pre-labeling with eFluor670 and excluding the effector cells labeled with eFluor450 (second row). The viable target cells are then identified by exclusion of the non-viable target cell population labeled with Aquavivid (Third row). Flow cytometry particles are identified according to their morphology (first row). The normalized number of viable target cells was calculated relative to $1x10^3$ flow cytometry particles (**B**) Formula used to calculate the percentage of ADCC using the normalized number of viable target cells. Data shown is representative of at least 4 experiments.

During viral infection, ADCC contributes to viral clearance trough specific recognition and, most importantly, targeted elimination of virus-infected cells. In contrast to previous ADCC assays using gp120-coated target cells, this method directly measures ADCC-mediated elimination of target cells rather than NK cell activation (Stratov et al., 2008; Chung et al., 2009; Madhavi et al., 2013), delivery of granzyme B into target cells (Pollara et al., 2011; Smalls-Mantey et al., 2012) or assessment of the percentage of apoptotic target cells (Kantakamalakul et al., 2006). Staining target and effector cells allows for precise calculation of target cells number; excluding effector cells that could have internalized eFluor670 membrane fragments or engulfed intact target cells (eFluor670+eFluor450+ effector cells) (Fig.3A), as previously reported for the RFADCC assay (Kramski et al., 2012). Notably, in contrast to flow-cytometry based method evaluating ADCC-mediated elimination of HIV-1 infected cells (Alpert et al., 2012; Smalls-Mantey et al., 2012; Pham et al., 2014; Veillette et al., 2014), this assay does not require pre-infection of target cells and a biosafety level containment laboratory to ensure safety. In that context, the proposed experiments outlined in Figure 2 allow a rapid evaluation of ADCC responses, which takes 6-8 h from target cell preparation to flow cytometry acquisition.

To determine whether the results obtained with gp120-coated target cells could reproduce the ADCC response mediated against CEM.NKr cells infected with HIV-1 lacking Nef and Vpu, the assay was performed as described above using gp120-coated or HIV-1 infected cells as target cells. With the exception that viable HIV-1-infected target cells were determined as eFluor670+eFluor450-Aquavivid-GFP+ cells, the gating strategy remained the same as shown in Figure 3. Figure 4A-B shows that no significant differences in the percentage of ADCC-mediated killing were observed between gp120-coated or HIV-1-infected target cells. This is likely due to the fact that gp120 Env coats CEM-NKr cells through CD4 interaction, thus forcing gp120 Env to sample the CD4-bound conformation, which results in the exposure of CD4-induced ADCC-mediating epitopes such as those recognized by A32, CH54 and L9-i1 (Fig. 1C) and mimicking conformations achieved by infecting cells with HIV-1 viruses lacking Nef and Vpu accessory proteins (Veillette et al., 2014). Indeed, introduction of a mutation in the HIV-1 gp120 CD4-binding site (D368R), known to impair gp120-CD4 interaction, completely abrogated binding of gp120 to target

cells (Fig.S2A) and consequently their ADCC-mediated elimination (Fig.S2B). As expected, the A32-induced ADCC-mediated killing of either HIV-1-infected or gp120-coated cells was significantly reduced when A32 Fab fragments were added prior to incubation with the full A32 antibody (Fig.4A-B).

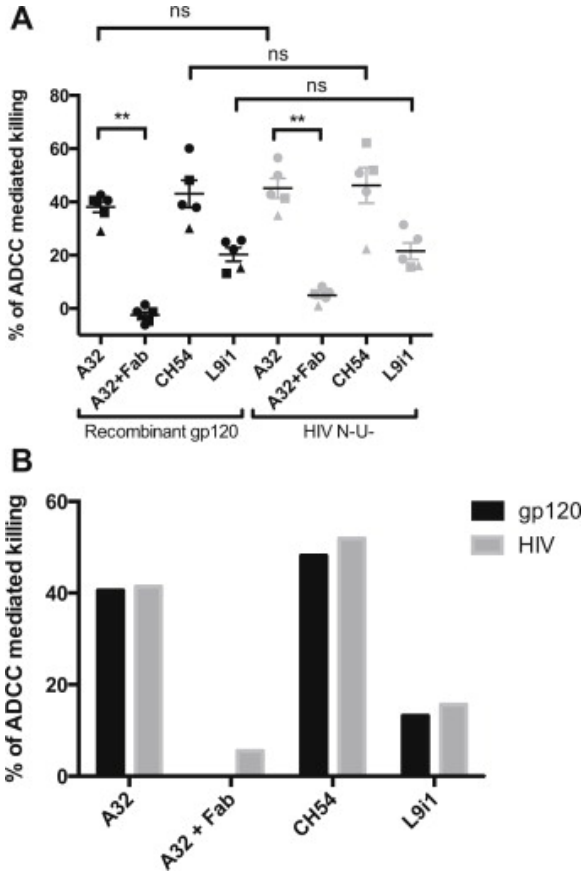

Figure 2.8 **(Article 3 Figure 4) Comparison of the ADCC response mediated against gp120-coated cells and HIV-1 infected cells.**

CEM.NKr cells were either coated with 50 ng/ml of recombinant HIV-1 gp120 for 30 min or were infected with HIV-1 lacking Nef and Vpu (N-U-) for 48h. The ADCC assay was performed as described in Fig.2 using the ADCC-mediating mAbs A32, CH54 and L9-i1 at 5 µg/ml. Alternatively, target cells were pre-incubated 30 min with 5 µg/ml of A32-Fab prior incubation with effector cells and the A32 mAbs. Viable gp120-coated target cells were identified as presented in Fig.3, while viable HIV-1-infected target cells were determined as eFluor670+ eFluor450- Aquavivid- GFP+ cells. (**A**) Data shown are the results of at least 5 independent experiments +/- SEM. ADCC was measured using PBMCs from three different healthy donors using the formula presented in Fig.3B. The different symbols depicted in the figure represent the three different donors used. Statistical significance was tested using a Mann-Whitney test (** p< 0,01, ns : non-significant). (**B**) Data shown represent the

percentage of ADCC-mediated killing obtained in a single experiment with the same PBMCs donor.

The capacity of PBMCs and purified NK cells to mediate an ADCC-response using this method was next compared. The assay was performed as described above using $1x10^5$ CEM.NKr cells coated with gp120 as target cells and $1x10^6$ PBMCs or $1X10^5$ purified NK cells obtained from the same donor, as effector cells. NK cell were isolated from cryopreserved PBMCs by negative selection using magnetic beads (EasySep™ Human NK Cell Enrichment Kit, StemCell, Vancouver, BC, Canada) after an overnight rest. As shown in Figure 5, only a small reduction in the A32-induced ADCC-mediated killing was observed when purified NK cells were used as effector cells relative to PBMCs. Thus, these results suggest that NK cells play a major role in the ADCC response mediated against gp120-coated target cells in this assay but also indicates that other cells present in PBMCs such as monocytes could contribute to a smaller extent to target cell killing, in agreement with previous studies (Pollara et al., 2011; Smalls-Mantey, Connors, and Sattentau, 2013).

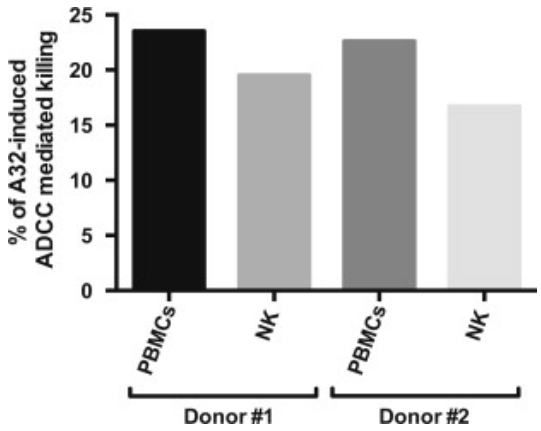

Figure 2.9 **(Article 3 Figure 5) Comparison of the ADCC response mediated by PBMCs and purified NK cells.**
The ADCC assay was performed as described in Fig.2 with the ADCC-mediating mAb A32 using $1x10^5$ cells coated with gp120 as target cells and $1x10^6$ PBMCs or $1X10^5$ purified NK cells as effector cells. Data shown represents the percentage of ADCC-mediated killing obtained in a single experiment with two different PBMCs/NK cell donors.

This assay was next applied to determine the capacity of sera collected from 18 individuals infected with HIV-1 and 5 healthy subjects (non-infected individuals) to mediate a specific HIV-1 gp120 ADCC response. Individuals infected with HIV-1 were recruited from the Canadian Cohort of HIV Infected Slow Progressors (Peretz et al., 2007; Pereyra et al., 2010; Kamya et al., 2011). Informed consent was obtained from all study participants and research adhered to the ethical guidelines of CRCHUM. The percentage of ADCC-mediated killing induced by human sera (using a 1:1000 dilution) was normalized to the ADCC-mediated killing obtained when using the A32 mAb. As shown in Fig.6A, sera from individuals infected with HIV-1 were found to induce moderate to potent HIV-1 specific gp120 ADCC, whereas background level lysis was detected using sera from, healthy subjects, demonstrating the specificity of the method. It is important to mention that ADCC responses were also detected using higher dilutions of HIV+ sera (Figure S1B), but none when target cells were not coated with gp120 (Figure S1C), highlighting the sensitivity of the assay . To test the reproducibility of the assay, HIV-1 gp120 specific ADCC responses mediated by the A32 mAb and sera obtained from individuals infected or not with HIV-1, were measured in four consecutive assays using PBMCs, as effector cells, from the same donor (Fig.6B). Very little variation between experiments was observed, with a coefficient of variation of 8% to 12% for A32 and sera from individuals infected with HIV-1, demonstrating the reproducibility of the assay. ADCC responses mediated by HIV+ sera against gp120-coated cells were next compared to those mediated against cells infected with HIV-1 lacking Nef and Vpu (Fig.6C). As obtained with ADCC-mediating mAbs (Fig.4A), no significant differences in the percentage of ADCC-mediated killing were observed between gp120-coated or HIV-1-infected target cells when using HIV+ sera (Fig.6C). Thus, suggesting that most of the ADCC-mediating Abs present in HIV+ sera are directed against HIV-1 gp120 Env. Altogether, these results demonstrate the reliability and potential of this method to screen for ADCC activity directed against the HIV-1 gp120 glycoprotein.

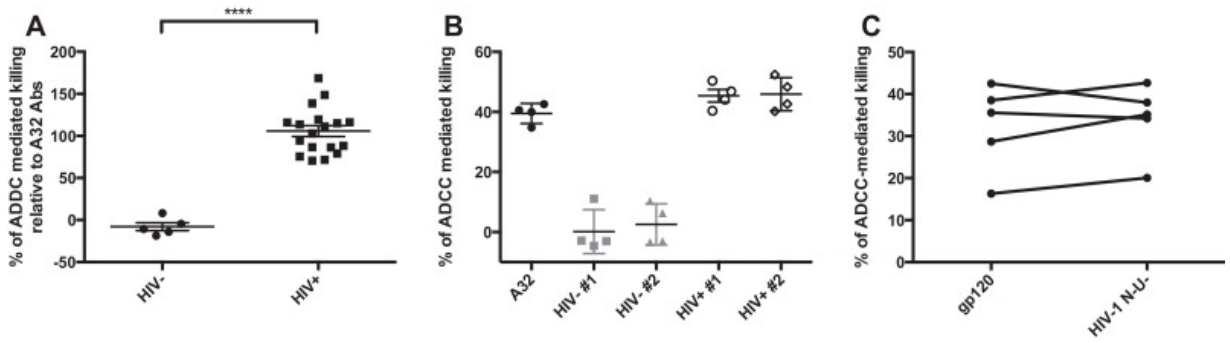

Figure 2.10 (Article 3 Figure 6) ADCC killing mediated by sera from individuals infected or not with HIV-1.

(A) The ADCC assay was performed as described in Fig.2, with the ADCC-mediating A32 mAb, sera from five healthy subjects (HIV-) and eighteen individuals infected with HIV-1 (HIV+),using gp120-coated target cells. ADCC was measured using PBMCs from a unique donor in at least three experiments. The percentage of ADCC-mediated killing induced by human sera was normalized to the ADCC-mediated killing induced by the A32 mAbs in each experiment. Statistical significance was tested using a unpaired t-test (**** p< 0,0001). (**B**) To test the reproducibility of the assay, ADCC was performed using A32, sera from two healthy subjects (HIV-) and two individuals infected with HIV-1 (HIV+) in four consecutive assays using the same healthy PBMCs donor. **(C)** Data shown represent paired values of HIV+ sera-mediated ADCC against cells coated with gp120 or infected with HIV-1 lacking Nef and Vpu (N-U-). Statistical significance was tested using a Wilcoxon matched-pairs signed rank test.

In conclusion, this method represents a simple, rapid and reliable FACS-based assay to study HIV-1 gp120 specific ADCC responses that recapitulate those obtained using HIV-1-infected cells.

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Supplementary data

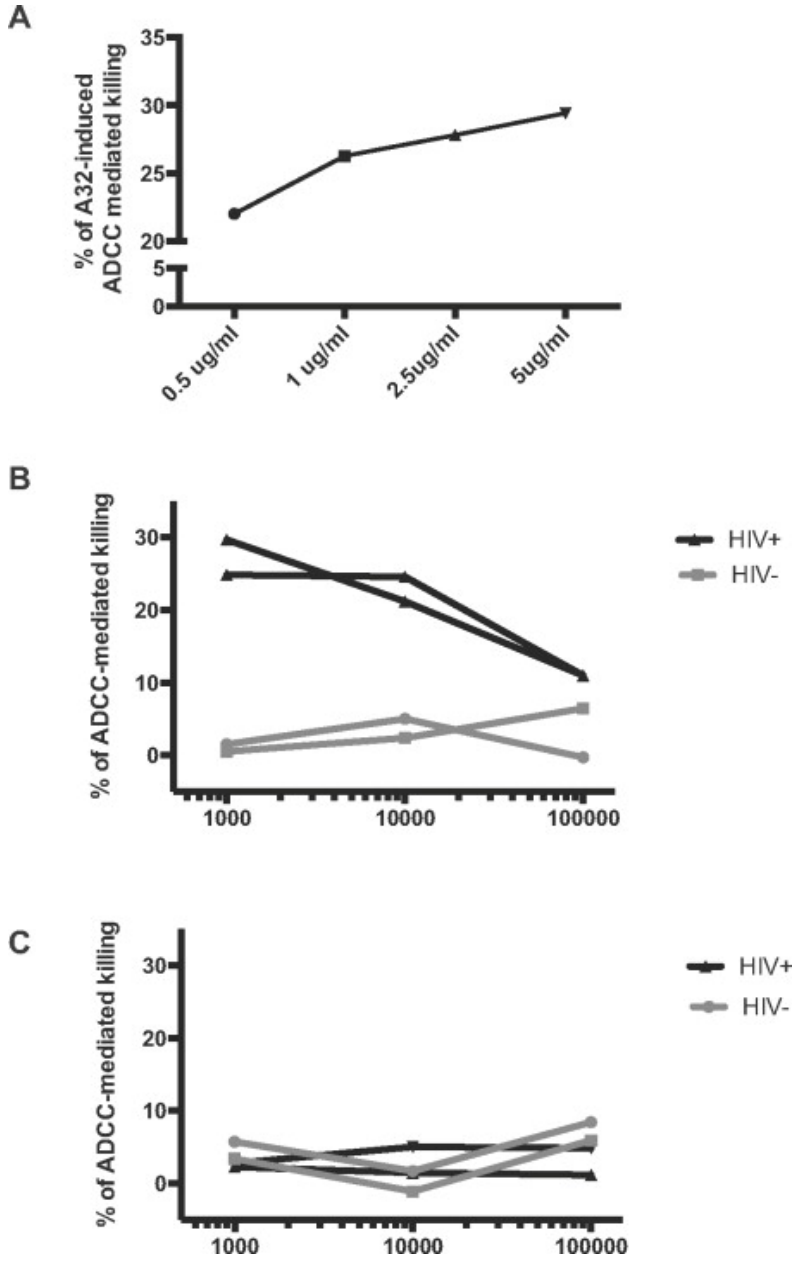

Figure 2.11S (Article 3 Figure S1) ADCC responses detection with decreased concentrations of ADCC-mediating antibody or increased dilutions of human sera.

The ADCC assay was performed as described in Fig.2 with decreasing concentrations of the ADCC-mediating A32 mAb **(A)** or increasing dilutions of sera from individual infected or not with HIV-1 (B-C), using cells coated **(A-B)** or not (**C**) with HIV-1 recombinant gp120 as target cells and PBMCs as effector cells.

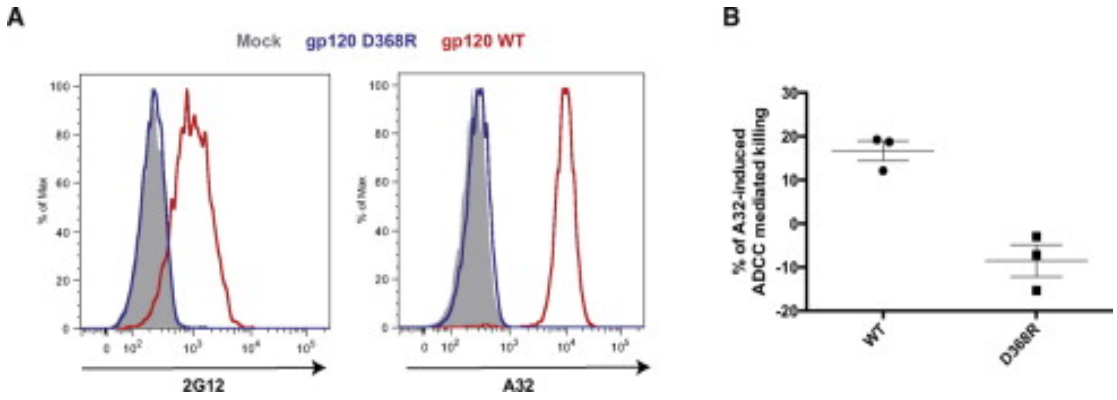

Figure 2.12S (Article 3 Figure S2) HIV-1 gp120 must interact with CD4 in order to bind target cells and mediate an ADCC response.

(A) The ability of HIV-1 recombinant gp120 wild-type (WT) or a CD4 binding site gp120 variant that is not longer able to interact with CD4 (D368R), to bind target cells was evaluated by flow cytometry using the anti-gp120 2G12 or A32 mAbs and appropriate secondary mAbs. **(B)** The ADCC assay was performed as described in Fig.2 with the ADCC-mediating A32 mAb at 5 µg/ml, using cells coated with HIV-1 recombinant gp120 WT or D368R as target cells.

CHAPITRE III

LES PROTÉINES ACCESSOIRES NEF ET VPU MODULENT INDIRECTEMENT LA CONFORMATION D'ENV

Introduction

Alors qu'il était décrit dans la littérature comment les glycoprotéines d'Env du VIH-1 changent de conformation après leur interaction avec CD4, jusqu'à présent aucune étude n'avait démontré que cette interaction pouvait se faire à la surface de cellules infectées et qu'elle pouvait avoir d'importantes conséquences sur la réponse immunitaire exercée contre celles-ci. Ce chapitre décrit les récents travaux démontrant le rôle majeur que l'interaction Env – CD4 joue dans la modulation de la réponse ADCC exercée non seulement par des anticorps monoclonaux mais également par des anticorps hautement prévalents dans le sérum d'individus infectés. De plus, il est démontré dans ce chapitre que les protéines virales accessoires Nef et Vpu, en limitant à la fois la présence de CD4 et d'Env à la surface des cellules infectées, confèrent une protection aux cellules infectées contre cette réponse immunitaire. Des résultats préliminaires y sont également présentés à savoir que les fonctions des protéines accessoires Nef et Vpu dans la modulation de la réponse ADCC contre les cellules infectées au VIH-1 seraient hautement conservées dans des isolats primaires du virus et même dans des groupes phylogénétiquement distincts, renforçant ainsi la notion que la réponse ADCC exerce une pression immunitaire majeure sur le virus.

La première section de ce chapitre fut l'objet d'une publication dans *The Journal of Virology* en 2014. Maxime Veillette a mis au point la technique de mesure de l'ADCC par cytométrie en flux, réalisé les expériences des figures 6 et 7 et supplémentaire 3, préparé toutes les figures du manuscrit et participé à la rédaction de celui-ci. Anik Désormeaux a réalisé les expériences des figures 1, 2, 3, 4 et 5. Andrés Finzi a réalisé les expériences de la figure 8 et participé à la rédaction et préparation du manuscrit. Halima Medjahed, Nour-

Elhouda Gharsallah, Mathieu Coutu, Joshua Baalwa, Yongjun Guan, George Lewis, Guido Ferrari, Beatrice H. Hahn, Barton F. Haynes, James Robinson, Daniel E. Kaufmann, Mattia Bonsignori, Joseph Sodroski ont contribué des réactifs.

Ensuite, une publication encore une fois dans *The Journal of Virology* en 2015 est présentée. Maxime Veillette a réalisé toutes les expériences du manuscrit à l'exception de celles utilisant la technique d'ÉLISA sur base cellulaire, préparé les figures du manuscrit et participé à son écriture. Laurie-Anne Batraville et Olina Dagher ont réalisé les expériences d'ÉLISA sur base cellulaire. Jonathan Richard a contribué à optimiser la technique d'ADCC utilisée dans ce manuscrit et participé au criblage des sérums d'individus infectés. Mathieu Coutu, Nicole Bernard, Cécile Tremblay, Daniel E. Kaufmann et Michel Roger ont contribué des réactifs. Andrés Finzi a participé à la préparation du manuscrit.

La troisième section de ce chapitre est un manuscrit en publication dans *The Journal of Virology*. Maxime Veillette a mis au point la méthodologie pour compétitioner les anticorps de sérums de patients infectées par de la gp120 recombinante, optimisé l'utilisation d'isolats primaires du VIH-1 dans les techniques de marquage de surface et de mesure de l'ADCC par cytométrie en flux, réalisé des expériences préliminaires avec les anticorps ciblant la gp41 d'Env et émis l'hypothèse de travail que ces anticorps de par leur méthode de reconnaître Env à la surface des cellules infectées ne pourraient pas induire une réponse ADCC optimale. Shilei Ding a réalisé la majorité des expériences de ce manuscrit. Mathieu Coutu, Jérémie Prévost, Louise Scharf, Pamela J. Bjorkman, Guido Ferrari, James E. Robinson, Christina Stürzel, Beatrice H. Hahn, Daniel Sauter, Frank Kirchhoff, George K. Lewis et Marzena Pazgier ont contribué des réactifs. Andrés Finzi a écrit le manuscrit.

INTERACTION WITH CELLULAR CD4 EXPOSES HIV-1 ENVELOPE EPITOPES TARGETED BY ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

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Résumé

L'étude des corrélats de protection du récent essai vaccinal anti-VIH-1 RV144 a démontré que des anticorps contre les glycoprotéines d'Env virale sans activité de neutralisation à large spectre corrélaient avec un risque diminué d'acquisition du virus. Ces résultats ont stimulé l'intérêt d'étudier d'autres fonctions des anticorps, notamment les fonctions effectrices telles que la réponse ADCC. Les épitopes d'Env reconnus par une majorité des anticorps capable d'induire une réponse ADCC sont que très faiblement exposés dans le trimère d'Env natif. Dans cet article, nous avons étudié le mécanisme d'exposition des épitopes reconnus par des anticorps induisant une réponse ADCC et nous démontrons que l'interaction entre Env et CD4 à la surface d'une même cellule infectée induit l'exposition de ces épitopes. Notamment, Env doit pouvoir transiter vers sa conformation liée à CD4 pour être reconnue par les anticorps induisant une réponse ADCC. De manière importante, le retrait de

CD4 de la surface des cellules infectées par les protéines accessoires virales Nef et Vpu en plus de l'antagonisme du facteur de restriction BST-2 par Vpu modulent l'exposition des épitopes d'Env et donc réduisent la susceptibilité des cellules infectées à la réponse ADCC. De plus, les changements conformationnels d'Env induits par CD4 à la surface des cellules infectés sont conservés chez les Env du VIH-1 et des lignées du VIH-2/VIS_{mac}. Prises ensemble, nos observations décrivent un mécanisme hautement conservé et requis pour l'exposition d'épitopes d'Env reconnus par des anticorps capables d'induire une réponse ADCC et ainsi pourraient expliquer l'avantage évolutif pour le VIH-1 de retirer CD4 de la surface cellulaire par ses protéines accessoires Nef et Vpu.

Abstract

Anti-HIV-1 envelope glycoprotein (Env) antibodies without broadly-neutralizing activity correlated with protection in the RV144 clinical trial, stimulating interest in other protective mechanisms involving antibodies, such as antibody-dependent cell-mediated cytotoxicity (ADCC). Env epitopes targeted by many antibodies effective at mediating ADCC are poorly exposed on the unliganded Env trimer. Here we investigate the mechanism of exposure of ADCC epitopes on Env, and show that binding of Env and CD4 within the same HIV-1-infected cell effectively exposes these epitopes. Env capacity to transit to the CD4-bound conformation is required for ADCC epitope exposure. Importantly, cell-surface CD4 down-regulation by Nef and Vpu accessory proteins and Vpu-mediated BST-2 antagonism modulate exposure of ADCC-mediating epitopes and reduce the susceptibility of infected cells to this effector function in vitro. Significantly, Env conformational changes induced by cell-surface CD4 are conserved among Env from HIV-1 and HIV-2/SIVmac lineages. Altogether, our observations describe a highly-conserved mechanism required to expose ADCC epitopes that might help explain the evolutionary advantage of downregulation of cell-surface CD4 by the HIV-1 Vpu and Nef proteins.

Importance

HIV-1 envelope epitopes targeted by many antibodies effective at mediating Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) are poorly exposed on the unliganded envelope trimer. Here we investigate the mechanism of exposure of these epitopes and found that envelope interaction with the HIV-1 CD4 receptor is required to expose some of these epitopes. Moreover, our results suggest that HIV-1 CD4 downregulation might help avoid the killing of HIV-1-infected cells by this immune mechanism.

Introduction

Human immunodeficiency virus (HIV-1) entry, mediated by the trimeric viral envelope glycoproteins (Env) complex, is a critical step of the viral infectious cycle. Env trimer is the only virus-specific antigen present at the surface of viral particles; as such, it is highly exposed and thus elicits neutralizing and non-neutralizing antibodies. The mature HIV-1 Env trimer is derived from proteolytic cleavage of a trimeric gp160 precursor (1, 2) and is composed of the exterior gp120 and transmembrane gp41 subunits. The gp120 is retained on the trimer via non-covalent interactions with the gp41 ectodomain (3-5). The gp120 glycoprotein is responsible for interactions with the initial receptor, CD4 (6, 7). CD4 binding triggers conformational changes in gp120 that promote its interaction with one of the chemokine receptors, CCR5 or CXCR4 (8-15). CD4 binding also induces conformational changes within the HIV-1 Env trimer that result in the exposure of a helical heptad repeat (HR1) segment of the gp41 ectodomain (16-19). Eventually, the conformational transition of the gp41 ectodomain into a six-helix bundle composed of the HR1 and HR2 heptad repeat regions results in the fusion of the viral and target cell membranes (20-22). As the major viral determinant recognized by anti-HIV-1 antibodies, the HIV-1 Env trimer represents a likely candidate for a vaccine immunogen. Interestingly, there is increasing evidence suggesting a role of Fc-mediated effector function in controlling or preventing HIV-1 transmission. Studies in macaques infected with simian immunodeficiency virus (SIV) show an inverse correlation between Fc-mediated effector functions and viral loads or rate of disease progression (23-25). Of note, this inverse correlation was also observed in several studies on HIV-1-infected individuals (26-30).

It has been recently suggested that antibody-dependent cellular cytotoxicity (ADCC) responses apply significant immune pressure on HIV-1 (31), highlighting the potential impact of ADCC on viral progression. Recently, a potential correlation between high levels of ADCC-mediating antibodies and HIV-1 acquisition in the RV144 trial was suggested for a subset of individuals with low plasma IgA anti-Env antibody levels (32, 33). Indeed, Env-specific plasma IgA/IgG ratios were shown to be higher in infected than in uninfected RV144 vaccine recipients (34). Accordingly, efficient ADCC-inducing mAbs were isolated from a subset of RV144 subjects (33). Therefore, ADCC-mediating antibodies may contribute to the partial protection observed in the RV144 trial, stimulating renewed interest in the mechanism of recognition of these antibodies (33, 35). Here we investigate whether interaction of CD4 and Env modulates some of the ADCC-mediating antibody recognition.

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 5% fetal bovine serum (Sigma) and 100 µg/ml of penicillin-streptomycin (Wysent). CEM.NKR cells (obtained from Dr David Evans) (25) were grown at 37°C and 5% CO_2 in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (R10).

Ficoll density gradient-isolated and cryopreserved human PBMCs from healthy donors (HIV and HCV seronegative), who gave written informed consent under research protocols approved by the CRCHUM, 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 16h before subsequent experiments. CD4+ T cells were isolated using a negative isolation kit (EasySep™ Human CD4+ T Cell Enrichment Kit, Stemcell) according to manufacturer's specifications and were then activated for 48h with 5 µg/ml PHA (Sigma) and 100 U/ml IL-2 (NIH AIDS Reagent).

Activated CD4+ T cells were maintained in culture in presence of 100 U/ml IL-2 for another 48h before infection.

Plasmids and Site-directed mutagenesis

Mutations were introduced individually into the previously described pSVIIIenv vector expressing the HIV-1_{YU2} or pcDNA3.1 expressing the codon-optimized HIV-1_{JRFL} envelope glycoproteins (5, 36) or into a previously reported pcDNA3.1 human CD4 expressor (37). Site-directed mutagenesis was performed using the QuikChange II XL site-directed mutagenesis protocol (Stratagene). When indicated, a stop codon was introduced to replace the codon for Gly 711, truncating the cytoplasmic tail (ΔCT) and enhancing cell-surface expression of selected HIV-1_{YU2} and HIV-1_{JRFL} envelope glycoproteins. The HIV-1_{JRFL} Env-eYFP expressor was generated by inserting the codon-optimized sequence of HIV-1_{JRFL} Env (residues 1-711) at the N-terminus of eYFP (from BD Clontech). The CD4-eCFP fusion protein was previously described (38). Transmitted/Founder envelope expressors from clades C (C1086) (39-41) and D (190049) were previously described (42), HIV-2 7312A (43, 44) and SIV (45) envelope expressors were previously described. The ADA Env was introduced into the pNL4.3 infectious molecular clone (46) through insertion of the SalI-BamHI fragment from NLHXADA (47). Subsequently, the BamHI-GFP-IRES.Nef-XhoI portion of the HxBru.ADA.GFP.IRES.Nef- construct (48) was transferred to the NL4.3 ADA intermediate to generate NL4.3.ADA.GFP.IRES.Nef. The isogenic NL4.3.ADA.GFP.IRES.Nef- Vpu- variant was generated by overlapping PCR using BamHI and SalI as cloning sites and the NL4.3.ADA.GFP.IRES.Nef and NL4.3Udel (49) as templates, respectively. An XhoI frameshift was made to the Nef sequence of NL4.3.ADA.GFP.IRES.Nef to generate NL4.3.ADA.GFP.IRES.Nef- and of NL4.3.ADA.GFP.IRES.Nef Vpu- to make NL4.3.ADA.GFP.IRES Nef- Vpu-.

Antibodies and ligands

Anti-HIV-1 gp120 monoclonal antibodies directed against the inner domain (A32, C11) were previously described (50, 51). Antibodies directed against the outer-domain (2G12,

PGT121), CD4-induced epitopes (17b, 412d, 48d), the CD4-binding site (VRC01, VRC03, b12) and quaternary-dependent epitopes (PG9, PG16) were obtained from Dr. Peter Kwong (VRC, NIAID), Dr. Dennis Burton (Scripps) and IAVI. The monoclonal antibody against a CD4-induced epitope (1.4H) was previously reported (52). The anti-cluster A Abs (L9-i1, L9-i2, N5-i5, N12-i3, N26-i1) were previously reported (35). The A32 Fab and the A32-blockable antibodies isolated from RV144 vaccinees (CH29, CH38, CH40, CH51, CH52, CH54, CH55, CH57, CH77, CH80, CH81, CH89, CH91, CH92, CH94) were recently described (33). CD4-Ig is a fusion protein in which the N-terminal two domains of CD4 are linked to the Fc component of immunoglobulin G (53). The monoclonal antibody anti-CD4 OKT4 (BioLegend) binds to the D3 domain of CD4 and was used to measure cell surface levels of CD4. The secondary goat anti-mouse and anti-human antibodies coupled to Alexa Fluor 594 and 647 (Invitrogen), respectively, were used in flow cytometry experiments.

Viral production and infection of T cells

VSVG-pseudotyped NL4.3 GFP ADA-based viruses were produced by calcium phosphate transfection of 293T cells with our panel of pNL4.3-GFP-ADA-based HIV-1 proviral vectors and VSVG-encoding vector. Two days after transfection, cell supernatants were harvested and concentrated by ultra-centrifugation for 1 h at 29000 rpm on a 20% sucrose cushion. Pellets were harvested in fresh DMEM and aliquots were stored at −80°C until use. These viruses were then used to infect approximately 20% of CEM.NKR and primary CD4+ T cells by spin-infection at 300 x g for 1-2 h in 96-well plates.

Cell-surface staining and antibody-dependant cellular cytotoxicity measurement by flow cytometry

For cell-surface staining, CEM.NKR or primary CD4+ T cells were incubated for 30 min at room-temperature (RT) 48 h post-infection with 1 µg/ml OKT4, 2G12, A32 and CH54 Abs in PBS. Cells were then washed once with PBS and stained with 1 µg/ml goat anti-mouse (AF-594) and anti-human (AF-647) secondary antibodies for 20 min in PBS. After one more PBS washing, cells were fixed in a 2% PBS-formaldehyde solution. For evaluation of A32-mediated ADCC cytotoxicity, CEM.NKR infected cells were stained with viability

(AquaVivid, Invitrogen) and cellular (eFluor670, eBiosciences) markers for 20 min and then washed twice in R10 (Gibco). Target cells were then mixed with PBMC effectors cells at an effector (E) target (T) ratio of 10:1 in 96-wells V-bottom plates (Corning), preincubated for 5 min at RT before adding 5 µg/ml of the monoclonal A32 antibody. After 15 min incubation at RT, co-cultures were centrifuged for 1 min at 300 x g and incubated at 37°C for 6 h before being fixed in a 2% PBS-formaldehyde solution. Samples were analyzed on a LSRII cytometer (BD Biosciences) and data analysis was performed using FlowJo vX.0.6 (Tree Star). Percentage (%) of cytotoxicity was calculated with the following formula: (% of GFP+ cells in T + E) – (% of GFP+ cells in T + E + A32) divided by (% of GFP+ cells in T). Supplementary figure 3 shows the gating strategy and formula allowing the calculation of A32-mediated cytotoxicity.

Cell-based ELISA

Detection of trimeric Env on the surface of HOS cells was performed by cell-based ELISA, as described (54). Briefly, HOS cells were seeded in 96-well plates ($2x10^4$ cells per well) and transfected the next day with 150 ng of envelope expressors or proviruses together with 1.7, 3.5 or 7 ng per well of a pcDN3.1 vector expressing human wild-type or mutant CD4 molecules. When the pSVIII envelope expressor was used, it was cotransfected with 0.01 µg of a Tat-expressing plasmid per well, using the standard polyethylenimine (PEI, Polyscience Inc, PA, USA) transfection method. Two days later, cells were washed twice with blocking buffer (10 mg/ml non-fat dry milk, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris,pH 7.5 and 140 mM NaCl) and then incubated for 1 h at room temperature with 20 nM of CD4-Ig or anti-HIV-1, HIV-2/SIV Env monoclonal antibodies. All ligands were diluted in blocking buffer. A horseradish peroxidase-conjugated antibody specific for the Fc region of human IgG (Pierce) was then incubated with the samples for 45 minutes at RT. For all conditions, cells were washed 5 times with blocking buffer and 5 times with washing buffer. HRP enzyme activity was determined after the addition of 30 µl per well of a 1:1 mix of Western Lightning oxidizing and luminol reagents (Perkin Elmer Life Sciences). Light emission was measured with an LB 941 TriStar luminometer (Berthold Technologies).

Immunoprecipitation of envelope glycoproteins

For pulse-labeling experiments, $3X10^5$ 293T cells were transfected by the calcium phosphate method with codon-optimized vectors expressing the HIV-1_{YU2} envelope glycoproteins Layer 3 variants (5, 54). One day after transfection, cells were metabolically labeled for 16 h with 100 μCi/mL [^{35}S]methionine-cysteine ([^{35}S] Protein Labeling Mix; Perkin-Elmer) in Dulbecco's modified Eagle's medium lacking methionine and cysteine and supplemented with 5% dialyzed fetal bovine serum. Precipitation of radiolabeled HIV-1_{YU2} envelope glycoproteins from medium was performed with various amounts of A32 for 1 hour at 37°C in the presence of 50 μl of 10% Protein A-Sepharose (American BioSciences).

Statistical analyses.

Statistics were analyzed using GraphPad Prism version 6.01. Unpaired t-tests were used to test the significance of differences between means.

Results

Exposure of Env ADCC-mediating epitopes by co-expressed CD4

It was recently reported that recipients of the ALVAC-HIV / AIDSVAX B/E vaccine in the RV144 trial induced ADCC responses mediated by antibodies that were competed by the A32 Fab fragment (33); these results indicate that these ADCC-mediating antibodies may recognize an Env epitope similar to or overlapping the A32 epitope. The A32 antibody recognizes a discontinuous epitope on the surface of the inner domain of the gp120 exterior Env (33). Interestingly, we observed that the interaction of A32, C11 (an antibody that recognizes the gp120 β-sandwich and N- and C-termini) (50, 51), RV144 mAbs (33), and the recently-described anti-cluster-A class of Abs (35) with Env is greatly increased upon co-expression of the CD4 receptor in a dose-dependent manner (see Materials and Methods) (Figure 1 A-D). We interpreted this observation as an indication that this increased antibody binding was dependent on the ability of Env and CD4 to interact, because changes in the HIV-

1 gp120 CD4-binding site (D368A) or in the gp120-binding site (F43H) of CD4 known to decrease gp120-CD4 interaction (55, 56) decreased the exposure of epitopes recognized by those antibodies (Figure 1 A-D). For some RV144 antibodies (CH29, CH38) expression of the CD4 F43H variant did not decrease Env recognition as much as for A32. This could be due to the fact that this CD4 variant, albeit it decreases Env interaction, it does not completely abrogate CD4/Env interaction (55) and therefore, a suboptimal Env-CD4 interaction might be sufficient to expose epitopes recognized by these antibodies. Expression levels of Env at the cell surface were not affected by the co-expression of the wild-type or mutated (F43H) CD4 receptor (Supplementary Figure 1) indicating that the observed differences in binding were not a result of variable levels of surface Env expression.

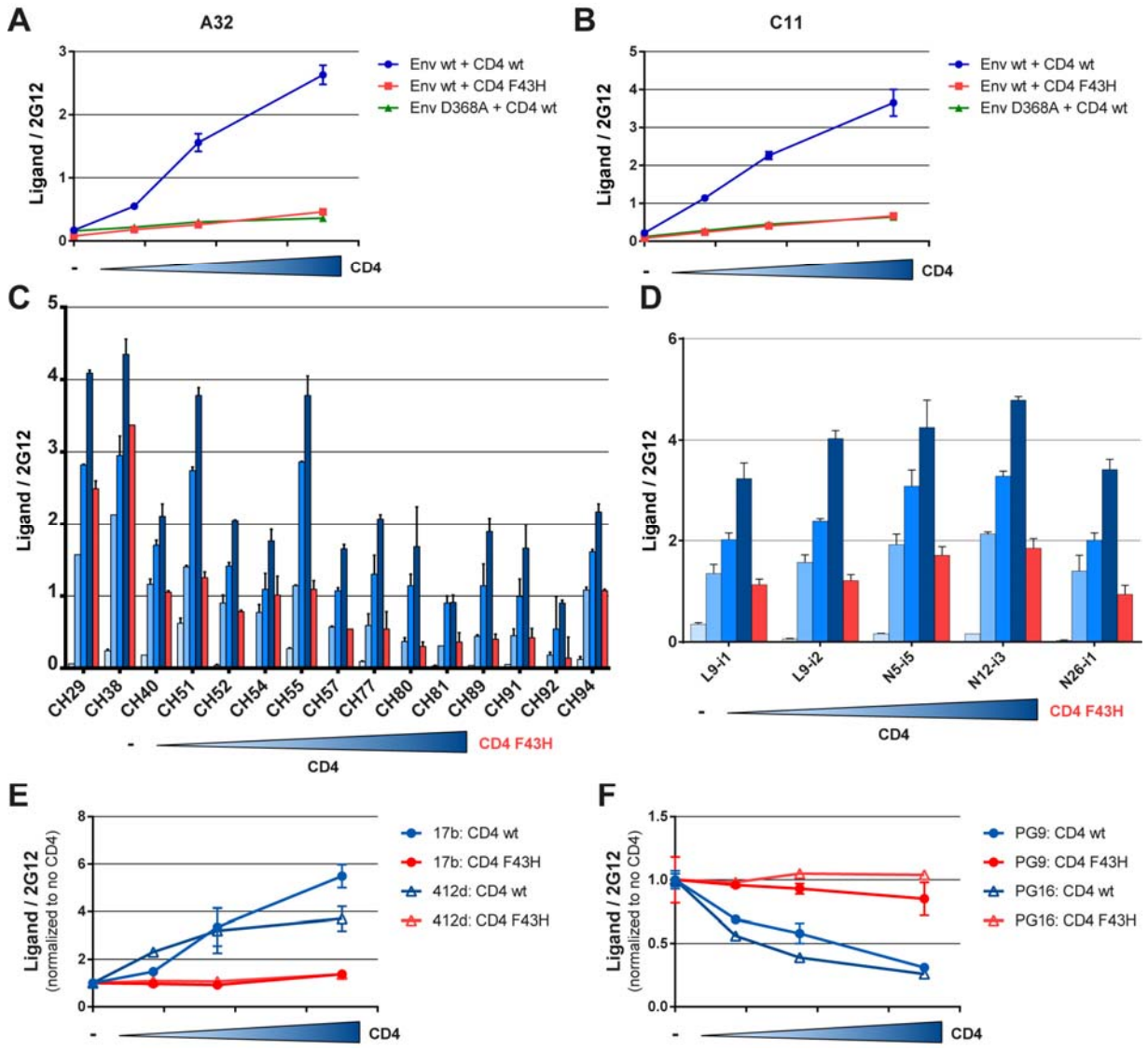

Figure 3.1 **(Article 4 Figure 1) Interaction of HIV-1 Env with co-expressed CD4 induces conformational changes that expose inner domain as well as complex CD4-induced epitopes.**

Interaction of co-expressed CD4 with HIV-1$_{YU2\Delta CT}$ Env enhances recognition by (**A**) A32, (**B**) C11, (**C**) ADCC-mediating antibodies isolated from RV144 trial recipients (33) and (**D**) cluster A-recognizing antibodies (35). Epitope exposure requires CD4-gp120 interaction as shown by decreased recognition of these epitopes with either a mutant of CD4 (F43H) with decreased capacities to interact with gp120 or a CD4-binding site Env variant (D368A). (**E**) Coexpression of Env and CD4 also induces conformational changes in Env that enhance CD4i epitopes (17b, 412D) and decrease recognition by quaternary-dependent Abs such as PG9 and PG16 (**F**). Data shown are representative of those obtained in at least 3 independent experiments performed in triplicate +/- SD. Env signals were normalized to that obtained with the gp120 outer domain-

recognizing antibody 2G12 (A,B,C,D) and this ratio was normalized to the absence of co-expressed CD4 for panels E and F. "-" indicates in absence of CD4. The increasing blue bar indicates a step-wise increase in the amount of CD4 expressor being transfected.

These conformational changes were not limited to the exposure of inner-domain epitopes, but also resulted in the exposure of complex CD4i (17b, 412d) epitopes comprising elements of the outer domain and bridging sheet of gp120 Env (Figure 1E). The coexpression of CD4 apparently promoted the opening of the Env trimer, as indicated by decreased recognition by quaternary-dependent Abs such as PG9 and PG16 (57) (Figure 1F). These conformational changes were dramatically decreased by introducing the F43H binding site mutation in CD4, indicating that proper Env-CD4 interaction is required for these changes to occur. Meaningfully, cell-surface CD4 competed for ligands that recognize the CD4-binding site such as CD4-Ig, b12, VRC03 and VRC01 (Figure 2), suggesting that CD4 recognizes its binding site in a manner similar to the recognition of Env in the context of viral particles.

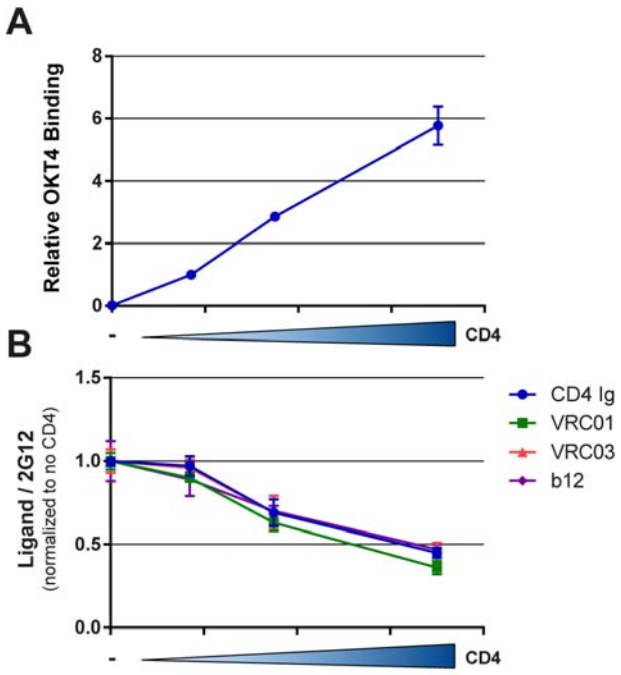

Figure 3.2 **(Article 4 Figure 2) Co-expressed CD4 competes for ligands that recognize the Env CD4-binding site.**
Cells expressing HIV-$1_{Yu2\Delta CT}$ Env together with increasing concentrations of human CD4 were stained by the anti-CD4 OKT4 antibody (**A**) or the CD4-binding site ligands CD4-Ig, b12, VRC03 and VRC01 (**B**), using the cell-based ELISA described in the materials and methods section. Data are representative

of those obtained in at least three independent experiments performed in triplicate +/-SD. Signals were normalized to that obtained with the gp120 outer domain-recognizing antibody 2G12 in absence of co-expressed CD4.

The transition of Env to the CD4-bound conformation is required for efficient interaction with ADCC-mediating antibodies

Consistent with the necessity that Env interacts with cell-surface CD4 to expose CD4i epitopes, these conformational changes required the ability of Env to reach the CD4-bound conformation. Indeed, a mutation (H66A) in Layer 1 of the gp120, known to impede the transition to the CD4-bound conformation (5, 54), decreased the CD4-induced exposure of A32 and C11 epitopes. Conversely, an S375W substitution, which fills the Phe43 cavity and predisposes to a CD4-bound conformation (58), enhanced exposure of these epitopes and was sufficient to revert the phenotype of the Layer 1 variant (Figure 3). Thus, the ability of Env to transition from the unbound to the CD4-bound conformation is a prerequisite to expose inner domain ADCC epitopes.

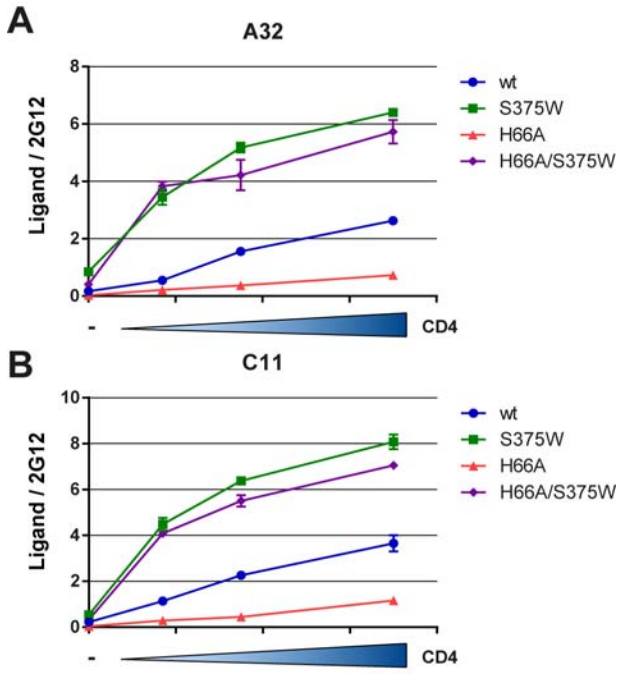

Figure 3.3 (Article 4 Figure 3) Conformational changes induced by co-expressed CD4 require Env to transit to the CD4-bound conformation.
HIV-$1_{YU2\Delta CT}$ Layer 1 (H66A) Env variant with a decreased propensity to sample the CD4-bound conformation (5, 54) exhibits decreased exposure of (**A**) A32 and (**B**) C11 epitopes upon co-expression of CD4. However, a gp120

change that fills the Phe43 cavity and favors a conformation closer to the CD4-bound conformation (58) enhances the CD4-induced exposure of these epitopes and is sufficient to revert the phenotype of the Layer 1 variant. Data shown are representative of those obtained in at least 3 independent experiments performed in triplicate +/-SD. Signals were normalized to that obtained with the gp120 outer domain-recognizing antibody 2G12.

Noteworthy, Env conformational changes induced by co-expressed CD4 were observed in Envs from laboratory-adapted, primary and transmitted/founder (T/F) HIV-1 isolates as well as Envs from the HIV-2/SIV lineage (Figure 4), indicating that this mechanism is highly-conserved among primate immunodeficiency virus Envs. In addition, and consistent with previous reports indicating that CD4 could interact with Env within the endoplasmic reticulum (ER) (59, 60), we observed that Env-CD4 interaction occurs within the same cell, as evaluated by FRET analysis (Supplementary Figure 2). Moreover, we obtained evidence suggesting that CD4 coexpression results in the formation of Env-CD4 complexes at the cell surface since these complexes are recognized by the anti-cluster A N26-i1 antibody (Figure 1 D), which selectively binds gp120-CD4 complexes (Supplementary Figure 4).

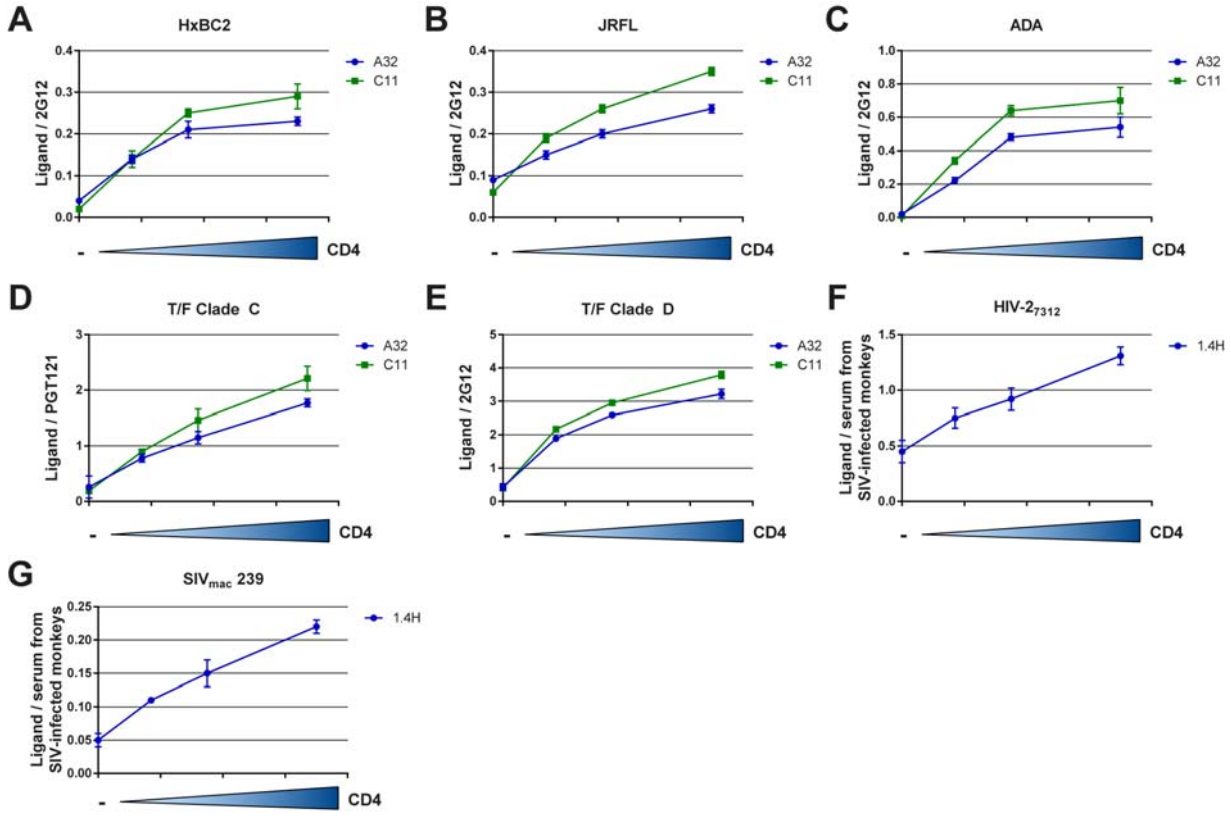

Figure 3.4 **(Article 4 Figure 4) Envelope conformational changes induced by co-expressed CD4 are conserved among HIV-1 and HIV-2/SIVmac Env.** Laboratory-adapted HIV-1$_{HxBc2}$ (**A**), primary HIV-1$_{JRFL}$ (**B**), HIV-1$_{ADA}$ (**C**), transmitted/founder HIV-1 clades C (C1086) (**D**), D (190049) (**E**) and HIV-2$_{7312}$ (**F**) and SIVmac239 (**G**) envelope glycoprotein expressors were transfected into HOS cells together with increasing concentrations of a human CD4 expressor. Forty-eight hours post-transfection, Env conformation was assessed by cell-based ELISA with antibodies A32 and C11 recognizing the HIV-1 gp120 inner domain, as described in the materials and methods section. For HIV-2 and SIVmac239 envelopes, the recently described CD4i (1.4H) antibody was used (52). Data are representative of those obtained in at least three independent experiments performed in triplicate +/-SD. Signals were normalized to that obtained with the gp120 outer domain-recognizing antibody 2G12 or PGT-121 (for C1086) for HIV-1 Env. For HIV-2 and SIVmac239 Envs, signals were normalized to that obtained with serum from SIV-infected macaques.

HIV-1 accessory proteins Nef and Vpu prevent the exposure of epitopes recognized by anti-Env ADCC-mediating antibodies at the surface of infected cells.

HIV-1 accessory proteins Nef and Vpu are known to decrease cell-surface levels of CD4 (2,3). We therefore asked whether these proteins could indirectly affect Env conformation by modulating CD4 levels at the cell surface. We first evaluated whether Env conformational changes induced by CD4 were observed when the envelope glycoproteins were expressed by replication-competent proviruses in our cell-based ELISA assay. As expected, Env conformational changes depended on proper Env-CD4 interactions since the D368A Env mutant expressed in the proviral context was unable to be recognized by A32 or C11 despite high-levels of cell-surface CD4 (Figure 5). Notably, proviruses lacking the ability to downregulate CD4, due to deletions of their viral accessory proteins Nef and Vpu, presented Envs with higher exposure of the A32 and C11 epitopes (Figure 5). We then asked whether Env conformational changes at the surface of T cells could be modulated by cell-surface levels of CD4. We therefore infected a T cell line (CEM-NKR)(25) with viruses lacking *nef*, *vpu* or *nef* and *vpu*. While deletion of *vpu* alone modestly affected cell-surface CD4 levels, *nef* deletion had a more pronounced effect (Figure 6A). Nef and Vpu are known to act together, through different cellular mechanisms, to decrease cell-surface levels of CD4 (2,3). Accordingly, deletion of both genes impaired the ability of HIV-1 to downregulate CD4 to extents that were not achieved by deleting *nef* or *vpu* alone. HIV-1-mediated CD4 downregulation was completely abrogated only when, in addition to deleting *nef* and *vpu*, the ability of Env to interact with CD4 was decreased by the D368A mutation. This is in agreement with the notion that Env-CD4 interaction plays a role in CD4 downregulation (22). Deletion of the *vpu* gene also resulted in enhanced levels of Env at the cell surface as measured by the outer-domain-specific 2G12 antibody (Figure 6B). In addition to its role in CD4 degradation, Vpu also antagonizes a restriction factor, Tetherin/BST-2, which normally inhibits retroviral release (29,30). Therefore, viruses produced in the absence of Vpu remain trapped at the cell surface by Tetherin/BST-2 and likely account for the observed enhancement of 2G12 staining.

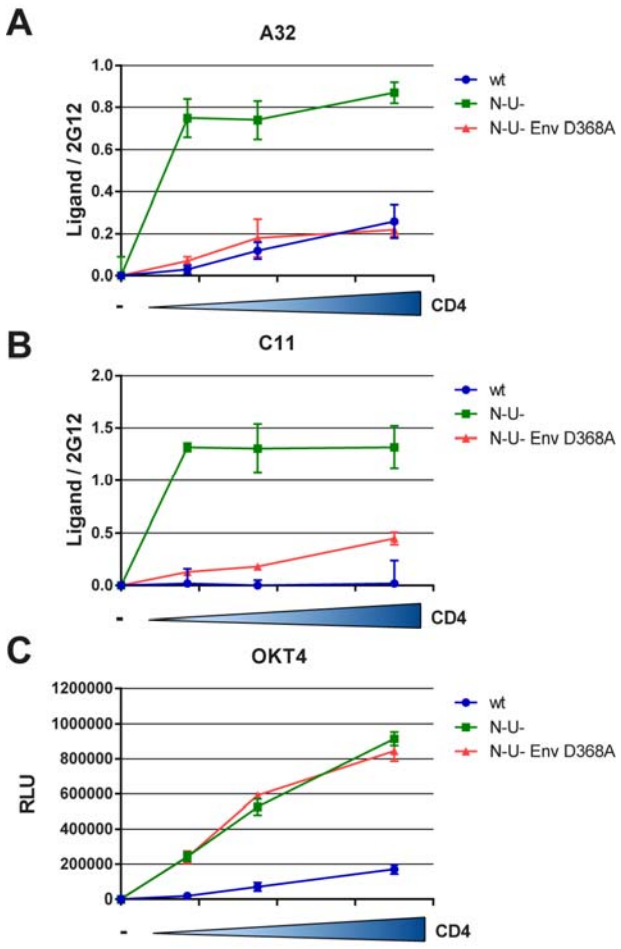

Figure 3.5 **(Article 4 Figure 5) Env conformational changes induced by co-expressed CD4 are conserved when Env is expressed within replication-competent proviruses and are indirectly modulated by Nef and Vpu proteins.**
pNL4.3 GFP ADA and pNL4.3 GFP ADA Vpu- Nef- were transfected into permissive, BST2-free, HOS cells (66) together with increasing concentrations of a human CD4 expressor. Forty-eight hours post-transfection, Env conformation was assessed by cell-based ELISA with (**A**) A32 and (**B**) C11 Abs, as described in the methods section. Importantly, Env conformational changes require CD4-gp120 interaction, as shown by decreased recognition by these Abs of an Env variant with a change (D368A) in the CD4-binding site. (**C**) Anti-CD4 OKT4 antibody was used to monitor levels of CD4. Data are representative of those obtained in at least three independent experiments performed in triplicate +/-SD. Signals were normalized to that obtained with the gp120 outer domain-recognizing antibody 2G12 (A,B).

We hypothesized that an adequate level of CD4 and Env at the cell surface must be achieved and, in addition, Env must be able to engage CD4 efficiently in order to detect Env conformational changes with inner domain-recognizing Abs such as CH54 and A32. Indeed,

deletion of *nef* and *vpu* alone resulted in a significant but only modest increase in CH54 and A32 staining (Figure 6C and D). However, when both genes were deleted in combination, both CH54 and A32 staining were significantly enhanced. Moreover, this enhancement depended on the ability of Env to engage CD4 because CH54 or A32 staining of cells infected with a *nef- vpu-* virus encoding the D368A Env variant was decreased (Figure 6D) despite having similar levels of Env at the cell surface as measured by 2G12 (Figure 6B). Importantly, infection of activated primary CD4+ T cells from three different healthy donors with our panel of viruses resulted in a similar pattern of A32, CH54, and anti-cluster A antibodies cell-surface staining (Figure 7) supporting the hypothesis that efficient interaction of Env ADCC-mediating Abs requires a threshold of CD4 and Env at the surface of infected cells as well as their ability to interact together.

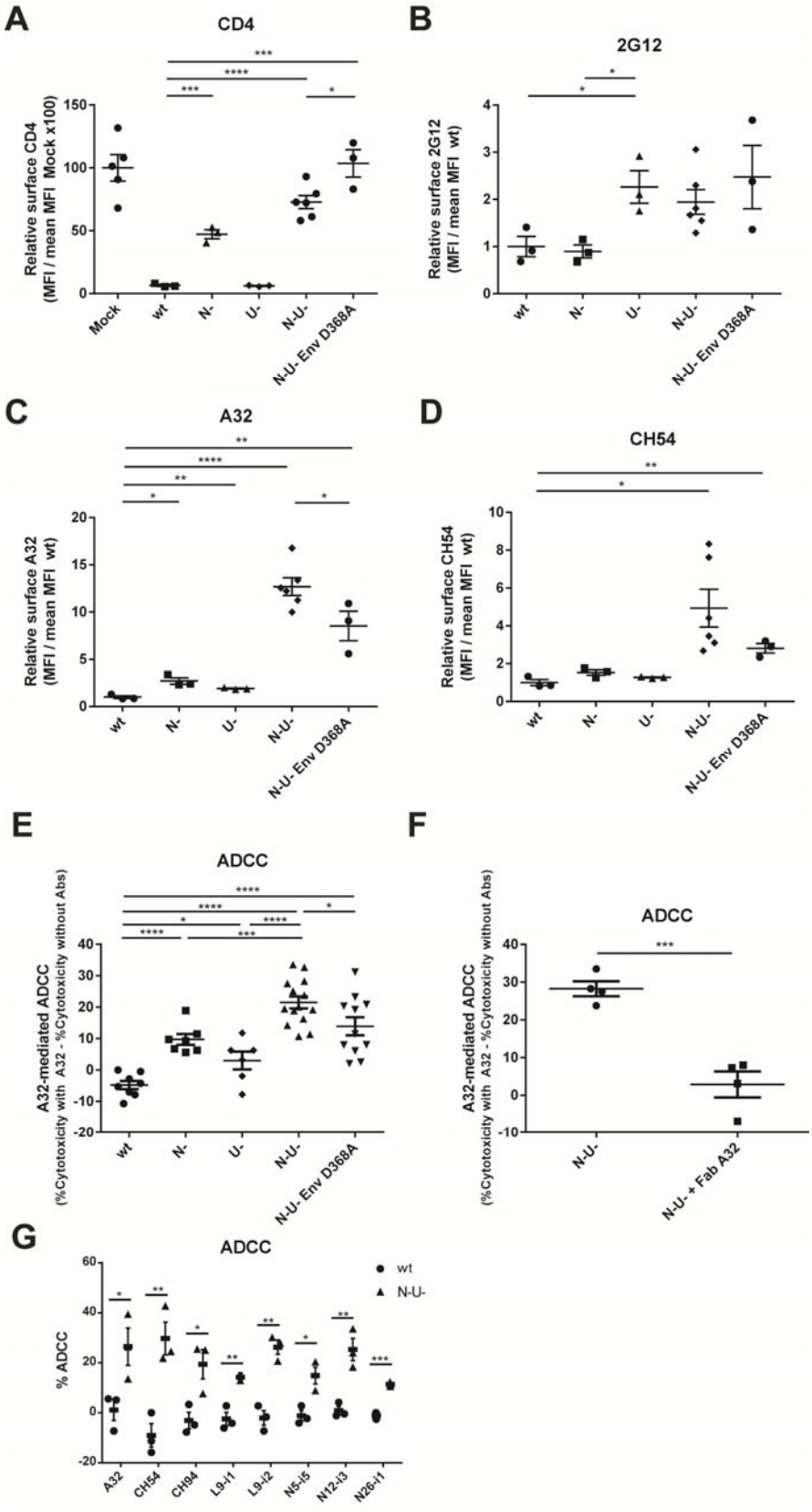
A
CD4
Relative surface CD4 (MFI / mean MFI Mock x100)
Mock
wt
N-
U-
N-U-
N-U- Env D368A
B
2G12
Relative surface 2G12 (MFI / mean MFI wt)
C
A32
Relative surface A32 (MFI / mean MFI wt)
D
CH54
Relative surface CH54 (MFI / mean MFI wt)
E
ADCC
A32-mediated ADCC (%Cytotoxicity with A32 - %Cytotoxicity without Abs)
F
ADCC
N-U- + Fab A32
G
ADCC
% ADCC
wt
N-U-
A32
CH54
CH94
L9-i1
L9-i2
N5-i5
N12-i3
N26-i1

Figure 3.6 (Article 4 Figure 6) Env conformational changes induced by surface CD4 increase susceptibility of HIV-infected cells to antibody dependent cellular cytotoxicity.

CEM.NKR cells infected with VSV-G pseudotyped NL4.3 GFP ADA either wildtype (wt), Nef- (N-), Vpu- (U-) or Nef-Vpu- (N-U-) encoding wt or D368A Env were stained against (**A**) surface CD4 (OKT4 Ab) or Env: (**B**) 2G12, (**C**) CH54 and (**D**) A32 at 48h post-infection and analyzed by flow cytometry as described in the materials and methods section. Signals were normalized to the mean signal of the wt virus (fold-change) for Env epitopes and to the mock control for surface CD4. Alternatively, the susceptibility to A32-mediated lysis by PBMC effector cells of those cells was analyzed by flow cytometry (**E**) as described in the materials and methods section. (**F**) Pre-incubating with the Fab fragment of A32 prevented A32-mediated lysis. (**G**) Susceptibility to A32, CH54, CH94, L9-i1, L9-i2, N5-i5, N12-i3 and N26-i1-mediated lysis by PBMC effector cells of wt vs N-U-infected CEM.NKR cell was analyzed as described in panel (E). Data shown are the results of at least three independent infections +/- SEM. ADCC was measured using PBMCs from three different healthy donors using the gating and formula presented in Supplementary Figure 3. Statistical significance was tested using an unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

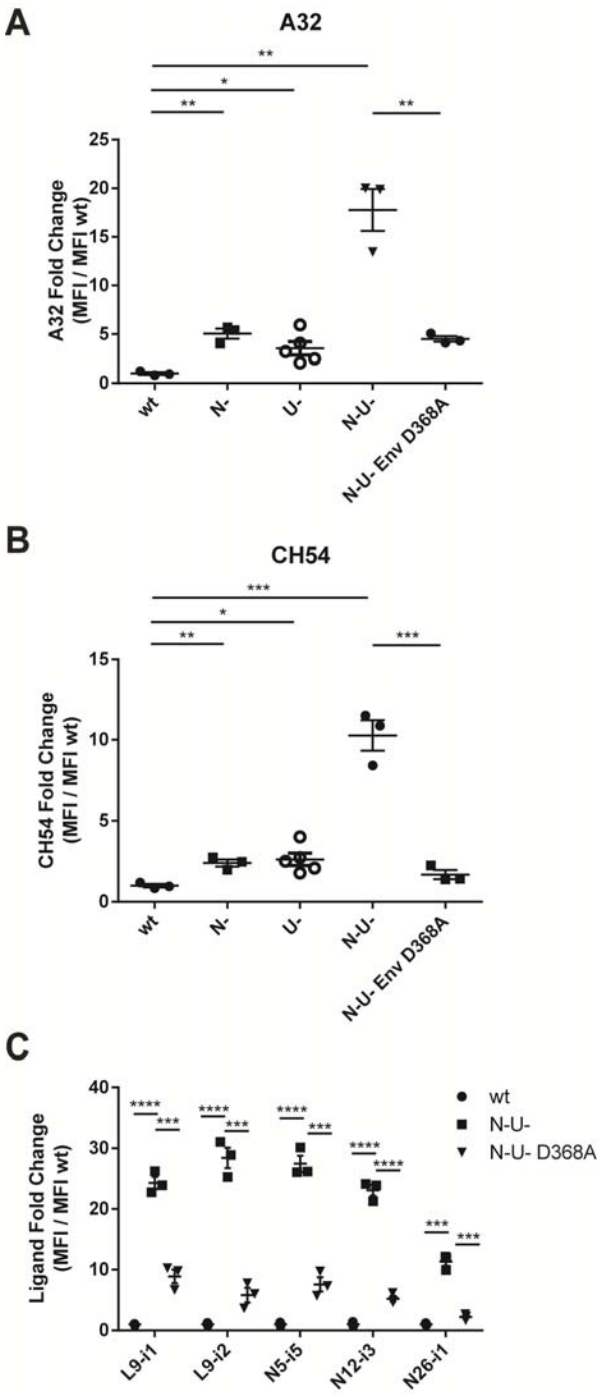

Figure 3.7 (Article 4 Figure 7) Nef and Vpu prevent the exposure of A32-like epitopes at the surface of infected primary CD4+ T cells.

PHA activated CD4+ T cells isolated from PBMCS of three healthy donors were infected with VSV-G pseudotyped NL4.3 GFP ADA either wildtype (wt), Nef- (N-), Vpu- (U-) or Nef-Vpu- (N-U-) encoding wt or D368A Env and were stained with (**A**) A32, (**B**) CH54 and (**C**) anti-cluster A Abs at 48h post-infection and analyzed by flow cytometry as described in the materials and

methods section. Signals were normalized to the mean signal of the wt virus (fold-change). Data shown are the results of at least three independent infections +/- SEM. Statistical significance was tested using an unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

We then asked whether the observed enhancement of A32 staining at the surface of HIV-1-infected cells correlated with enhanced susceptibility to ADCC mediated by PBMCs from healthy individuals. While deletion of *nef* or *vpu* only modestly enhanced A32 recognition at the surface of HIV-1-infected cells (Figure 6D), it resulted in a significant increase in ADCC (Figure 6E) suggesting that a low threshold of ADCC-mediating Abs recognition at the surface of HIV-1-infected cells is sufficient to sensitize cells to ADCC. Interestingly, the ability of PBMCs to mediate ADCC was at its maximum only when they were in contact with cells infected with the *nef-vpu-* virus (Figure 6E). This was also observed for RV144 CH54, CH94 as well as anti-cluster A class of antibodies, indicating that this result is not limited to A32 (Figure 6G). Remarkably, the interaction between Env and CD4 was critical for A32-mediated ADCC because cells infected with the *nef-vpu-* but coding for Env D368A were significantly less susceptible to be killed by the same PBMCs. Of note, the observed A32-mediating killing was specific to A32 since it was completely blocked by pre-incubation of the target cells with an A32 Fab fragment (Figure 6F).

The A32 epitope is potentially accessible in the CD4-bound Env trimer

Finally, we asked how A32 could recognize Env at the surface of infected cells. A recent report (35) supported our previous observations indicating that A32 interaction with HIV-1 gp120 was modulated by the inner domain Layers 1 and 2 (5). Here we expanded this analysis by analyzing the contribution of the recently-described Layer 3 (5, 54, 61). Interestingly, two highly-conserved residues located at the interface between Layers 2 and 3 (T248, W479) also modulated A32 interaction with gp120. Notably, these residues were shown to be critical for the transition of Env to the CD4-bound conformation (54). This is in agreement with the notion that the epitope recognized by A32, and perhaps by other inner-domain-directed Abs such as RV144 Abs, are occluded in the unliganded trimer and must be exposed by cell-surface CD4 in order to be recognized efficiently (Figure 8).

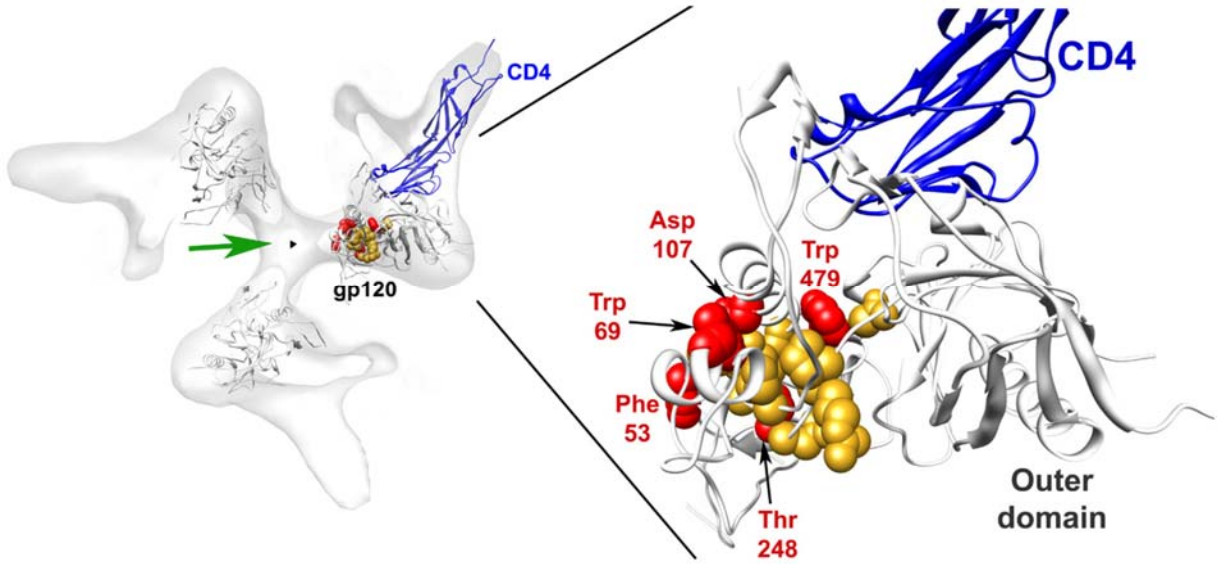

Figure 3.8 **(Article 4 Figure 8) The A32 epitope is potentially accessible in the CD4-bound Env trimer.**
The cryoelectron tomographic map of the CD4-bound and 17b Fab-bound HIV-1 Env trimer (EMDB 5020) (67) is viewed from the perspective of the target cell. The density associated with the 17b antibody Fab has been removed for clarity. The Env trimer axis is designated with a black triangle. Three CD4-bound gp120 cores (PDB 3DNO) were fit to the density map, and the CD4-bound gp120 with complete N- and C-termini (PDB 3JWO) (61) was aligned with one subunit. On this subunit, CD4 domains 1 and 2 are shown (blue ribbon). The gp120 residues implicated by mutagenesis in binding the A32 antibody in this and a previous study (5) are depicted in CPK mode (red = significant effects on A32 binding, orange = moderate effects on binding). The hypothesized angle-of-approach of A32-like antibodies that mediate ADCC is represented by the green arrow.

Discussion

ADCC utilizes the IgG Fc-gamma receptor (FcγR, FCγRIIIa or CD16a) expressed on natural killer (NK) but also in other immune cell types (eg., macrophages, neutrophils) as a means of bringing these cells into contact with antibody-coated cells (62). The Fab portion of the antibody binds the antigen at the surface of the target cell and the Fc portion binds FcγR on the effector cells. Because FcγR is able to form a complex with the ITAM-containing CD3ξ and/or FCεRI, the interaction of FcγR with cell-bound IgG Abs can result in the activation of these cells to mediate cytolysis by releasing perforin and granzymes (63). Interestingly, there

is increasing evidence supporting a role of Fc-mediated effector function in controlling or preventing HIV-1 transmission. Studies in macaques infected with simian immunodeficiency virus (SIV) show an inverse correlation between Fc-mediated effector functions and viral loads or decreased disease progression (23-25). Noteworthy, this inverse correlation was also observed in several studies with HIV-1-infected individuals (26-30, 64). Importantly, it has been recently suggested that antibody-dependent cellular cytotoxicity (ADCC) responses apply significant immune pressure on HIV-1 (31), highlighting the potential impact of ADCC on viral progression. Recently, a potential correlation between high levels of ADCC-mediating Abs and HIV-1 acquisition in the RV144 trial was identified for a subset of individuals presenting low plasma IgA anti-Env antibody levels (32, 33). Accordingly, high plasma levels of IgA Abs specific for the C1 region of Env were shown to block binding and effector function of known ADCC-mediating Abs (34). Moreover, efficient ADCC-inducing mAbs were isolated from a subset of RV144 subjects, suggesting that at least partial protection in the RV144 trial might be due to ADCC-mediating Abs (33), thus driving renewed interest in the mechanism of recognition of these Abs (33, 35). However, additional work is needed to understand how these ADCC-mediating antibodies work. RV144 ADCC-mediating monoclonal Abs have been described to preferentially use the VH1 gene family and their effector function is largely blocked by competition with the A32 Fab fragment (33), which recognizes a discontinuous epitope on the surface of the inner domain of gp120 (65), thus suggesting that they all recognize a common/overlapping epitope. Accordingly, a recently reported group of mAbs targeting Env epitopes exposed by CD4 binding (anti-Cluster A Abs) and able to mediate ADCC were shown to recognize an epitope blocked by A32 Fab (35) and by the specific gp120 inner domain C11 mAb (50, 51). Detailed structural information on the epitope recognized by A32 is still missing; however, we recently mapped the A32 epitope to the inner domain Layers 1 and 2 and identified residues W69 and D107 as key players in this interaction (5). We have now extended these results to some Layer 3 residues (T248, W479) involved in the transition to the CD4-bound conformation (54), which is consistent with our findings that the epitope recognized by this antibody is not well exposed in the unbound trimeric Env since its interaction depends on the exposure of the inner domain layers, which are only exposed upon CD4 interaction (Figure 8).

We observed that interaction of A32, C11 and RV144 mAbs with Env was greatly increased upon co-expression of the CD4 receptor in a dose-dependent manner. Importantly, this was dependent on the ability of Env and CD4 to engage efficiently since changes in CD4 or Env that altered this interaction decreased exposure of these epitopes (Figure 1). Of note, cell-surface CD4 competed for ligands that recognize the CD4-binding site, suggesting that cell-surface CD4 recognizes its binding site in a manner similar to the recognition of Env in the context of viral particles (Figure 2). Strikingly, Env conformational changes induced by cell-surface CD4 were observed among several primate immunodeficiency virus Envs, suggesting that this mechanism is highly conserved (Figure 4).

Viruses lacking the ability to downregulate CD4 or that presented high levels of Env at the surface of infected cells, due to deletions of their viral accessory proteins Nef and Vpu, presented Envs with higher exposure of Env ADCC-mediating epitopes (Figure 5, 6 and 7) and resulted in a significant increase of elimination of these cells by ADCC (Figure 6). Maximal sensitization of HIV-1-infected cells to elimination by ADCC required both: high levels of cell-surface CD4 as well as enhanced expression of Env at the surface of infected cells. Finally, the ability of Env and CD4 to efficiently engage was also required for efficient elimination of HIV-1-infected cells by ADCC.

Altogether, these observations support the notion that efficient viral release and cell-surface CD4 downregulation result in the removal of Env-CD4 complexes targeted by ADCC-mediating antibodies. Thus, interaction of Env and CD4 is important for exposure of ADCC-mediating epitopes, suggesting that one consequence of Vpu and Nef activity might be downregulation of potential ADCC epitopes from the surface of HIV-1-infected cells. Therefore, enhancing Env levels at the surface of infected cells and targeting Vpu and Nef ability to downregulate CD4 could potentially render HIV-1-infected cells susceptible to ADCC and thus have therapeutic utility. Additional efforts are required to address the role that ADCC plays in decreasing viral progression.

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Supplemental Materials and Methods

FRET analysis by flow cytometry.

HEK 293T cells were transfected with plasmids expressing CD4-eCFP and HIV-1_{JRFL}-eYFP. eCFP and eYFP were used as controls. All transfected cells were fixed with 2% PFA and analyzed by FRET as described (1) in a LSRII flow cytometer (BD Biosciences) with 405, 488, 561 and 633 lasers. Double-positive cells were gated and false positive FRET signals resulting from eYFP excitation by the 405 nm laser were excluded. Remaining cells were evaluated for FRET by adjusting a gate on cells that were cotransfected with eCFP and eYFP only and should therefore be FRET-negative (1).

ELISA

ELISA was carried out as described in (3, 4, 6). Briefly, recombinant proteins were captured onto ELISA trays using an affinity purified goat antibody, D7324 (Aalto Bio Reagents, Dublin, Ireland), specific for the C-terminus of gp120 followed by washing and blocking prior to incubation with the stated concentrations of N26-i1 or N5-i5. After incubation, the plates were washed and binding was detected using polyclonal goat-anti-human IgG-alkaline phosphatase (Southern Biotechnology, Birmingham, AL) and the Blue Phos Microwell Phosphatase Substrate System (KPL, Gaithersburg, MD). Recombinant proteins were produced and purified as described previously (2). The YU2-Core and YU2-Core-V3 proteins were produced as described (7).

Supplemental Figures

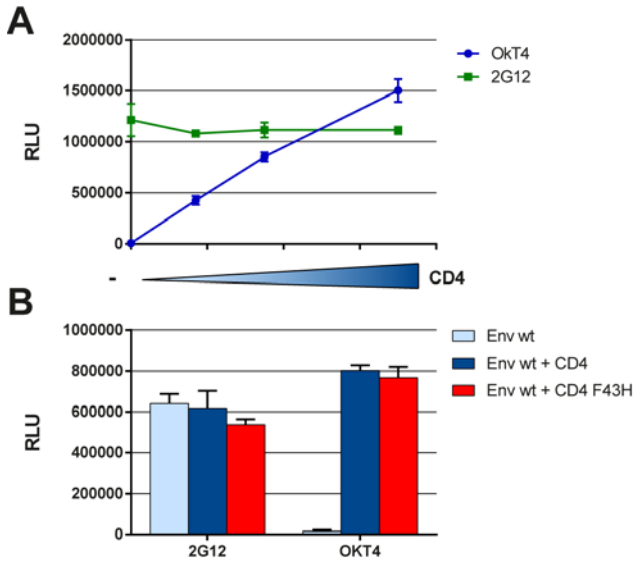

Figure 3.9S (Article 4 Figure S1) Coexpression of CD4 does not affect Env surface levels.
HOS cells co-transfected with HIV-1$_{YU2\Delta CT}$ Env and increasing concentrations of CD4 shows similar levels of surface Env as measured by cell-based ELISA using 2G12 Ab (**A**). The CD4 F43H mutation does not affect CD4 or Env expression (**B**). Data shown are representative of those obtained in at least 3 independent experiments performed in triplicate +/- SD.

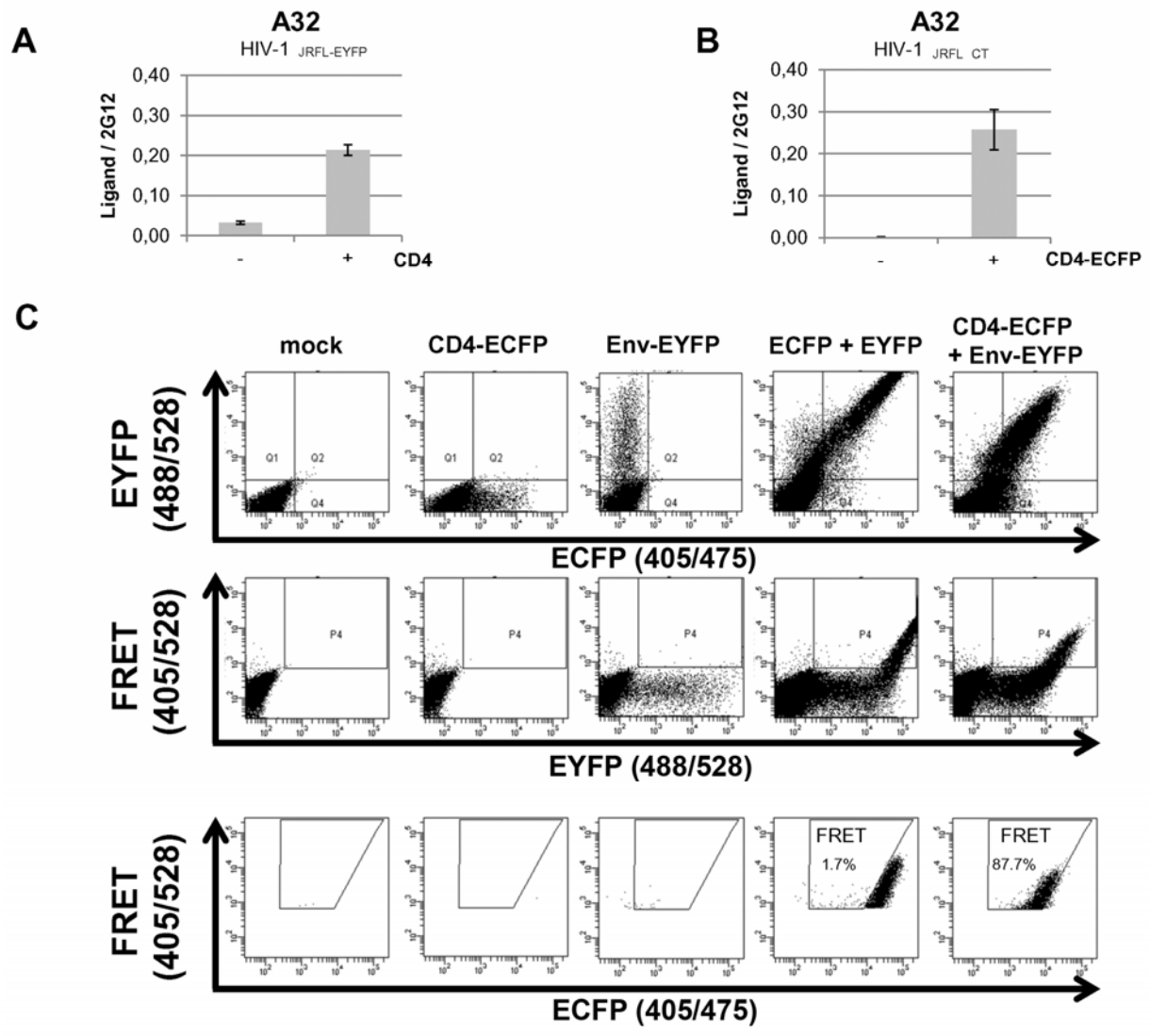

Figure 3.10S (Article 4 Figure S2) CD4 and HIV-1 Env interact within the same cell. (**A**) Fusion of eYFP (from BD Clontech) at position 711 of HIV-1$_{JRFL}$ Env does not affect the conformational changes induced by co-expressed CD4. Briefly, HOS cells were transfected with HIV-1$_{JRFL}$-eYFP with or without a human CD4 expressor. Env conformation was measured by cell-based ELISA with A32 as described in the methods section. (**B**) Fusion of eCFP (from BD Clontech) to the C-termini of CD4 does not affect its ability to induce HIV-1 Env$_{JRFL}$ conformational changes. HOS cells were transfected with HIV-1$_{JRFL}$ with and without a previously described CD4-eCFP fusion protein (5); conformational changes were measured as described in (**A**); data are representative of those obtained in at least three independent experiments performed in triplicate and were normalized to signals obtained with 2G12. (**C**) Setup of FRET measurements by flow cytometry. HEK 293T cells were transfected with plasmids expressing CD4-eCFP and HIV-1$_{JRFL}$-eYFP. eCFP and eYFP were used as controls. All transfected cells were fixed with 2% PFA

and analyzed by FRET as described (1) in a LSRII flow cytometer (BD Biosciences) with 405, 488, 561 and 633 lasers. Double-positive cells were gated (panel 1) and false positive FRET signals resulting from eYFP excitation by the 405 nm laser were excluded (panel 2). The remaining cells were evaluated for FRET by adjusting a gate on cells that were cotransfected with eCFP and eYFP only and should therefore be FRET-negative (panel 3) (1). Data are representative of those obtained in at least three independent experiments.

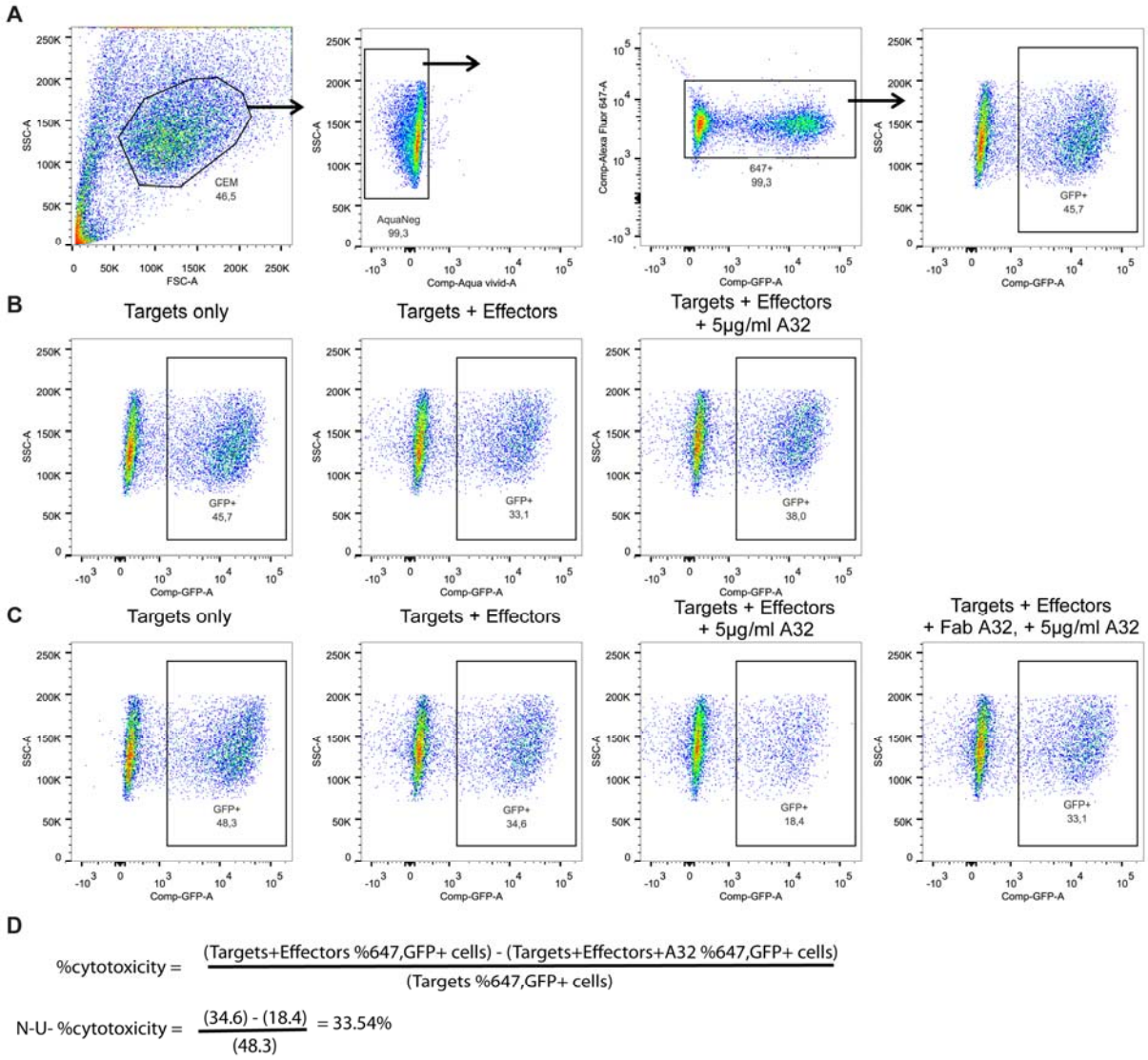

Figure 3.11S (Article 4 Figure S3) Determination of A32-mediated cell lysis by flow cytometry: gating strategy and calculations.

CEM.NKR cells infected with VSVg-pseudotyped wt or N-U- virus (encoding a GFP reporter gene) were analyzed for their susceptibility to A32-mediated lysis by PBMC effectors cells at 2 days post infection. Briefly, target CEM.NKR infected cells were stained with viability (AquaVivid) and cellular (eFluor670) markers and then mixed with unstained PBMC effectors cells at an effector/target ratio of 10:1. Cocultures were incubated at 37°C for 6 h in presence or not of A32 mAb. (**A**) Gating strategy by which target cells (according to cell morphology, FSC/SSC) that are AquaVivid-negative (i.e. living cells), eFluor670-positive (target cells) and GFP-positive (infected cells) were determined. (**B, C**) Determination of A32-mediated cell lysis using the gating strategy presented in panel A. One representative experiment is shown,

using HIV-1 wt (**B**) or HIV-1 N-U- (**C**) infected cells. Numbers shown represent the percentage of GFP-positive live target cells after incubation. (**D**) Formula used to calculate the percentage (%) of ADCC. As an example, % of ADCC was calculated by using the data presented in panel C.

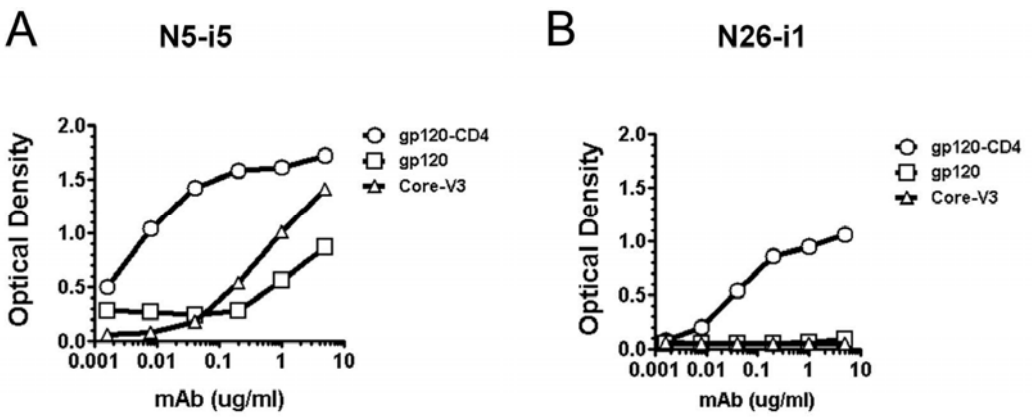

Figure 3.12S (Article 4 Figure S4) Demonstration that N26-i1 selectively binds gp120-CD4 complexes.

Panel **A** shows the binding of N5-i5 to gp120-CD4 complexes (FLSC, open circles), gp120 (open squares) or YU2-Core-V3 (open diamonds). Panel **B** shows the binding of N26-i1 to the same ligands (symbols are as in panel A). Binding was determined by ELISA as described in Supplemental Materials and Methods. FLSC and gp120 were from the HIV-$1_{Ba\text{-}L}$ isolate, YU2-Core-V3 were from the HIV-1_{YU2} isolate.

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THE HIV-1 GP120 CD4-BOUND CONFORMATION IS PREFERENTIALLY TARGETED BY ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY-MEDIATING ANTIBODIES IN SERA FROM HIV-1-INFECTED INDIVIDUALS

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Résumé

Des études récentes ont démontré un lien entre les réponses effectrices des anticorps et une protection ou contrôle des infections au VIH-1 et VIS. Fait intéressant, la présence d'anticorps avec une forte capacité à induire une réponse ADCC chez les individus vaccinés lors de l'essai vaccinal anti-VIH-1 RV144 a semblé corréler avec un risque diminué d'infection au VIH-1. Ces anticorps ont récemment été caractérisés et reconnaissent la forme d'Env liée à CD4. Le retrait de CD4 de la surface des cellules infectées par les protéines accessoires Nef et Vpu, en plus de l'antagonisme de BST-2 par Vpu, modulent l'exposition d'épitopes d'Env CD4-induits et donc protègent les cellules infectées de la réponse ADCC. Dans cet article, nous démontrons une forte prévalence des anticorps capables d'induire une réponse ADCC et reconnaissant des épitopes d'Env CD4-induits dans le sérum d'individus infectés. Cette présence d'anticorps corrèle avec le potentiel des sérums à induire une réponse ADCC contre des cellules infectées exposant les épitopes CD4-induits d'Env. De plus, nos résultats démontrent que les régions variables d'Env V1, V2, V3 et V5 ne représentent pas un déterminant majeur de la réponse ADCC observée chez les individus infectés au VIH-1. Pris ensembles, nos résultats suggèrent que le VIH-1 exerce un contrôle strict sur l'exposition

d'épitopes d'Env à la surface des cellules infectées afin d'empêcher leur élimination par les réponses effectrices déclenchées par des anticorps.

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.

Introduction

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

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 (2×10^4 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).

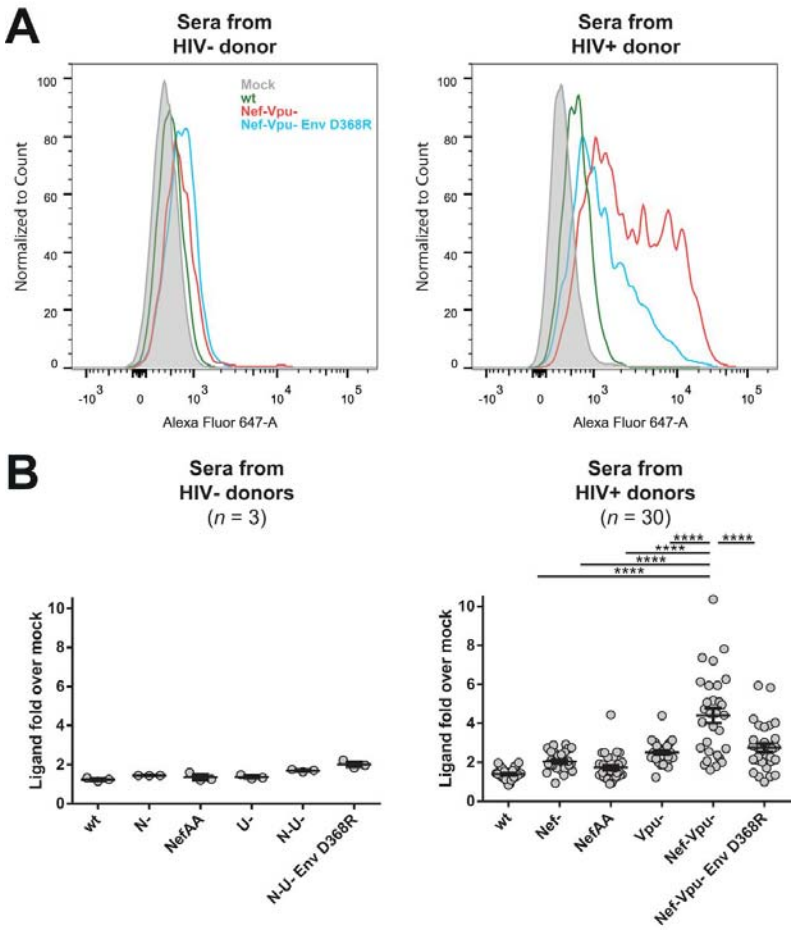

Figure 3.13 (Article 5 Figure 1) Env – CD4 interaction is required for efficient recognition of infected cells by sera from HIV-1-infected individuals.
CEM.NKr cells infected with a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing wild-type (wt) or a CD4-binding site (D368R) Env variant, lacking Nef (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 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

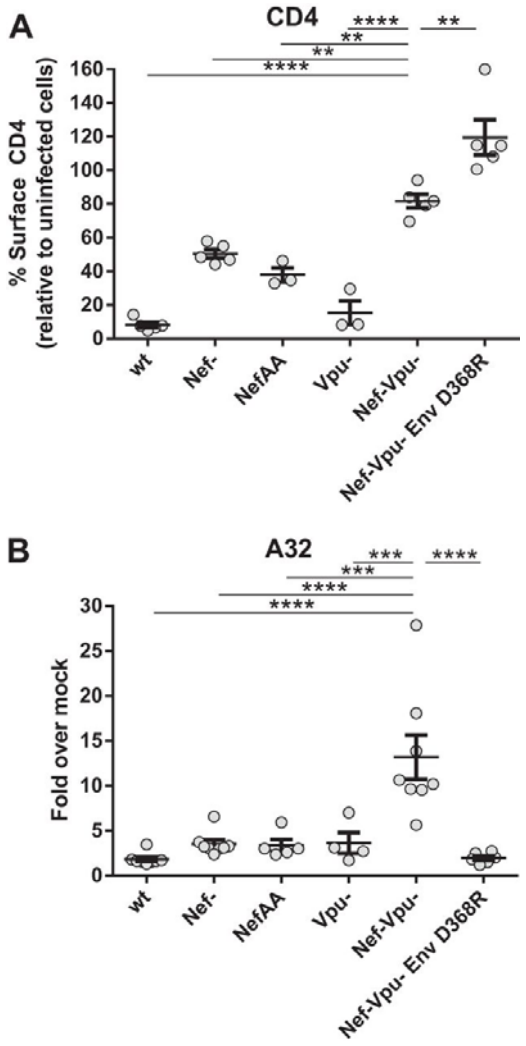

Figure 3.14 (Article 5 Figure 2) Env – CD4 interaction modulates the exposure of Env ADCC-mediating A32 epitope at the surface of infected cells.
CEM.NKr cells infected with a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing wild-type (wt) or a CD4-binding site (D368R) Env variant, lacking Nef (Nef-) 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 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

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.

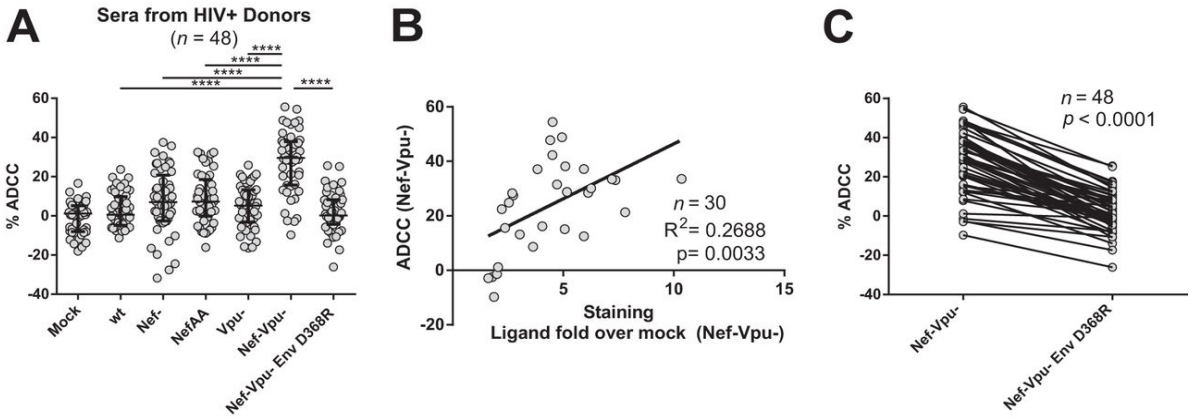

Figure 3.15 (Article 5 Figure 3) Env – CD4 interaction modulates susceptibility of infected cells to ADCC killing mediated by sera from HIV-1-infected individuals.

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 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

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

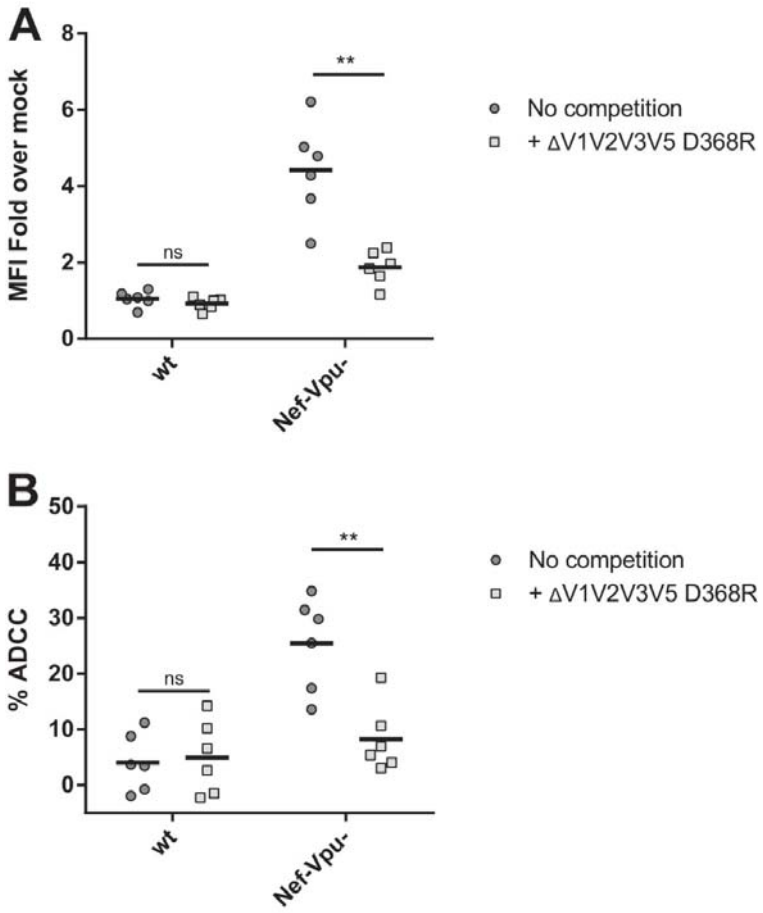

Figure 3.16 (Article 5 Figure 4) A recombinant gp120 protein lacking V1V2V3 and V5 variable regions is sufficient to adsorb the majority of ADCC activity present in sera from HIV+-infected individuals.
CEM.NKr cells infected with wt or Nef-Vpu- VSV-G pseudotyped NL4.3 GFP ADA were used at 48h post-infection for surface staining (**A**) or FACS-based ADCC assay (**B**) using sera from HIV-1 infected individuals pre-incubated in absence or presence of 83.3 pmol ΔV1V2V3V5 D368R / µl sera for 30 min at room temperature. Data shown are representative of at least two different experiments. Statistical significance was tested using paired one-way ANOVAs (** $p<0.01$, ns, not significant).

Discussion

Renewed interest on Fc-mediated functions such as ADCC stems in part from correlations in controlling or preventing HIV-1 infection. Fc-mediated effector functions were found to inversely correlate with viral loads or decreased disease progression in SIV-infected macaques (7-9) as well as in HIV-1-infected individuals (10-14). Furthermore, analysis of

immune protection correlates in the recent RV144 vaccine trial suggested that high levels of ADCC mediating Abs correlated with decreased HIV-1 acquisition when combined with low plasma IgA anti-Env Ab levels (16, 44) thus warranting further studies on the viral determinants modulating ADCC.

Recent observations suggested that Vpu antagonism of BST2 was sufficient to protect HIV-infected cells from ADCC (45, 46). In this study, we found no significant increase in the susceptibility of cells infected with a virus lacking Vpu to sera-mediated ADCC (Figure 3). However, in agreement with previous work done with CD4i Abs (18), decreasing Env – CD4 interaction by introducing an Env CD4 binding site mutation (D368R) in the context of a virus lacking both Nef and Vpu was sufficient to dramatically diminish killing of infected cells by sera-mediated ADCC (Figure 3C). This is also supported by our soluble gp120 competition assay suggesting that gp120 CD4i Abs represent the major determinant of sera-mediated ADCC in HIV-1-infected individuals (Figure 4) since this recombinant protein could not absorb Abs directed against the gp41, quaternary-dependent Abs or V1V2V3 and V5 variable regions. In this study we only tested sera from HIV-1-infected individuals (not from vaccinees); therefore, we do not exclude the possibility that additional types of ADCC-mediating Abs such as anti-V1V2, elicited through vaccination, could have therapeutic utility and/or help in preventing infection.

Furthermore, stratifying the patients sera in clinical disease progression rates (classic, rapid progressors or long-term non-progressors, as described in (25, 26)) did not reveal any significant differences between them as to the requirement of Env – CD4 interaction to promote sera-mediated ADCC against infected cells (Suppl. Table I).

Recent observations suggested that the angle of approach of the Ab is important in order to mediate ADCC (47). Whether anti-gp120 CD4i Abs bind Env with an angle of approach that promotes the recruitment of Fc-bearing effector cells is unclear but warrants further studies.

Altogether, these data suggest that ADCC-mediating Abs are elicited in the course of natural HIV-1 infection and that a significant proportion of them require Env to interact with CD4 in order to be effective at mediating ADCC. This supports a major role of CD4i Env epitopes in the susceptibility of infected cells to ADCC mediated by sera from HIV-1-infected individuals. Our data suggest that HIV-1, via Nef and Vpu, tightly controls cell-surface levels of CD4 and Env in order to limit the exposure of potential epitopes recognized by ADCC-mediating Abs elicited in the course of natural HIV-1 infection. Therefore, targeting Vpu and Nef ability to downregulate CD4 and BST-2 or 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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Supplemental Data

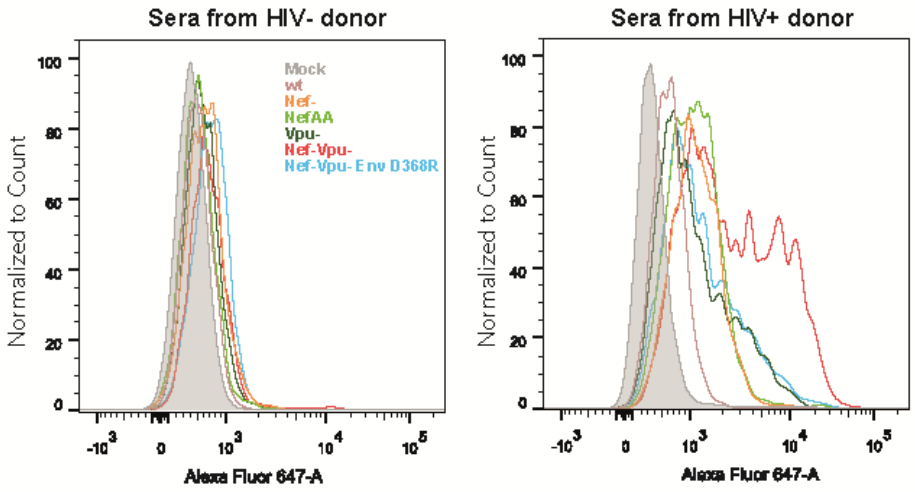

Figure 3.17S (Article 5 Figure S1) Surface staining by sera from HIV-infected individuals on cells infected with a panel of different viruses.
FACS histograms presenting the staining of representative sera from infected individuals or healthy donors against CEM.NKr cells infected with a panel of VSV-G 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 (NefAA), or lacking Vpu (Vpu-) or both Nef and Vpu (Nef-Vpu-).

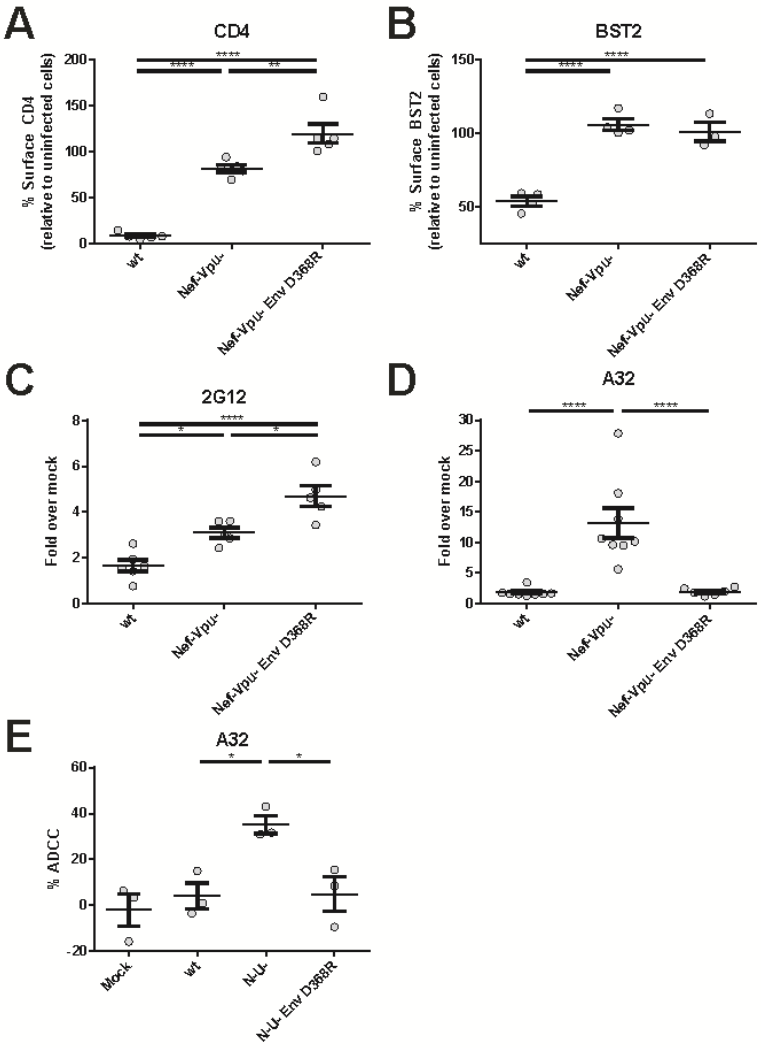

Figure 3.18S (Article 5 Figure S2) Env mutant D368R prevents exposure of CD4i epitopes and A32-mediated killing of infected cells.

CEM.NKr cells infected with VSV-G pseudotyped NL4.3 GFP ADA expressing wild-type (wt) or a CD4-binding site (D368R) Env variant lacking both Nef and Vpu (Nef-Vpu-) were stained for (**A**) surface CD4, (**B**) BST2 or (**C**) anti-Env 2G12 and (**D**) A32 mAbs. Alternatively, infected cells were used in a FACS-based ADCC assay (**E**) (1) to determine their susceptibility to A32-mediated ADCC killing by PBMCs (see Materials & Methods for details). Data shown are the results of at least three different experiments, with mean ± SEM. Statistical significance was tested using one-way ANOVA tests (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

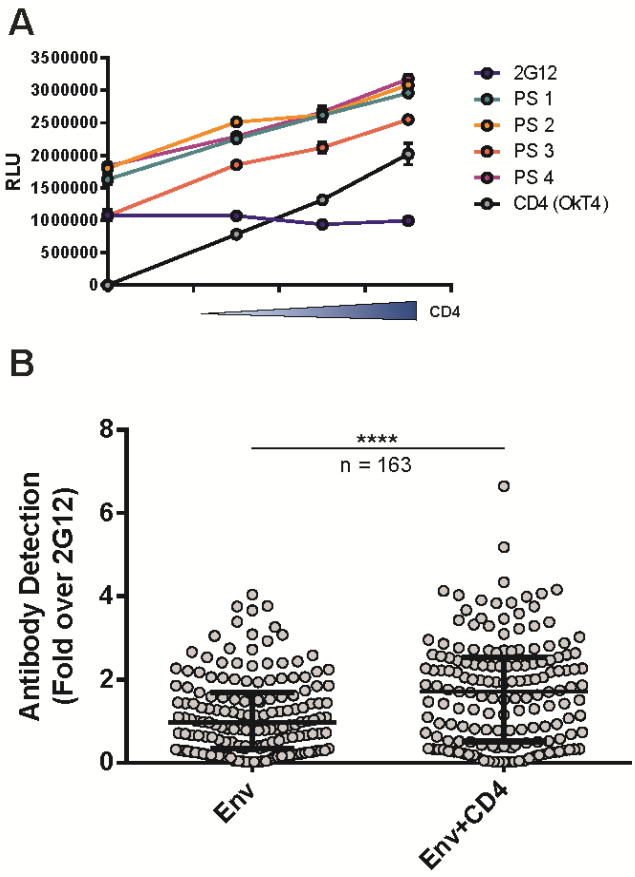

Figure 3.19S (Article 5 Figure S3) Cellular CD4 increases binding of IgG from HIV-infected individuals sera.

Hos cells expressing HIV-1_{YU2} $Env_{\Delta CT}$ bound IgGs present in sera from HIV-1-infected individuals at greater extents in presence of human CD4, as measured by cell-based ELISA (1, 2). Panel (A) shows relative luminescence units (RLU) values obtained from 4 different patients' sera and the gp120 outer domain-recognizing 2G12 as well as the anti-CD4 OkT4 mAbs. No signal was obtained with sera from healthy donors (data not shown). As previously described, 2G12 detection was not affected by the presence of cellular CD4 (1). (B) Binding of IgGs present in sera from HIV-1 infected individuals acquired in triplicates was normalized to signal values obtained for 2G12. Shown is the mean value for each serum, with median ± interquartile range. Statistical significance was tested using Wilcoxon matched-pairs signed rank test (**** $p<0.0001$).

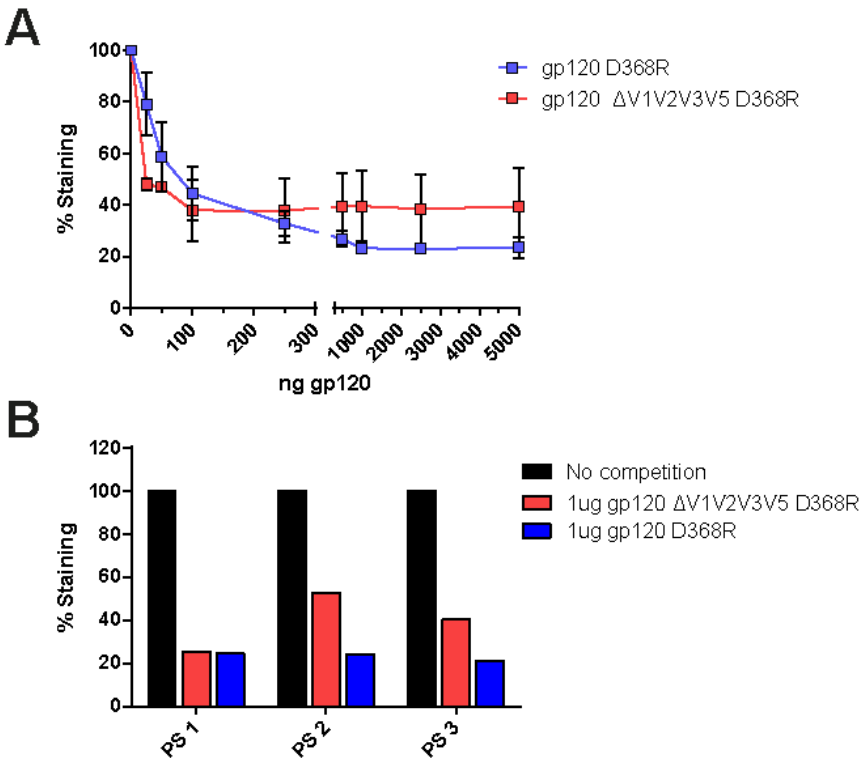

Figure 3.20S (Article 5 Figure S4) Soluble gp120 competes for anti-Env antibodies binding to gp120-coated cells.

CEM.NKr cells were coated with 50 ng of recombinant HIV-1_{YU2} gp120 for $7.5x10^5$ cells/ml, as previously described (3). Sera from HIV+ donors were pre-incubated for 30 minutes at room temperature with increasing amounts (**A)** or 1 µg (**B**) of recombinant D368R gp120 proteins (unable to interact with cell-surface CD4 (3)) either full length (wild-type) or lacking the V1, V2, V3 and V5 variable regions, before being used as a source of antibody for surface staining of the gp120-coated cells. Shown is the percentage of remaining staining intensity (MFI) compared to conditions in absence of competition. Panel (**A**) depicts the mean with SD of three different randomly-selected sera from HIV+ donors while panel (**B**) shows the extent of staining inhibition for each of the three individual sera.

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A HIGHLY-CONSERVED RESIDUE OF THE HIV-1-GP120 INNER DOMAIN IS IMPORTANT FOR ADCC RESPONSES MEDIATED BY ANTI-CLUSTER A ANTIBODIES

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Résumé

Des études précédentes ont démontré que le sérum de patients infectés au VIH-1 contient des anticorps capables d'induire une réponse ADCC contre les cellules infectées. Ces anticorps reconnaissent de manière préférentielle les épitopes des glycoprotéines d'Env qui sont induits lors de l'interaction Env – CD4. Ici, nous démontrons qu'un résidu tryptophane en position 69 du domaine interne de la gp120 est important pour la réponse ADCC médiée par des anticorps du groupe A et par le sérum d'individus infectés au VIH-1.

Abstract

Previous studies have shown that sera from HIV-1-infected individuals contain antibodies able to mediate antibody-dependent cellular cytotoxicity (ADCC). These

antibodies preferentially recognize envelope glycoproteins (Env) epitopes induced upon CD4 binding. Here, we show that a highly conserved tryptophan at position 69 of the gp120 inner domain is important for ADCC mediated by anti-cluster-A antibodies and sera from HIV-1-infected individuals.

Text

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

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

a panel of human antibodies targeting several well-defined epitopes in gp120 and gp41 and sera from randomly-selected chronically HIV-1-clade B-infected individuals (HIV+ sera).

We infected CEM.NKr cells with a panel of HIV-1 NL4-3-GFP constructs containing the ADA-Env and either wild-type or defective nef and vpu genes, as described (2, 5). Furthermore, we examined a well characterized infectious molecular HIV-1 clone constructed from a transmitted/founder (T/F) virus (CH77) (11-14) containing intact or defective nef and vpu genes. Two days post-infection, the cells were evaluated for cell-surface levels of CD4 and stained with HIV+ sera or anti-Env antibodies targeting well-known epitopes in gp120, gp41 or both (Figure 1A and Table 1). Nef and Vpu are known to synergistically decrease cell-surface levels of CD4 (2,3). Accordingly, defects in both genes impaired the ability of HIV-1 to downregulate CD4 to extents that were not achieved by either nef or vpu alone. The highest surface CD4 levels were observed for cells infected with virus lacking intact nef and vpu genes and containing a mutation of D368R in Env that abrogates its interaction with CD4 (15, 16) (Figure 1A, Table 1). The latter observation is in agreement with the notion that Env-CD4 interaction plays a role in CD4 downregulation (17, 18). HIV+ sera and the anti-cluster-A antibodies (these antibodies target conformational CD4i epitopes mapped to the C1-C2 regions of gp120 (10, 19, 20)) recognized wild-type-infected cells with low efficiency (Figure 1C and D). Our results are in agreement with previous reports indicating that the highly-conserved region recognized by anti-cluster A antibodies is buried inside the Env trimer where it is not readily accessible for binding in the ligand-free closed state (10, 21-27). Accordingly, anti-cluster-A and HIV+ sera recognized more efficiently cells infected with a virus lacking Nef and Vpu and hence expose Env at higher levels and in its CD4-bound conformation (2, 5, 28). Nevertheless, our results are also consistent with previous reports indicating that anti-cluster A antibodies, such as A32, can recognize a large proportion of cells infected with a wild-type virus (9, 29, 30). Indeed, A32 recognized ~32% of pNL4.3 ADA and ~54% of CH77 wild-type-infected cells (Figure 2). However, the intensity of recognition (i.e. the amount of antibody binding per cell) was dramatically increased for cells presenting Env in its CD4-bound conformation (i.e., Nef-Vpu- infected cells), as previously reported (2-5, 28, 31). Similar to anti-cluster A antibodies, co-receptor binding site (CoRBS) (17b and LF17) (Figure 1E) as well as anti-V3 antibodies (19b and GE2 JG8) (Figure 1F) recognized cells

infected with Nef-Vpu- HIV-1 cells most efficiently. This suggests that their epitope was formed upon Env-CD4 interaction and that they all belong to the CD4i family of antibodies. We noted, however, that the overall recognition of CoRBS and anti-V3 Abs was lower than that observed for HIV+ sera and anti-cluster A Abs.

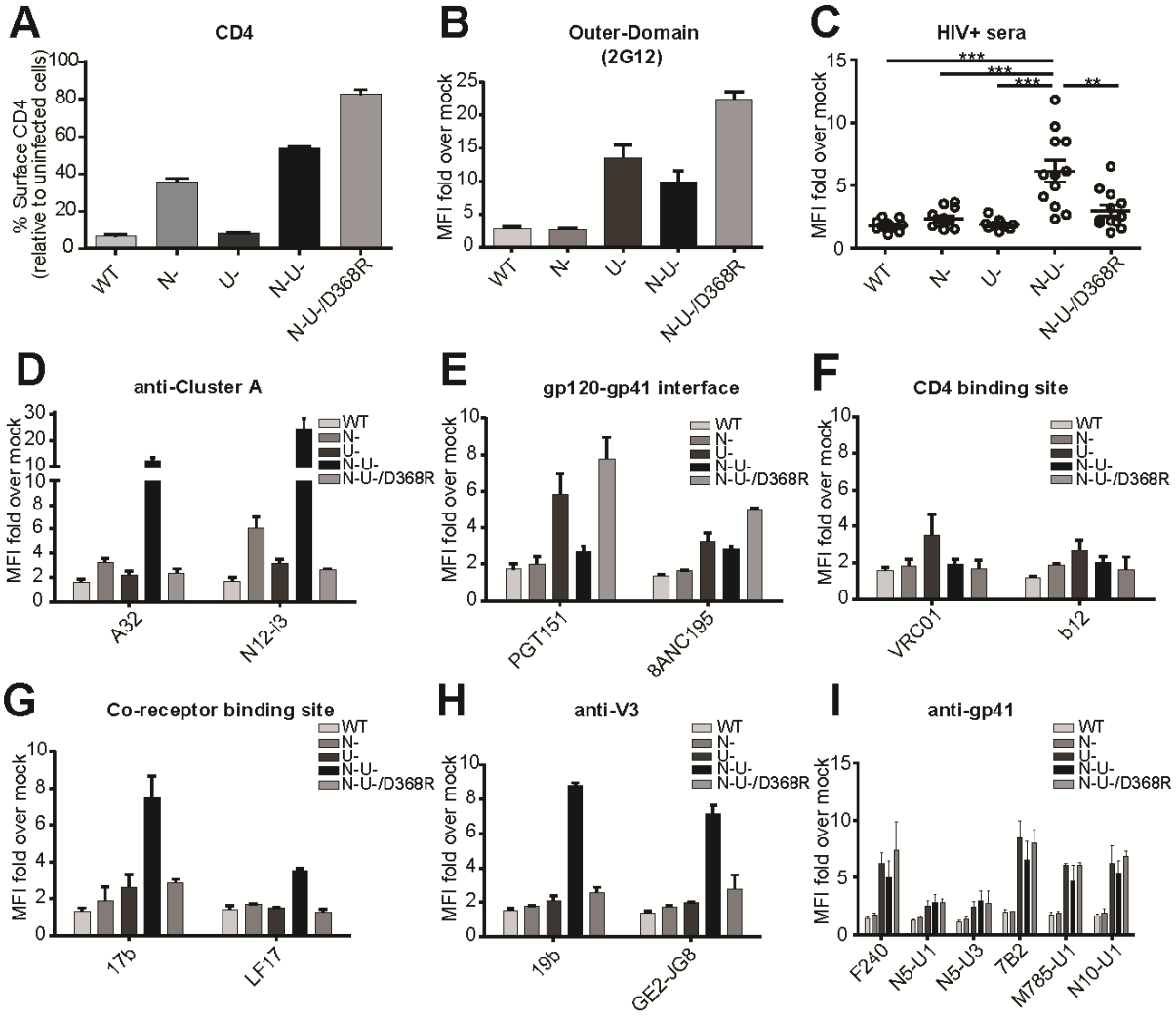

Figure 3.21 (Article 6 Figure 1) Effect of Nef, Vpu and Env-CD4 interaction on recognition of infected cells by HIV+ sera and a panel of monoclonal antibodies.

CEM.NKr cells infected with a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing wild-type (WT) or a CD4-binding site (D368R) Env variant, lacking Nef (N-), Vpu (U-) or both Nef and Vpu (N-U-) were stained at 48 h post-

infection with (**A**) an anti-CD4 antibody (OKT4) or (**B**) 1/1000 dilution of sera from 12 HIV-1-infected individuals (HIV+ sera), or (**C**) 1ug/mL of anti-cluster-A (A32, N12-i3), (**D**) anti-gp120-gp41 interface (PGT151, 8ANC195), (**E**) anti-CD4-binding site (VRC01, b12,), (**F**) anti-CoRBS (17b, LF17), (**G**) anti-V3 (19b, GE2 JG88), (**H**) anti-gp41 (F240, N5-U1, N5-U3, 7B2, M785-U1, N10-U1) and (**I**) CD4-independent outer-domain recognizing 2G12 antibodies and then fluorescently labeled with an Alexa-Fluor-647 conjugated anti-human IgG secondary Ab. Shown is the fold increase of staining relative to mock for all tested sera and antibodies. Data shown are the results of 3 different experiments and error bars depict the SEM. Statistical significance was tested using paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$).

In the absence of Vpu there is more Env at the cell surface, as measured by the CD4-independent outer-domain recognizing 2G12 antibody (Figure 1B); likely due to tetherin/BST-2 trapping of viral particles. Cells infected with viruses lacking Nef and Vpu but containing the D368R mutation in Env that impairs CD4 binding (2, 4, 5, 15, 16) were poorly recognized by HIV+ sera, anti-cluster-A, anti-coreceptor binding site and anti-V3 antibodies (Figure 1 C-F). When we tested PGT151 and 8ANC195, two antibodies that recognize the interface between gp120 and gp41 (32, 33), we observed a different phenotype. Both antibodies efficiently recognized cells infected with a virus lacking Vpu or expressing Env D368R, but not lacking Vpu and Nef (Figure 1G). 8ANC195 and PGT151 have been shown to bind to CD4-bound Env (34), but in our system CD4 and Env are interacting on the same membrane and CD4 domains D3-D4 may block access of these Abs to their epitopes on Env, which are located underneath the CD4 binding site.

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

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

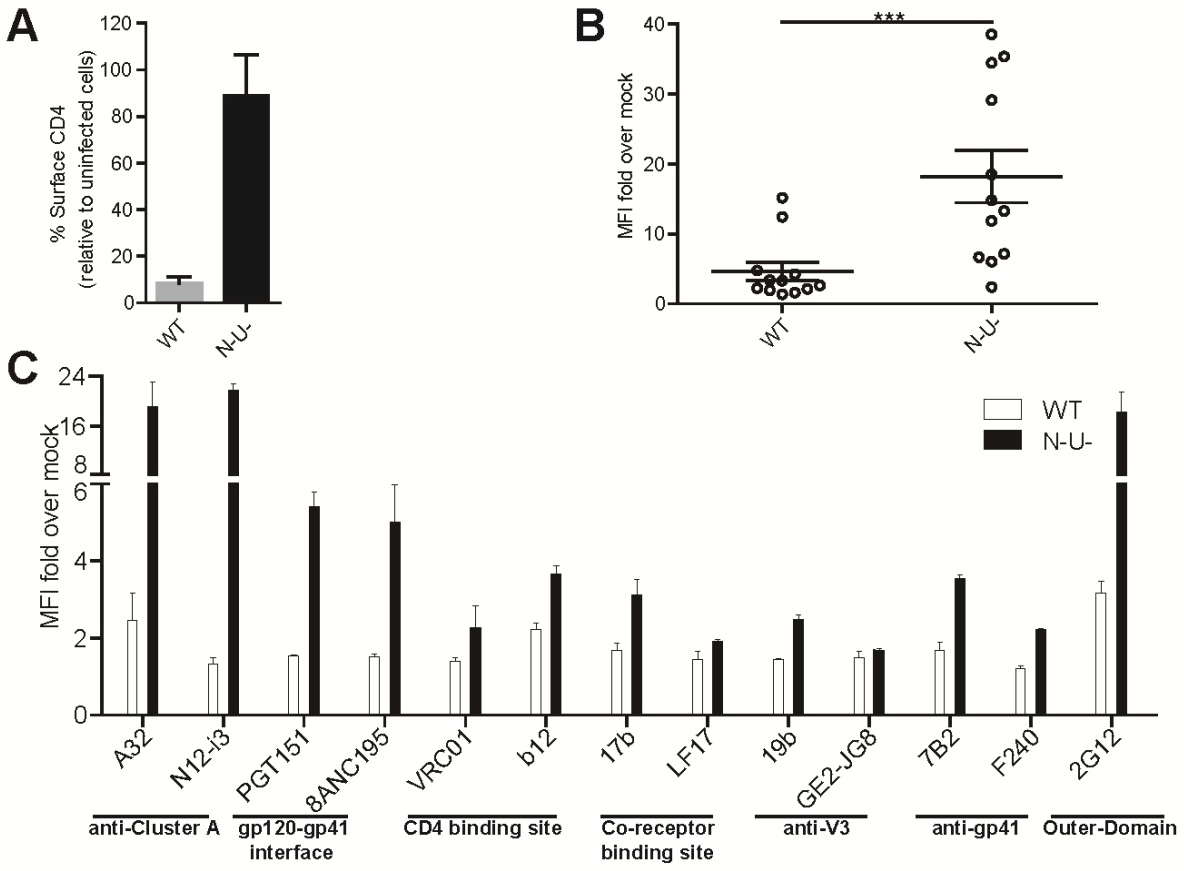

Figure 3.22 (Article 6 Figure 2) Effect of Nef and Vpu on recognition of cells infected with a primary isolate by HIV+ sera and a panel of monoclonal antibodies. CEM.NKr cells infected with VSV-G pseudotyped T/F CH77 expressing wild-type (WT) or lacking Nef and Vpu (N-U-) were stained at 48 h post-infection with (**A**) an anti-CD4 antibody (OKT4) or (**B**) 1/1000 dilution of sera from 12 HIV-1-infected individuals (HIV+ sera). (**C**) CH77-infected cells were also

stained with a panel of anti gp120 and gp41 antibodies (1ug/mL) and then fluorescently labeled with an Alexa-Fluor-647 conjugated anti-human IgG secondary Ab. Data shown are the results of 3 independent experiments, with median ± interquartile range. Statistical significance was tested using paired one-way ANOVAs (** $p<0.01$, **** $p<0.0001$).

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

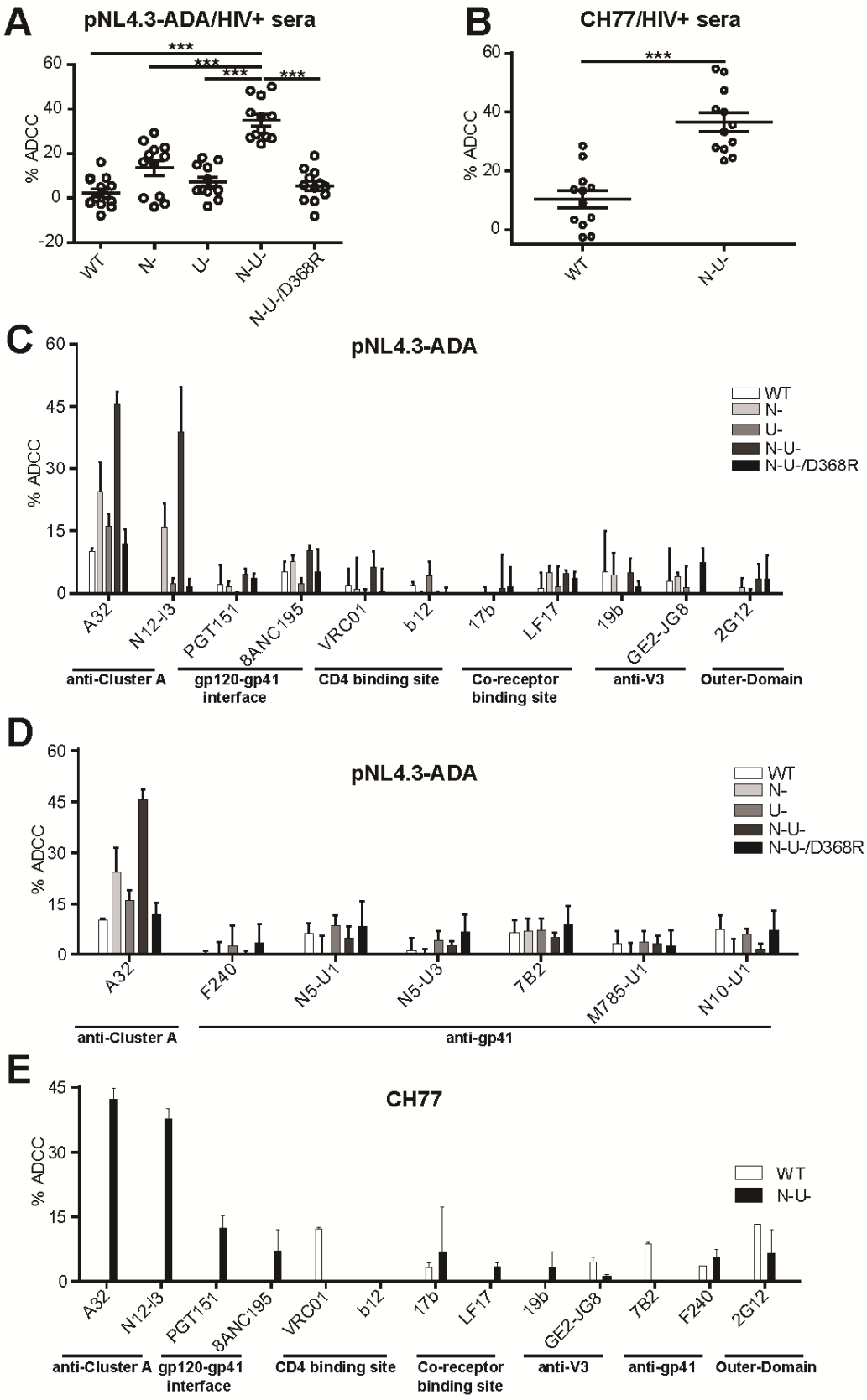
A
pNL4.3-ADA/HIV+ sera
% ADCC
WT
N-
U-
N-U-
N-U-/D368R
B
CH77/HIV+ sera
C
pNL4.3-ADA
A32
N12-i3
PGT151
8ANC195
VRC01
b12
17b
LF17
19b
GE2-JG8
2G12
anti-Cluster A
gp120-gp41 interface
CD4 binding site
Co-receptor binding site
anti-V3
Outer-Domain
D
F240
N5-U1
N5-U3
7B2
M785-U1
N10-U1
anti-gp41
E
CH77

Figure 3.23 (Article 6 Figure 3) Effect of Nef, Vpu and Env-CD4 interaction on ADCC responses mediated by HIV+ sera and a panel of monoclonal antibodies. 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 (N-), or lacking Vpu (U-) or both Nef and Vpu (N-U-) were used at 48h post-infection as target cells in our FACS-based ADCC assay (5) to determine their susceptibility to ADCC mediated by (**A**) 1/1000 dilution of sera from 12 HIV-1-infected individuals. (**B**) The susceptibility of VSV-G pseudotyped T/F CH77-infected cells expressing wild-type (WT) or lacking Nef and Vpu (N-U-) to ADCC mediated by the same panel of HIV+ sera was also evaluated. (**C**) The susceptibility of pNL4.3 ADA infected cells to ADCC mediated by 5ug/mL of anti-gp120 (anti-cluster A A32, N12-i3; anti-CD4-binding site 8ANC195, VRC01, b12; anti-CoRBS 17b, LF17; anti-V3 19b, GE2 JG88; anti-outer domain 2G12), anti-gp120-gp41 (PGT-151) or (**D**) anti-gp41 (F240, N5-U1, N5-U3, 7B2, M785-U1 andN10-U1) antibodies was also evaluated. (**E**) Susceptibility of CH77-infected cells to anti gp120 and anti-gp41 antibodies. PBMCs from healthy donors were used as effector cells. Data shown are the results of 3 independent experiments, with median ± interquartile range. Statistical significance was tested using paired one-way ANOVAs (** $p<0.01$, **** $p<0.0001$).

To investigate which region of the gp120 was targeted by ADCC-mediating Abs present in HIV+ sera, we used our previously-described antibody competition assay using purified soluble gp120Yu2 lacking variable regions V1, V2, V3, V5 with the D368R mutation (ΔV1V2V3V5/D368R) (2). As a control, we also tested the ability of these recombinant proteins to block recognition of infected cells by five anti-cluster A antibodies (A32, N5-i5, N12-i3, N26-i1, 2.2c). Our recent structural studies mapped cluster A epitope region into mobile Layer 1 and 2 of to the gp120 inner domain of the CD4-triggered gp120 (19, 20, 26). Residues of variable loops and outer domain of gp120 are not involved in anti-cluster A antibody binding. In addition, although cluster A mAbs are capable of binding unliganded gp120, CD4-binding enhances their exposure in context of full length gp120 antigen (10, 36, 37). As expected, pre-incubation of anti-cluster A antibodies (Figure 5A) or HIV+ sera (Figure 5B) with either full-length or ΔV1V2V3V5 D368R gp120 recombinant proteins captured anti-Env antibodies and prevented their recognition of infected cells. Decreased recognition correlated with decreased ADCC activity (Figure 5C and D). These data indicate that antibodies targeting the core of the gp120 are responsible for the majority of ADCC responses in HIV+ sera.

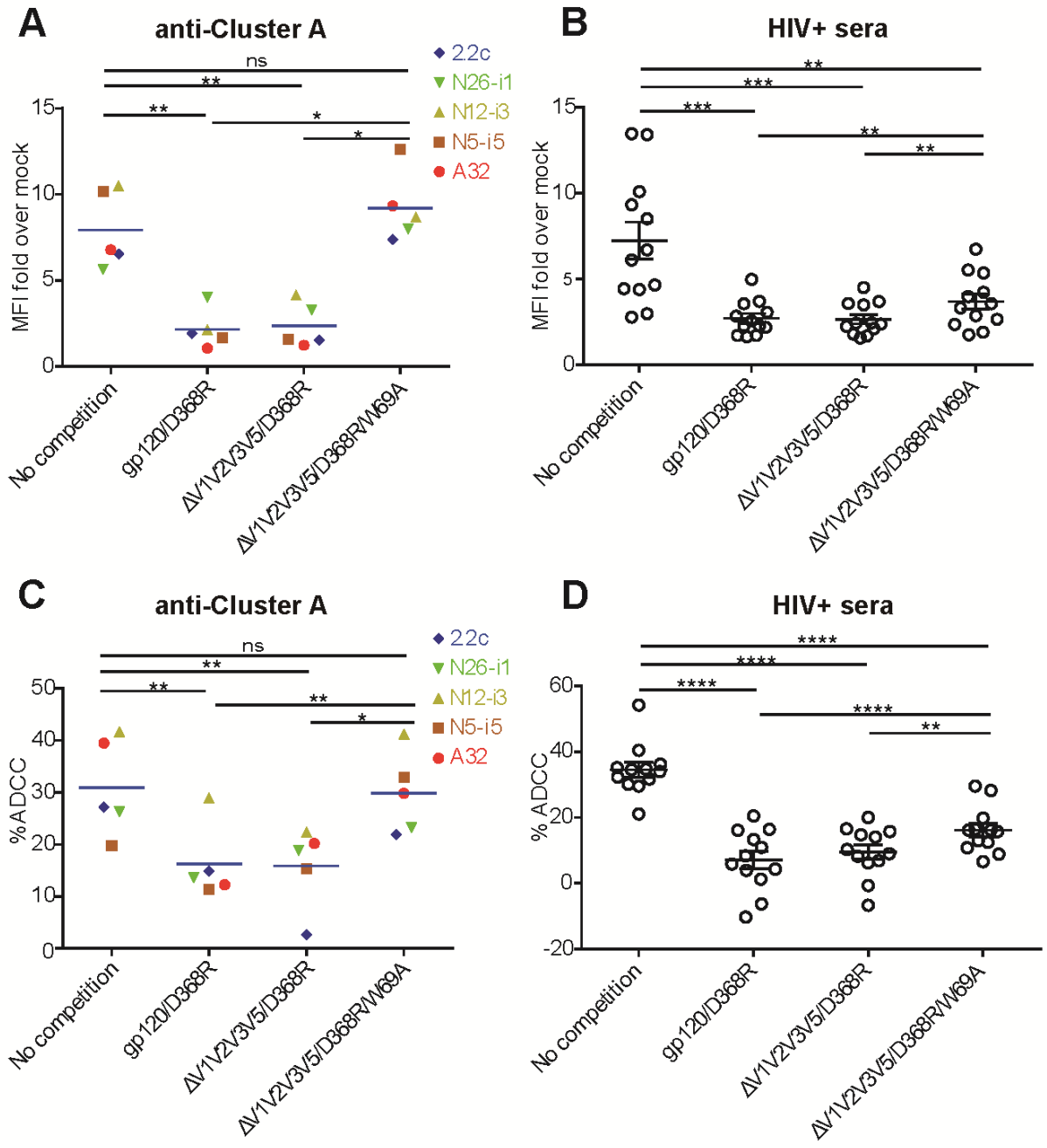

Figure 3.24 **(Article 6 Figure 4) Tryptophan 69 in Layer 1 is important for ADCC responses mediated by anti-cluster A antibodies and some HIV+ sera.** CEM.NKr cells infected with N-U- VSV-G pseudotyped NL4.3 GFP ADA virus were used at 48h post-infection for (**A**, **B**) surface staining or (**C**, **D**) FACS-based ADCC assay using 5 ug/mL of anti-cluster A antibodies (A32, N5-i5, N12-i3, N26-i1 and 2.2c) or 1/1000 dilution of sera from 12 HIV-1-

infected individuals (HIV+ sera) in absence or presence of recombinant gp120/D368R (10ug/ug of antibody), ΔV1V2V3V5/D368R (6ug/ug of antibody) or ΔV1V2V3V5/D368R/W69A (6ug/ ug of antibody) for 30 min at room temperature. Data shown are representative of at least 3 independent experiments. Statistical significance was tested using paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

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

Why does similar binding of infected cells by different classes of antibodies not translate into equivalent ADCC responses? For example, anti-cluster A and anti-co-receptor binding site antibodies recognize CD4-induced Env epitopes, which become unmasked by the interaction of Env trimers with CD4. Both cluster A and co-receptor binding site region epitopes should persist on infected cell surfaces for similar periods of time and therefore, both should constitute good targets for ADCC. Why then anti-cluster A antibodies are able to mediate potent ADCC responses whereas anti-co-receptor binding site antibodies are not? ADCC is mediated not only by antibody variable region binding to antigen on infected cells but also by the antibody constant region binding to Fc receptors on effector cells and therefore, even subtle differences in the glycosylation patter of the Fc portion of these antibodies could affect their ability to mediate ADCC. Nevertheless, our studies suggest that fine specificities among epitope targets at the surface of infected cells might also play a role in determining potency of ADCC response. We believe that targeting CD4i conformational, C1-C2 epitopes within cluster A region, which depend on W69, could allow for an efficient antigen

engagement and optimal angle of approach to engage with the Fcγ receptor of effector cell for effective ADCC immune-complex formation, as previously suggested (19, 20). Our results confirm that Nef and Vpu protect HIV-1-infected cells from ADCC but also show that recognition of infected cells by an antibody does not necessarily translate into ADCC. This raises the intriguing possibility that the angle of approach of a given class of antibodies could impact its capacity to mediate ADCC.

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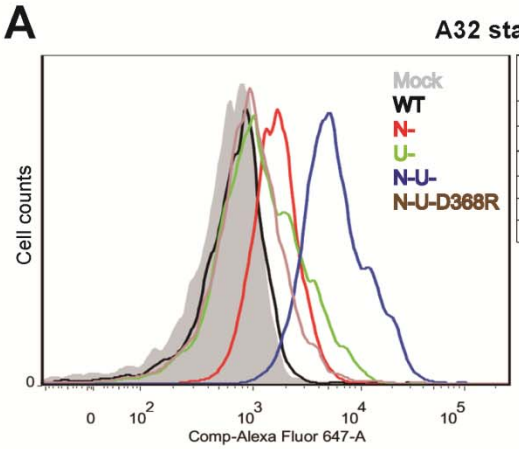
TABLE 1 Recognition of infected cells by a panel of anti-gp120 and anti-gp41 antibodies

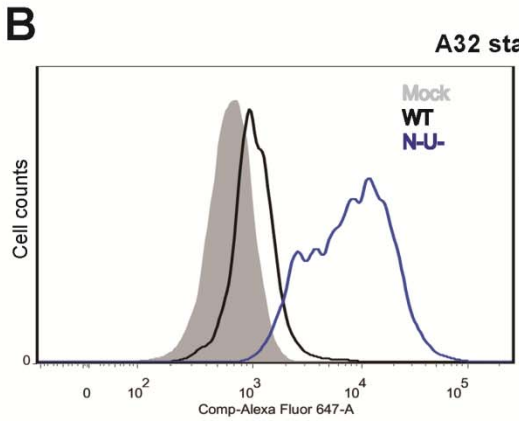
pNL4.3-ADA	CD4 (%)[a]	Staining[b]																
		anti-Cluster A		gp120-gp41 interface		CD4 binding site		Co-receptor binding site		anti-V3		anti-gp41						Outer-Domain
		A32	*N12-i3*	*PGT151*	*8ANC195*	*VRC01*	*b12*	*17b*	*LF17*	*19b*	*GE2-JG8*	*F240*	*N5-U1*	*N5-U3*	*7B2*	*M785-U1*	*N10-U1*	*2G12*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

a The relative percentage of surface CD4 on infected cells comparative to uninfected cells.
b The staining (MFI fold) of infected cells over uninfected mock cells.

Supplemental Data



pNL4.3-ADA	% infected cells	A32⁺ cells (%) (among infected GFP⁺ cells)	MFI	MFI fold over mock
Mock	0	0	718	1.0
WT	26.3	32.2	994	1.4
N-	21.5	78.6	1911	2.7
U-	29.6	63.5	1684	2.3
N-U-	23.3	100	7457	10.4
N-U-/D368R	28.1	43.4	1320	1.8



CH77	% infected cells	A32⁺ cells (%) (among infected p24⁺ cells)	MFI	MFI fold over mock
Mock	0	0	678	1.0
WT	14.7	54.4	1176	1.7
N-U-	17.7	100	10500	15.5

Figure 3.25S (Article 6 Figure S1) Recognition of HIV-1-infected cells by the anti-cluster A32 antibody.

CEM.NKr cells infected with (**A**) a panel of VSV-G pseudotyped NL4.3 GFP ADA expressing wild-type (WT) or a CD4-binding site (D368R) Env variant, lacking Nef (N-), or lacking Vpu (U-) or both Nef and Vpu (N-U-) or (**B**) with VSV-G pseudotyped primary T/F CH77 infectious molecular clone were stained at 48 h post-infection with the anti-cluster A A32 antibody (1ug/mL) and then fluorescently labeled with an Alexa-Fluor-647 conjugated anti-human IgG secondary Ab. Histograms depicting representative staining of infected (GFP+ in **A** or p24+ in **B**) cells are shown. Right panels show the percentage of infected cells, the percentage of infected (GFP+ in **A** or p24+ in **B**) cells that were recognized by A32 as well as the mean fluorescence intensity (MFI) of

these cells. MFI of infected cells over mock-infected cells is shown in the last column.

CHAPITRE IV

LA MODULATION DE LA CONFORMATION DE L'ENV PAR LE VIH-1 : COMMENT EN TIRER AVANTAGE ET IMPLICATIONS DANS LA PATHOGÉNÈSE

Introduction

Le chapitre précédent a démontré les mécanismes d'exposition d'épitopes d'Env lors de son interaction avec CD4. Il a également clarifié la notion que des anticorps capables d'induire une réponse ADCC suite à la reconnaissance de ces épitopes CD4-induits sont hautement prévalents lors d'infections naturelles au VIH-1. Il est primordial pour le virus d'empêcher la reconnaissance des cellules infectées par ces anticorps. Il a donc évolué des mécanismes hautement conservés pour y arriver : ses protéines accessoires Nef et Vpu. Dans cette présente section, il sera initialement abordé comment de petites molécules ciblant Env et capables de mimer la liaison à CD4 sont suffisantes pour induire des changements conformationnels d'Env et ainsi exposer des épitopes capables d'être reconnus par les anticorps du sérum d'individus infectés, travaux publiés dans le journal *PNAS*. De plus, un deuxième article, publié dans *EBioMedicine*, décrit comment la relâche de gp120 par les cellules infectées permet de rediriger la réponse ADCC vers des cellules CD4+ non-infectées, contribuant ainsi à protéger les cellules infectées de cette réponse effectrice. Prises ensemble, les données présentées dans ce chapitre contribuent à la fois à l'explication des mécanismes de pathogénèse du VIH-1 en plus de paver la voie vers d'éventuelles approches thérapeutiques pouvant cibler de manière efficace les cellules infectées tout en protégeant les cellules non-infectées.

CD4 MIMETICS SENSITIZE HIV-1-INFECTED CELLS TO ADCC

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Résumé

Les cellules infectées par le VIH-1 qui présentent les glycoprotéines d'Env sous leur conformation liée à CD4 sont préférentiellement reconnues par des anticorps capables d'induire une réponse ADCC. De ce fait, le VIH-1 a évolué un mécanisme complexe et hautement efficace afin de permettre d'empêcher l'exposition de ces épitopes d'Env susceptibles à la réponse ADCC. Pour se faire, il retire le récepteur CD4 de la surface cellulaire tout en limitant la quantité d'Env exposé. Dans cet article, nous démontrons que des petites molécules se liant à Env sont capables de mimer l'effet de la liaison de CD4 et ainsi induire la conformation liée à CD4 des glycoprotéines virales. Ceci a pour effet de sensibiliser des cellules infectées avec des isolats primaires du VIH-1 à la réponse ADCC induite par les

anticorps présents dans le sérum, le liquide cervico-vaginal ainsi que dans le lait maternel d'individus infectées au VIH-1. De plus, nous avons identifié que ces petites molécules peuvent sensibiliser des cellules infectées naturellement après expansion *ex vivo* de l'infection à la réponse ADCC induite par des anticorps et cellules effectrices autologues. Pris ensemble, nos résultats suggèrent que les petits composés mimant CD4 pourraient avoir un effet thérapeutique dans la prévention et le contrôle de l'infection au VIH-1.

Abstract

HIV-1-infected cells presenting envelope glycoproteins (Env) in the CD4-bound conformation on their surface are preferentially targeted by antibody-dependent cell-mediated cytotoxicity (ADCC). HIV-1 has evolved a sophisticated mechanism to avoid exposure of ADCC-mediating Env epitopes by downregulating CD4 and by limiting the overall amount of Env at the cell surface. Here we report that small-molecule CD4-mimetic compounds induce the CD4-bound conformation of Env and thereby sensitize cells infected with primary HIV-1 isolates to ADCC mediated by antibodies present in sera, cervico-vaginal lavages and breast milk from HIV-1-infected individuals. Importantly, we identified one CD4-mimetic with the capacity to sensitize endogenously-infected *ex-vivo*-amplified primary CD4 T cells to ADCC killing mediated by autologous sera and effector cells. Thus, CD4-mimetics hold the promise of therapeutic utility in preventing and controlling HIV-1 infection.

Significance

Prevention of HIV-1 transmission and progression likely requires approaches that can specifically eliminate HIV-1-infected cells. Rationally designed CD4-mimetic compounds (CD4mc) have been shown to efficiently inhibit viral entry and sensitize HIV-1 particles to neutralization by otherwise non-neutralizing CD4-induced antibodies. Here we found that CD4mc can also sensitize HIV-1-infected cells to antibody-dependent cell-mediated cytotoxicity (ADCC). Indeed, CD4mc induced the CD4-bound conformation of HIV-1 envelope glycoproteins, exposing CD4-induced epitopes recognized by easy-to-elicit antibodies present in sera, cervico-vaginal lavages and breast milk from HIV-1-infected

individuals. Importantly, we provide evidence that CD4mc can efficiently sensitize primary CD4 T cells from HIV-1-infected individuals to ADCC mediated by autologous sera and effector cells. Therefore, CD4mc might represent a novel approach to prevent and control HIV-1 infection.

Author Contributions

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

Introduction

Worldwide, it is estimated that more than 35 million people are living with human immunodeficiency virus (HIV). In 2013 alone, around 2.1 million people became newly infected with HIV and 1.5 million people died from AIDS (1). Measures to prevent HIV-1 transmission are desperately needed. Prevention of HIV-1 transmission and progression likely requires approaches that can specifically target and eliminate HIV-1-infected cells. Interestingly, there is increasing evidence supporting a role of Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) in controlling HIV-1 transmission and disease progression (2-8). Analysis of the correlates of protection in the RV144 vaccine trial suggested that increased ADCC activity was linked with decreased HIV-1 acquisition (9) and antibodies (Abs) with potent ADCC activity were isolated from some RV144 vaccinees (10). Recent studies reported that the viral accessory proteins Nef and Vpu protect HIV-1-infected cells from anti-HIV-1 envelope (Env)-mediated ADCC responses (11-14). Importantly, we and others reported that Env in the CD4-bound conformation was preferentially targeted by ADCC-mediating Abs and sera from HIV-1-infected individuals (11, 12, 15, 16), which represent a significant proportion of anti-Env Abs elicited during natural HIV infection (11, 17). However, the vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, which limit the

exposure of CD4-induced (CD4i) Env epitopes at the surface of infected cells likely preventing ADCC responses.

Theoretically, agents promoting the CD4-bound Env conformation should expose CD4i epitopes that are readily recognized by ADCC-mediating Abs and sera from infected individuals (11, 12, 15, 16, 18), resulting in the sensitization of HIV-1-infected cells to ADCC. Importantly, modulating Env conformation at the surface of HIV-1-infected cells has become feasible as a result of the availability of small CD4-mimetic compounds (CD4mc). The prototypes of such compounds, NBD-556 and NBD-557, were discovered in a screen for inhibitors of gp120-CD4 interaction (19). These small-molecule ~337-dalton compounds and recent derivatives (DMJ-I-228, JP-III-48) bind in the Phe 43 cavity (20-22), a highly conserved ~150-$Å^3$ pocket in the gp120 glycoprotein located at the interface of the inner domain, outer domain, the bridging sheet and the CD4 receptor (23). CD4mc block gp120-CD4 interaction and induce thermodynamic changes in gp120 similar to those observed upon CD4 or soluble CD4 (sCD4) binding (24). Accordingly, these small molecules as well as sCD4 can promote the transition of Env to the CD4-bound conformation, thus sensitizing HIV-1 particles to neutralization by otherwise non-neutralizing CD4i Abs (17, 25). Additional strategies using scaffolded miniproteins targeting critical gp120 elements required for CD4 interaction allowed the identification of CD4-mimetics with nanomolar affinity for gp120 (26). One of these variants, M48U1, displayed remarkably potent neutralization of three HIV-1 isolates (27); its crystal structure in complex with HIV-1 gp120 was recently solved, showing that M48U1 engages the Phe 43 cavity in a manner similar to that of CD4 (28); thus, M48U1 might induce gp120 to adopt the CD4-bound conformation and expose CD4i epitopes. Previous studies exploring the anti-viral properties of CD4mc were performed on viral particles (17, 25, 27). However, whether these compounds are able to engage the large amounts of Env present at the surface of infected cells and modulate Env conformation in a way that allows exposure of ADCC-mediating epitopes is currently not known. In this study, we show that CD4mc strongly sensitize HIV-1-infected primary CD4 T cells to ADCC mediated by sera, cervicovaginal fluids (CVLs) and breast milk from HIV-1-infected individuals, and also help eliminate infected, *ex-vivo*-expanded primary CD4 T cells from HIV-1-infected individuals. Therefore CD4mc possess three valuable complementary antiviral

properties: direct inactivation of viral particles, sensitization of viral particles to neutralization by otherwise non-neutralizing antibodies, and sensitization of HIV-1-infected cells to ADCC-mediated killing.

Results

Env – CD4 interaction enhances recognition of HIV-1-infected cells by sera from HIV-1-infected individuals

We recently reported that Env interaction with the CD4 receptor at the surface of infected cells is critical for efficient ADCC activity mediated by monoclonal Abs targeting CD4i Env epitopes (12) or by sera from HIV-1-infected individuals (11). Env-CD4 interaction is modulated by the HIV-1 accessory proteins Nef and Vpu, which are known to decrease cell-surface levels of CD4 (29, 30). In addition to its role in CD4 degradation, Vpu also antagonizes a restriction factor, Tetherin/BST-2, which normally inhibits retroviral release (31, 32). Viruses lacking Vpu remain trapped at the cell surface resulting in an accumulation of exposed Env (11-14). Therefore, Nef and Vpu can indirectly modulate Env–CD4 interaction at the surface of infected cells through CD4 and BST-2 downregulation (11, 12). 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 CD4i Env epitopes recognized by ADCC-mediating Abs such as A32 and HIV-1+ sera (11, 12) (Figure 1*A* and *B*). However, if the ability of Env to interact with CD4 is decreased by a change near the CD4-binding site (D368R) (23, 33), Env CD4i epitopes are poorly exposed, resulting in decreased interaction with CD4i Abs and HIV-1+ sera (11, 12) (Figure 1*A* and *B*).

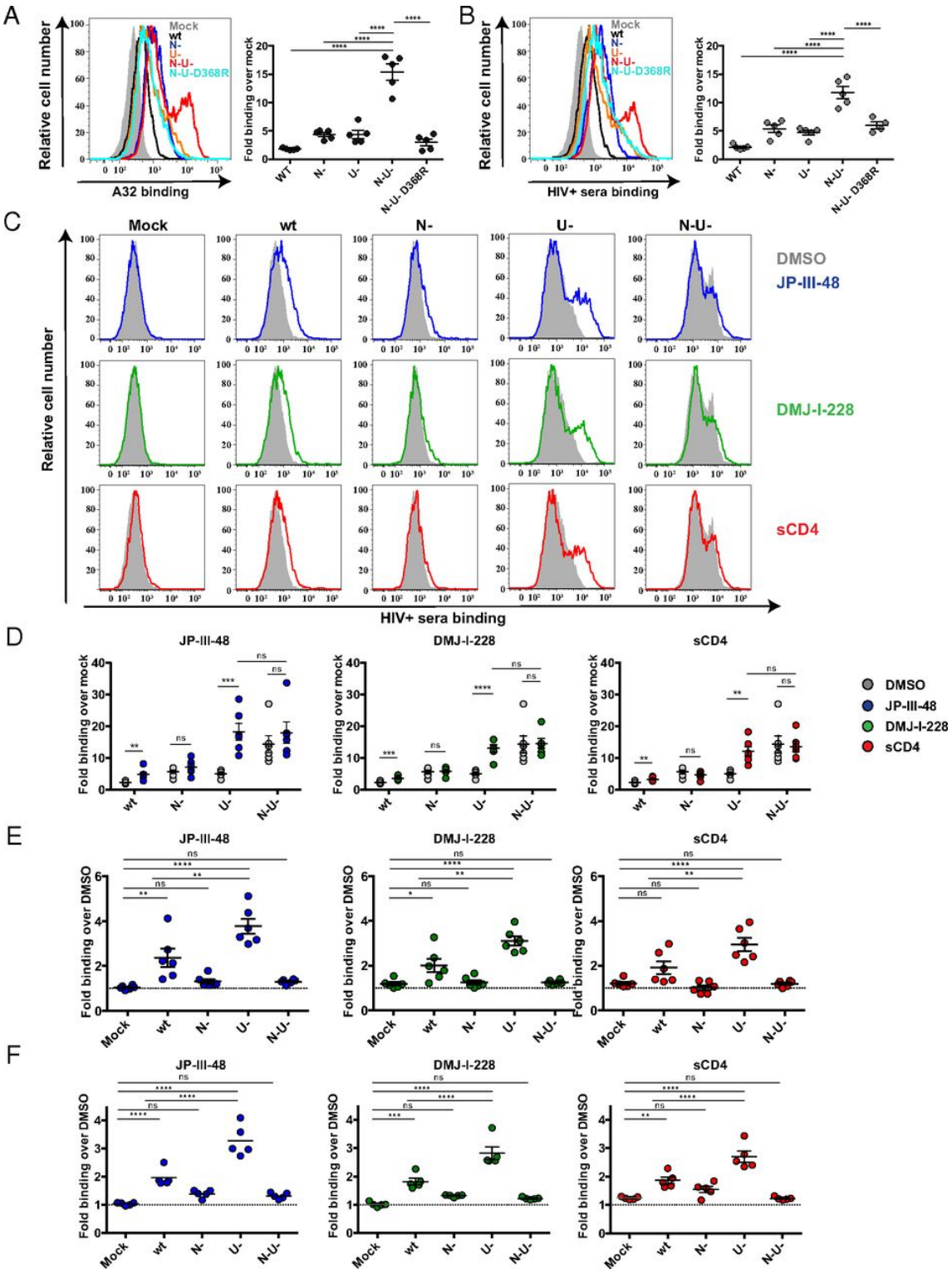
A
Relative cell number
A32 binding
Mock
wt
N-
U-
N-U-
N-U-D368R
Fold binding over mock
WT
N-
U-
N-U-
N-U- D368R
B
HIV+ sera binding
C
Mock
wt
N-
U-
N-U-
DMSO
JP-III-48
DMJ-I-228
sCD4
HIV+ sera binding
D
JP-III-48
DMJ-I-228
sCD4
Fold binding over mock
E
Fold binding over DMSO
F

Figure 4.1 (Article 7 Figure 1) Env interaction with CD4 or CD4mc enhances recognition of HIV-1-infected cells by sera from HIV-1-infected individuals.

Cell-surface staining of CEM.NKr cells infected with NL4.3 GFP ADA-based viruses, either wild-type (wt), lacking Nef (N-), Vpu (U-) or both Nef and Vpu (N-U-) and expressing wt Env or D368R Env (N-U- D368R) with (*A*) A32 mAb or (*B*) sera from an HIV-1-infected donor. Shown in (*A-B*) are histograms depicting representative staining of infected cells (GFP+), with right panels presenting the fold increase of staining relative to mock-infected cells. (*C*) Staining of mock-infected or HIV-1-infected cells with sera from an HIV-1-infected donor in presence of CD4-mimetics or vehicle (DMSO). (*D*) Quantification of data presented in (*C*) as fold binding over mock in 6 independent experiments. In (*E*) the data were calculated as a fold increase between CD4-mimetic-treated cells over cells treated with the vehicle (DMSO) only. (*F*) Effect of CD4-mimetics on the recognition of infected cells (as described in *C*) by sera from 5 additional HIV-1-infected individuals. Error bars indicate mean +/- SEM. Statistical significance was tested using (*A, B, E*) ordinary one-way ANOVAs, (*D*) an unpaired t-test or (*F*) paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

CD4-mimetics sensitize HIV-1-infected cells to ADCC mediated by sera from HIV-1-infected individuals

We explored the capacity of different CD4mc to promote the CD4-bound conformation of Env and thereby enhance Env recognition at the surface of HIV-1-infected cells by sera from HIV-1-infected individuals. Soluble CD4 is the recombinant human CD4 protein lacking the transmembrane domain and cytoplasmic tail, and is known to induce conformational changes in Env to some extent similar to those induced by CD4 expressed on target cells. Soluble CD4 induces formation of the bridging sheet and the co-receptor binding site, but certain gp120 epitopes, including potent ADCC targets in the C1-C2 region (A32-like epitopes), remain occluded in soluble CD4-triggered Env trimmers (34, 35). These epitopes become exposed on virions only upon the interaction of Env trimers with host CD4, indicating that binding membrane-anchored CD4 provides an additional energy component that is not provided by soluble CD4 (35). Rationally designed CD4mc (JP-III-48, DMJ-I-228) engage gp120 within the Phe43 cavity (22) and can act as CD4 agonists, inducing thermodynamic changes in the Env trimer more similar to those observed upon membrane CD4 binding (20, 24). Importantly, compounds of this class have been shown to sensitize HIV-1 particles to neutralization by CD4i and V3 non-neutralizing vaccine-elicited Abs (25). Figure 1

demonstrates that Env present at the surface of cells infected with a wild-type (wt) virus is barely recognized by HIV-1+ sera. This is due to efficient CD4 downregulation by the virus; Env cannot engage with CD4 and therefore remains in the unbound conformation, preventing CD4i epitope exposure (11, 12, 16). CD4mc (JP-III-48, DMJ-I-228) and sCD4 promote the exposure of Env CD4i epitopes, resulting in enhanced recognition of Env at the surface of HIV-1-infected cells by HIV-1+ sera. As expected, when the ability of the virus to downregulate CD4 is impaired by deleting *nef* (nef- or nef-vpu-), CD4mc do not enhance Env recognition by HIV-1+ sera. In the absence of Nef, CD4 accumulates at the cell surface and interacts with Env; thus, in this case, CD4 blocks access to the Phe43 cavity (11, 12), effectively competing for Env interaction. Cells infected with a wt virus express little Env at the cell-surface due to the BST-2-counteracting effect of Vpu (11, 12), explaining why the enhancement by CD4mc is small. Deletion of *vpu* results in enhanced Env expression at the cell surface, likely resulting from prevention of viral release by BST-2 (11-14) (Supplemental Figure 1); in this context, CD4mc can engage more Env at the cell surface, thus resulting in a more pronounced effect on Env recognition by HIV-1+ sera. Under these conditions, infected cells treated with CD4-mimetic compounds reach the same level of recognition as cells infected with a nef$^-$vpu$^-$ virus (Figure 1*C-D*). Similar results were observed with M48U1, a miniprotein CD4 mimic that also engages the gp120 Phe 43 cavity with nanomolar affinity (28) (Supplemental Figure 2). Interestingly, CD4mc JP-III-48 enhanced recognition of an Env variant (D368R) unable to efficiently engage CD4. Thus, Env adoption of the CD4-bound conformation but not CD4 interaction *per se* appears to be required to expose CD4i epitopes at the cell surface (Supplemental Figure 3).

Importantly, CD4mc enhancement of Env recognition by sera from several HIV-1-infected individuals (Figure 1*D-F*) is translated into higher ADCC killing of infected cells by effector PBMCs (Supplemental Figure 4). It is worth noting that the effect of CD4mc on Env detection and sensitization to ADCC is also observed when primary CD4 T cells from healthy individuals, rather than CEM-NKr cells, are used as targets cells (Figure 2). Interestingly, we observed that the effect of CD4mc was more pronounced when primary CD4 T cells were used. This could be due to enhanced levels of Env presented at the surface of these cells compared to CEM-NKr (Supplemental Figure 5).

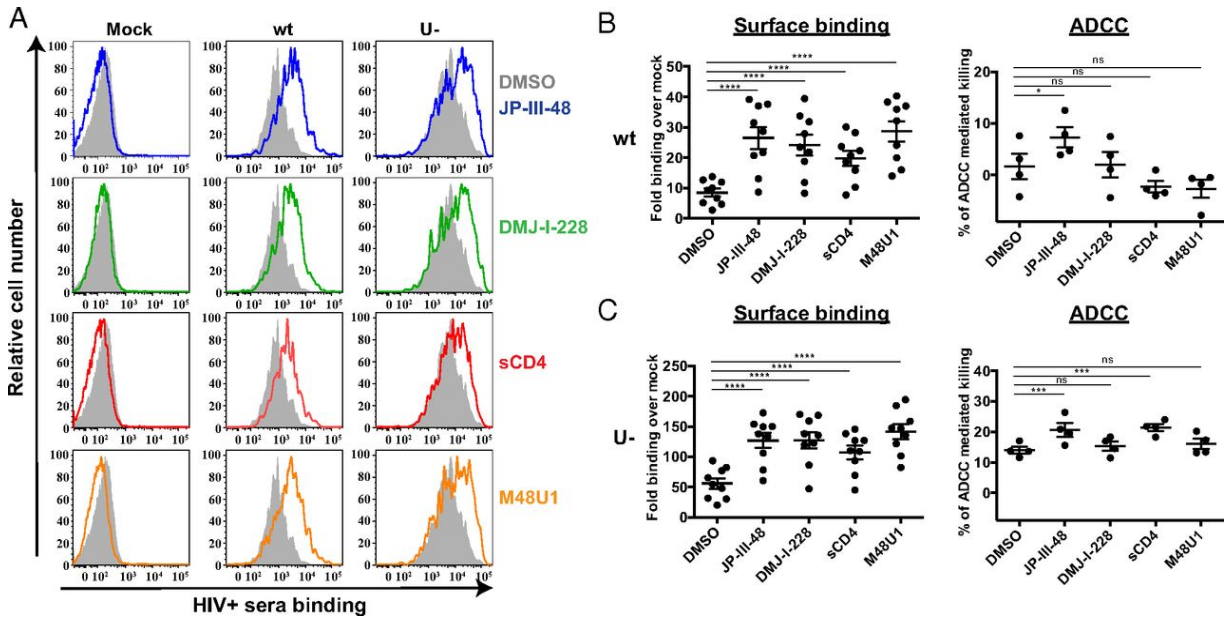

Figure 4.2 **(Article 7 Figure 2) CD4-mimetics enhance recognition and ADCC-mediated killing of HIV-1-infected primary CD4 T cells.**
Staining of primary CD4 T cells infected or not with NL4.3 GFP ADA-based viruses either wild-type (wt) or lacking Vpu (U-) with sera from nine HIV-1-infected individuals, in presence of CD4-mimetics or the vehicle (DMSO). Shown in (*A*) are histograms depicting representative staining of infected (GFP+) or mock infected cells with HIV-1+ sera. Quantification of cell-surface staining of wt (*B*) or U- (*C*) –infected cells, as fold binding over mock, are presented in left panels and the ability of the sera to mediate ADCC-mediated killing of infected primary CD4-T cells in presence of CD4-mimetics or the vehicle (DMSO) using autologous PBMCs is presented in the right panels. Error bars indicate mean +/- SEM. Statistical significance was tested using a paired one-way ANOVAs test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

CD4-mimetics enhance recognition and killing of cells infected with primary HIV-1 strains

To ensure that sensitization of HIV-1-infected cells by CD4-mimetics was also observed when using full-length clinically relevant primary HIV-1 isolates, we infected primary CD4 T cells with extensively characterized infectious molecular clones (IMCs) constructed from two transmitted/founder (T/F) and their corresponding 6-month (6-mo) consensus sequences (36-39). Primary viruses are known to exhibit low Env reactivity and as such have little or no intrinsic exposure of CD4i epitopes (40). JP-III-48 and DMJ-I-228 CD4-mimetics were able to significantly enhance recognition of cells infected with the four primary viruses by HIV-1+

sera (Figure 3*A*); cells infected with T/F and 6-mo IMCs from CH58 exhibited a greater enhancement of recognition when compared to the T/F and 6-mo IMCs from CH77 (Figure 3). This is likely related to the levels of Env present at the surface of infected cells; cells infected with CH58 T/F or 6-mo viruses expressed higher surface Env levels than cells infected with CH77 T/F or 6-mo strains (Supplemental Figure 6). Altogether, these results indicate that Env conformational changes induced by CD4mc are not just a function of TF viruses, but are also maintained during chronic infection.

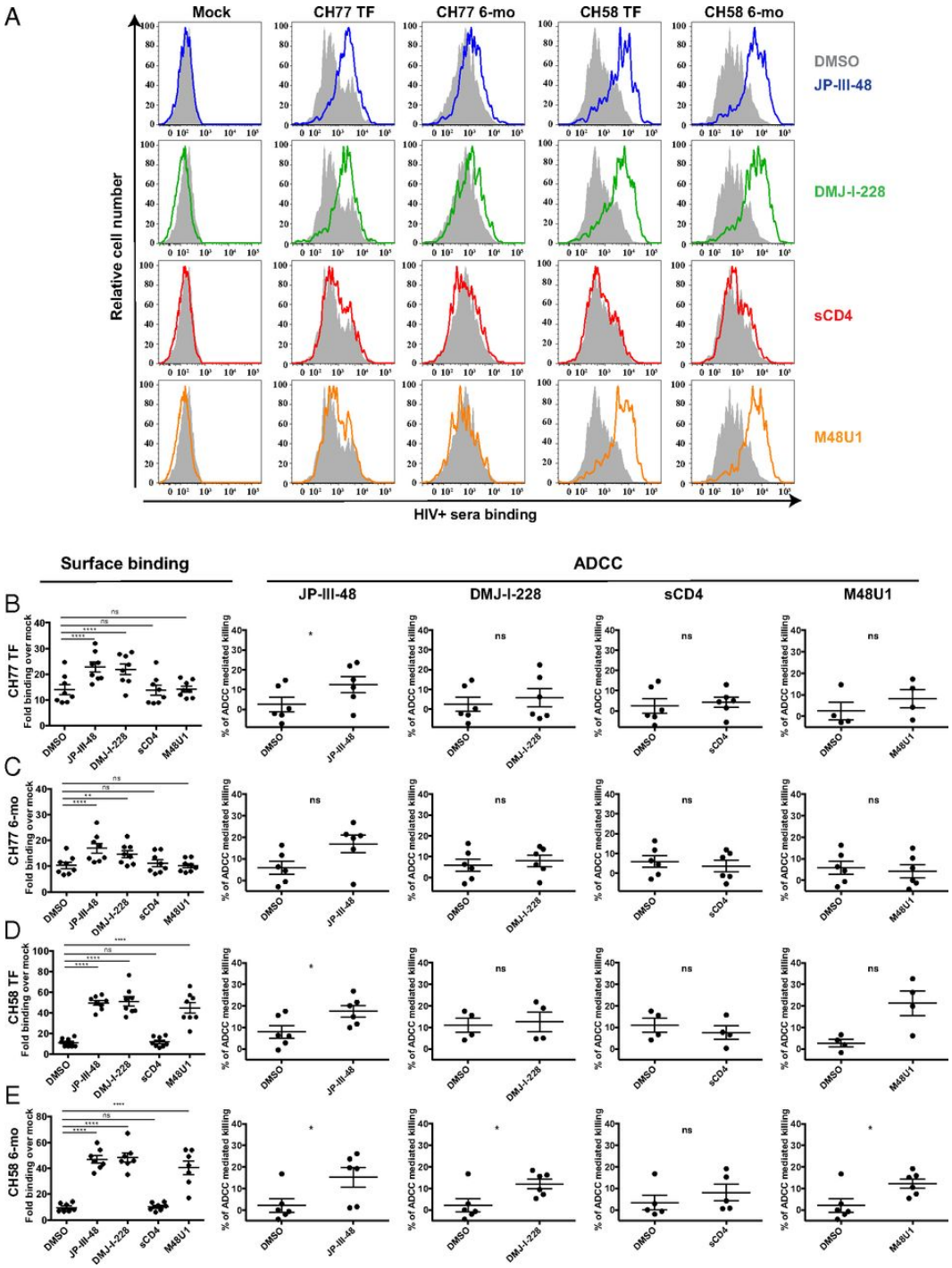

Figure 4.3 **(Article 7 Figure 3) CD4-mimetics sensitize primary CD4 T cells infected with HIV-1 primary isolates to ADCC.**
Staining of primary CD4 T cells infected or not with infectious molecular clones (IMCs) representing transmitted/founder (T/F) and corresponding 6-month (6-mo) consensus viruses from two longitudinally followed patients CH77 and CH58 with sera from eight HIV-1-infected individual in the presence

of CD4-mimetics or vehicle (DMSO). Shown in (*A*) are histograms depicting representative staining of infected (p24+) or mock-infected cells. Quantification of cell-surface staining of CH77 T/F (*B*), CH77 6-mo (*C*), CH58 T/F (*D*) and CH58 6-mo (*E*) -infected cells, as fold binding over mock, are presented in left panels and the ability of the sera to mediate ADCC-mediated killing of infected primary CD4-T cells in presence of CD4-mimetics or the vehicle (DMSO) using autologous PBMCs is presented in the right panels. Error bars indicate mean +/- SEM. Statistical significance was tested using a paired one-way ANOVAs test for the left panels and a paired t-test for the right panels (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

We found that JP-III-48 binds monomeric gp120 from the YU2 strain of HIV-1 with higher affinity than DMJ-I-228 (Supplemental Table 1). As a result, JP-III-48 exhibits much more potent inhibitory activity against two HIV-1 strains (Supplemental Table 1); this suggests that the ability of JP-III-48 to bind and/or induce conformational changes in the functional HIV-1 Env trimer is superior to that of DMJ-I-228. Accordingly, JP-III-48 was more effective at stimulating ADCC than DMJ-I-228 (Figure 3 and Supplemental Table 1). sCD4 did not enhance recognition or killing of infected cells. The effect of M48U1 was less than that observed with CD4mc for CH77 T/F or 6-mo strains. The quaternary architecture of some primary Envs might pose constraints against proteins engaging the Phe43 cavity; due to their smaller size (300-400 Da), CD4mc could bypass such constraints.

The CD4mc JP-III-48 sensitizes HIV-1-infected cells to ADCC mediated by antibodies present in cervico-vaginal fluids, breast milk and early-infection sera

Figures 1 to 3 indicate that CD4mc can sensitize HIV-1-infected cells to ADCC mediated by sera from chronically HIV-1-infected individuals. Previous studies (17) have suggested that some Abs against CD4i Env epitopes are elicited early during the course of HIV-1 infection. Therefore, we evaluated whether anti-Env Abs elicited during the first few weeks of infection could eliminate HIV-1-infected cells in the presence of CD4mc. Since we consistently observed enhanced sensitization of HIV-1-infected cells to ADCC by JP-III-48 compared to the other CD4-mimetics tested in this study (Figures 2 and 3), we selected JP-III-48 for the next set of experiments. We obtained sera from six early HIV-1 converters (within 4 weeks of the estimated date of infection) from the FRQS-SIDA Montreal Primary HIV-1-infection cohort and assessed their ability to mediate ADCC of primary CD4 T cells infected

with T/F CH58. Sera from early converters did not recognize Env at the surface of infected cells nor did they mediate ADCC in the absence of CD4-mimetics. However, JP-III-48 addition allowed Env recognition and significantly enhanced killing of infected cells by these sera (Figure 4*A*). These observations suggest that administration of CD4mc during the early phase of HIV-1 infection might benefit infected individuals by contributing to the elimination of infected cells. Antibodies able to mediate ADCC have also been reported in cervico-vaginal lavages (CVLs) (41) (18, 42) as well as in breast milk (7, 43) from HIV-1-infected women. CVLs from HIV-1-infected Beninese commercial sex workers (CSWs) and breast milk (BM) from HIV-1-infected Malawian women (43) were evaluated for their ability to mediate ADCC of primary infected CD4 T cells. As observed for sera from early seroconverters, in the absence of CD4-mimetics, CVLs and BM samples were unable to detect Env or mediate ADCC of HIV-1-infected primary CD4 T cells. However, addition of the CD4mc JP-III-48 significantly enhanced Env detection and killing of these cells (Figure 4*B-C*). This is in agreement with a previous report that found that HIV-1-Env specific IgG in genital mucosal compartments preferentially targets Env in the CD4-bound conformation and can mediate ADCC (18). Sera, CVL and breast milk samples from uninfected individuals failed to recognize Env in the presence or absence of the CD4mc JP-III-48 (Figure 4, left panels).

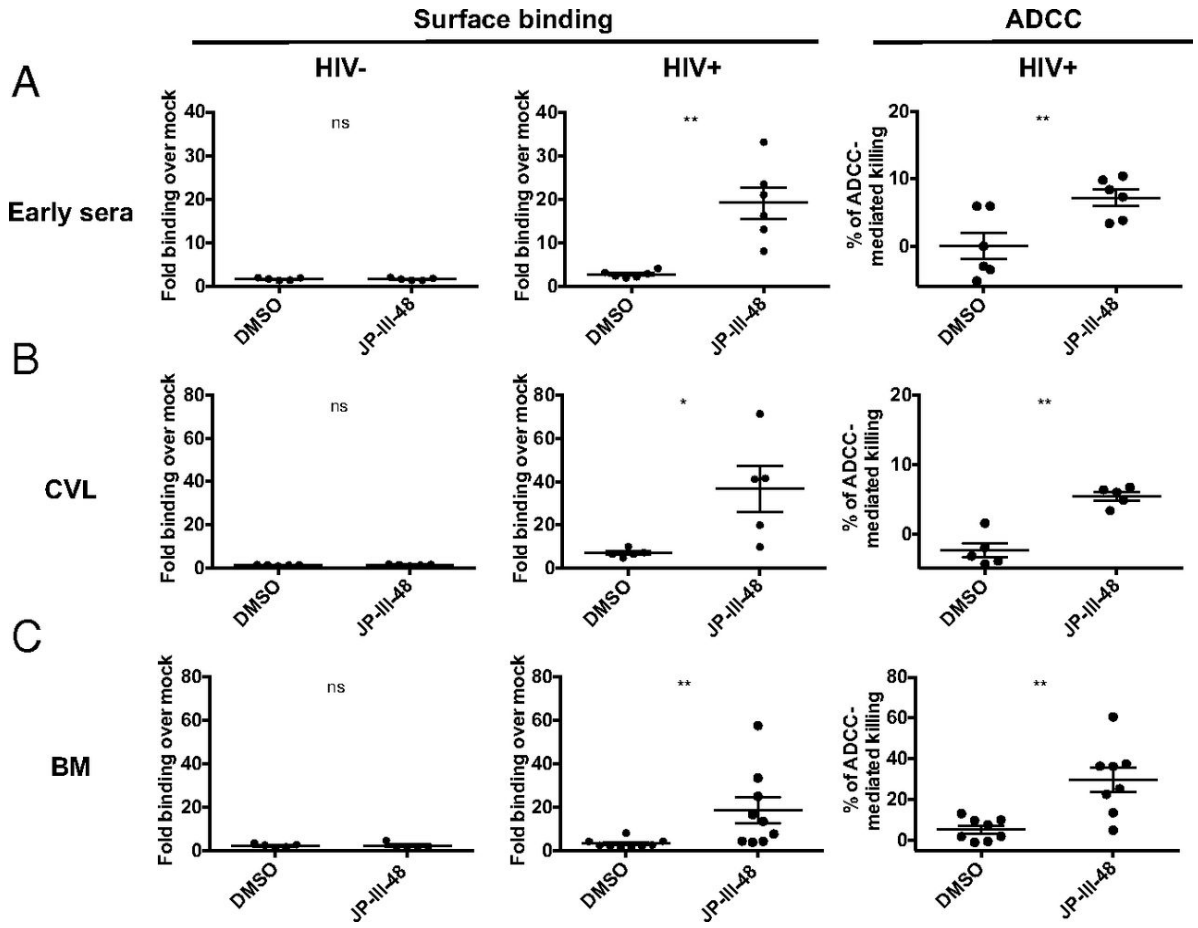

Figure 4.4 **(Article 7 Figure 4) The CD4mc JP-III-48 sensitizes primary CD4 T cells infected with primary HIV-1 isolates to ADCC mediated by sera from early-converters, cervico-vaginal lavages and breast milk from HIV-1-infected individuals.**

Staining of primary CD4 T cells infected or not with transmitted/founder CH58 virus (CH58 T/F) with samples obtained from uninfected (HIV negative) donors (first column) or from HIV-1-infected individuals (second and third columns). Sera from early converters (*A*), cervico-vaginal lavages (*B*) or breast milk (*C*) were used at 1/1000 (sera) and 1/100 (CVL and BM) dilutions to stain infected cells (first and second columns) or to mediate ADCC by autologous PBMCs in presence of vehicle alone or JP-III-48 CD4-mimetic (third column). Error bars indicate mean +/- SEM. Statistical significance was tested using a paired t-test or a Wilcoxon matched-pairs signed rank test for breast milk samples (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

A CD4mc sensitizes ex-vivo-amplified primary CD4 T cells from viremic untreated HIV-1-infected individuals to ADCC-mediated killing

To evaluate the potential of a CD4mc to sensitize endogenously-infected cells, we purified primary CD4 T cells from four viremic untreated HIV-1-infected individuals. Cells were activated with PHA for 36 h and then cultured in the presence of rIL-2. Six days after activation, viral replication was measured by intracellular p24 staining and Env detected at the surface of p24+ cells with autologous and heterologous HIV-1+ sera (Figure 5*A*-*D*). Significantly, in the four tested individuals, the CD4mc JP-III-48 enhanced Env detection by both autologous and heterologous sera; while activated primary CD4 T cells from uninfected subjects were not recognized in absence or presence of the CD4mc (Supplemental Figure 7). Importantly, enhanced recognition of infected cells by HIV+ sera resulted into a significant increase in ADCC killing mediated by autologous effector PBMCs (Figure 5*E*-*F*). These results highlight the potential of CD4mc to sensitize HIV-1-infected cells to ADCC in viremic HIV-1-infected individuals.

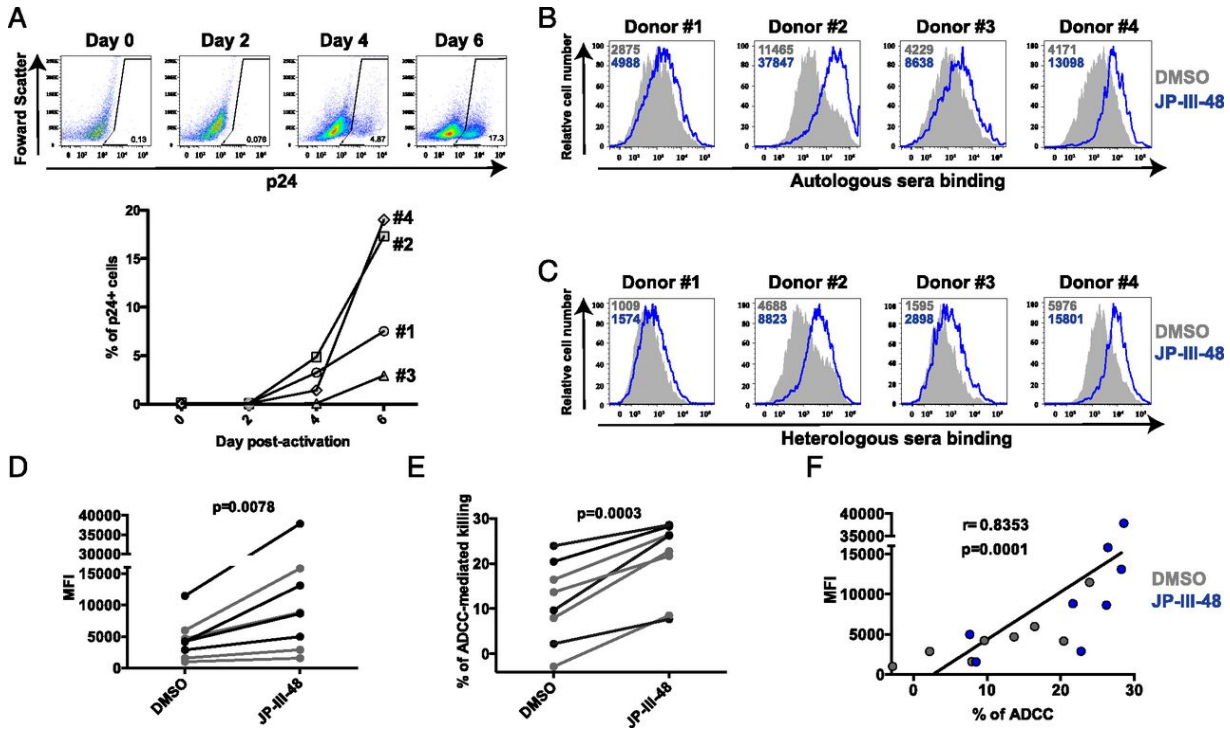

Figure 4.5 **(Article 7 Figure 5) The CD4mc JP-III-48 sensitizes *ex-vivo*-expanded endogenously infected primary CD4 T cells from viremic ART-naïve individuals to ADCC-mediated killing by autologous sera.**

(*A*) The top panel show percentages of p24+ CD4 T cells expanded after activation from a representative HIV-1-infected untreated viremic patient, while the bottom panel represents the percentages of p24+ CD4 T cells expanded after activation for four HIV-1-infected viremic patients (Supplemental Table 2). Six days post-activation, cells were stained with (*B*) autologous sera or (*C*) heterologous HIV+ sera in presence of the CD4mc JP-III-48 or the vehicle (DMSO). The data shown in (*B*-*C*) are histograms depicting staining of p24+ cells and are presented as mean fluorescence intensity (MFI). (*D*) Data shown represent paired values MFI obtained with autologous sera (depicted in black) and heterologous HIV+ sera (depicted in gray) in presence of vehicle alone (DMSO) or the CD4mc JP-III-48. (*E*) Six days post-activation, cells were used as target cells to evaluate their susceptibility to ADCC by autologous PBMCs in the presence of the CD4mc JP-III-48 or the vehicle (DMSO) by autologous sera (depicted in black) or heterologous HIV+ sera (depicted in gray). (*F*) A positive correlation was observed between the staining intensity of autologous and heterologous HIV+ sera on CD4 T cells and their ability to mediate ADCC. Statistical significance was tested using (*D*) the Wilcoxon matched-pairs signed rank test, (*E*) a paired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$) and (*F*) by a Spearman correlation.

Discussion

The HIV-1 accessory proteins Nef and Vpu help to protect HIV-1-infected cells from ADCC by decreasing the overall amount of Env at the cell-surface (11-14) and by down-regulating CD4 (11, 12), which otherwise engages Env, induces the CD4-bound conformation and exposes CD4i epitopes. These epitopes are readily recognized by several well-established ADCC-mediating Abs isolated from chronically-infected individuals or RV144 vaccinees (12), or by sera from HIV-1-infected individuals, which are known to have a large fraction of non-neutralizing CD4i Abs (11, 17). However, the vast majority of new HIV-1 infections worldwide are mediated by viruses coding for functional Nef and Vpu proteins and expressing Envs with low intrinsic reactivity (40); these factors limit the exposure of CD4i epitopes that would otherwise be recognized by CD4i Abs with the potential to mediate ADCC (11, 12, 16). It is becoming increasingly clear that HIV-1 has evolved multiple means to avoid exposure of Env CD4i epitopes at the surface of infected cells (11, 12). Therefore, strategies aimed at exposing these epitopes by either targeting Vpu and/or Nef or by engaging Env to induce the exposure of these epitopes might sensitize HIV-1-infected cells to ADCC. Recent advances in the development of a series of CD4mimetics that engage Env in a CD4-like manner and that induce similar conformational changes resulting in the exposure of CD4i epitopes permitted us to test this strategy. We observed that CD4mc can indeed expose CD4i epitopes at the surface of infected primary CD4 T cells and effectively sensitize them to ADCC killing mediated by CVLs, breast milk and sera from early and chronic HIV-1-infected individuals. This was observed with a variety of HIV-1 variants, notably including primary transmitted/founder viruses and their 6 month-counterparts. CD4mc were more efficient than protein-based CD4-mimetics (sCD4, M48-U1) at sensitizing cells infected with primary HIV-1-isolates to ADCC. The quaternary architecture of primary Envs may resist engagement with proteins such as sCD4 and M48-U1; the smaller size of the CD4mc might allow them to bypass such structural constraints. The resistance of most primary HIV-1 Envs to sCD4 engagement may explain why sCD4 exhibited only minimal *in vivo* potential as an antiviral agent (44). CD4mc have been shown to sensitize HIV-1 virions to neutralization by otherwise non-neutralizing CD4i Abs that can easily be generated through vaccination with CD4-bound stabilized gp120s (25, 45, 46). Importantly, these types of Abs were isolated from RV144 vaccinees and possessed

ADCC potential (10, 12). Therefore, one practical use of the observed ADCC and neutralization sensitization might be the enhancement of vaccine efficacy by CD4mc. A frustrating aspect of HIV-1 vaccine development is the difficulty of eliciting antibodies that potently neutralize diverse strains of virus (47-49). Therefore, the dual effect of these CD4mc (i.e., sensitization of HIV-1 viral particles to neutralization and infected cells to ADCC by CD4i antibodies) might help limit viral transmission. Such CD4i antibodies are elicited early and in a high proportion of infected individuals (11, 17) and can also be elicited by vaccination with "stabilized gp120 cores" that have been engineered to assume the CD4-bound state (45). One can envision a multicomponent vaccine regimen in which one of the immunogens is a stabilized gp120 core that elicits CD4i Abs and CD4mc administered orally or in a microbicide formulation that could sensitize a range of transmitted/founder viruses and infected cells to inhibition by the vaccine-elicited antibodies. Finally, our observation that endogenously-infected, *ex-vivo* amplified CD4 T cells can be sensitized to ADCC mediated by autologous sera suggest that CD4mcs could be useful for the "kill" part of the "shock and kill" strategy being pursued to purge the HIV reservoir (50). Moreover, by targeting both viral particles and infected cells, CD4 mimetics might represent an alternative and/or complementary approach to currently-available drugs for preventing viral transmission and treating HIV-1-infected individuals.

In summary, we report that CD4mc induce the CD4-bound conformation of Env, exposing CD4i epitopes recognized by easy-to-elicit Abs present in sera, CVLs and breast milk from HIV-1-infected individuals. CD4mc effectively sensitize HIV-1-infected cells to ADCC and thus, might have therapeutic utility for preventing transmission and decreasing the number of infected cells in HIV-1-infected individuals.

Materials and Methods

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 (12, 51). CD4 T lymphocytes were purified from rested PBMCs by negative selection and activated as previously described (11).

Plasmids and site-directed mutagenesis

pNL43-ADA(Env)-GFP.IRES.Nef proviral vectors (deleted for *vpu, nef, nef and vpu,* or expressing the D368R Env variant) and the VSVG-encoding plasmid (pSVCMV-IN-VSV-G) were previously described (12, 51). Transmitted founder (T/F) and corresponding 6-month consensus IMCs of patients CH58 and CH77 were inferred, constructed and biologically characterized as described (36-39).

Viral production, infections and ex vivo amplification.

Vesicular stomatitis virus G (VSVG)-pseudotyped NL4.3 GFP-encoding ADA-based viruses were produced and titrated as previously described (12). Viruses were then used to infect approximately 20 % to 30 % of 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 untreated HIV-1-infected individuals. Purified CD4+ T cells were activated with PHA-L at 10 µg /ml for 36 hours and then cultured for 6 days in RPMI-1640 complete medium supplemented with rIL-2 (100U/ml).

CD4-mimetics

Soluble CD4 and the mini-protein M48U1 were produced and purified as previously described (26, 52). The CD4-mimetic small molecules JP-III-48 and DMJ-I-228 were synthesized as described previously (20, 21). The compounds were analyzed, 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 µM in PBS for cell-surface staining or in RPMI-1640 complete medium for ADCC assays.

Flow cytometry analysis of cell-surface staining and ADCC responses

Cell-surface staining was performed as previously described (11,12). Binding of HIV-1 infected cells by sera (1:1000 dilution), breast milk (1:100 dilution) or concentrated CVLs (1:100 dilution) were performed 48-72 after *in vitro* infection or 6 days post-activation for endogenously-infected *ex-vivo*-amplified cells, at 37 °C in presence of the CD4-mimetics JP-III-48 (50 μM), sCD4 (10 μg/ml), M48U1 (100 nM) or with equivalent volume of vehicle (DMSO). Cells infected with HIV-1 primary isolates or for *ex vivo* amplification, cells were stained intracellularly for HIV-1 p24 using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) and then fluorescent anti-p24 mAb (PE-anti-p24, clone KC57; Beckman Coulter/Immunotech, Hialeah, FL) (1:100 final concentration). The percentage of infected cells (p24+ or GFP+ cells) was determined by gating the living cell population based on the viability dye staining. Samples were analyzed on an 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 (12, 15) after 48-72h for *in vitro* infection or 6 day post-activation for endogenously-infected *ex-vivo*-amplified cells using a 1:1000 final concentration of serum, 1:100 dilution of concentrated CVLs or breast milk samples. 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), as described (12, 15). For cells infected with HIV-1 primary isolates or for *ex vivo* amplification, infected cells were identified by intracellular staining for HIV-1 p24. In that context, the percentage of cytotoxicity was calculated with the following formula: (% of $p24^+$ cells in Targets plus Effectors) - (% of $p24^+$ cells in Targets plus Effectors plus serum) / (% of $p24^+$ cells in Targets) by gating infected lived target cells.

Statistical analyses

Statistics were analyzed using GraphPad Prism version 6.01 (GraphPad ,San Diego, CA, USA). P values <0.05 were considered significant; significance values are indicated as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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Supplemental Material and Methods

Antibodies

The following antibodies were used as first antibody for cell-surface staining: 1 µg/ml of mouse anti-CD4 mAb OKT4 (14-0048-82, eBiosciences, San Diego, CA, USA), 2 µg/ml of rabbit anti-BST-2 Ab (sc-99191, Santa Cruz), 1 µg/ml of human anti-HIV-1 Env mAbs 2G12 ,(AB002, Polymun), PGT-151 (kindly provided by Dr. P. Poignard) and A32 (kindly provided by Dr. J. Robinson), while 1 µg/ml of goat anti-mouse or anti-rabbit Alexa Fluor-594 (Invitrogen, San Diego, CA, USA) and anti-human Alexa Fluor-647 mAbs (Invitrogen, San Diego, CA, USA) were used as secondary Abs, and AquaVivid (Invitrogen, San Diego, CA, USA) as viability dye.

Sera, breast milk and cervico-vaginal lavages from HIV-infected individuals

Written informed consent was obtained from all study participants (the Montreal Primary HIV Infection Cohort (1, 2) and the Canadian Cohort of HIV Infected Slow Progressors (3-5)), research adhered to the ethical guidelines of CRCHUM and was reviewed and approved by CRCHUM institutional review board (ethics committee). Sera was collected during Ficoll isolation of PBMCs and conserved at -80 °C.

Cervico-vaginal lavages (CVLs) from female commercial sex workers (CSWs) were enrolled through a sex worker clinic in Cotonou, Benin. CSWs were untreated HIV-1 positive with less than 3 years of prostitution. This study was approved by the Ministère de la Santé du Bénin and by the CRCHUM research ethics boards. Written consent was obtained from every participant. Women who were less than 18 years of age, pregnant, menstruating during the visit, or taking oral contraceptives were excluded from the study. At enrollment, participants provided demographic and sexual behavioral information. Participants also underwent a genital examination by a physician. None of these women had vaginosis, candidiasis, or other sexually-transmitted infections at the time of the visit. The specimen collection and processing was performed using methods described previously (6). Briefly, CVLs samples were obtained from all study participants by a physician using a 10-ml syringe filled with sterile phosphate-

buffered solution and aimed directly into the cervical os. CVL fluids were then collected, transferred immediately into 20 ml of RPMI-1640, kept on ice, and processed within 1 h. CVL samples were centrifuged at 1500 r.p.m. for 10 min to remove cells and debris, and supernatants were stored at - 80°C until shipped on dry ice to Montreal, Canada. CVL samples were concentrated with Amicon Ultra-15 5 kDa (Millipore, Billerica, MA) prior to use for binding or ADCC measurement.

Breast milk (BM) samples were obtained from chronically HIV-1 infected lactating women enrolled between 2008 and 2009 in the CHAVI009 study in Blantyre Malawi (7). The subjects were consented for enrollment and followed throughout late pregnancy and the period of breastfeeding. All infants and all untreated pregnant women received a single dose of nevirapine during the peripartum period. Discarded breast milk samples from consenting, uninfected postpartum women were collected via the lactation support program at Children's Hospital Boston. All samples were deidentified prior to receipt by end user laboratories.

All samples 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, San Diego, CA, USA) was used to randomly select a number of sera, breast milk or CVLs from each cohort

Supplemental Data

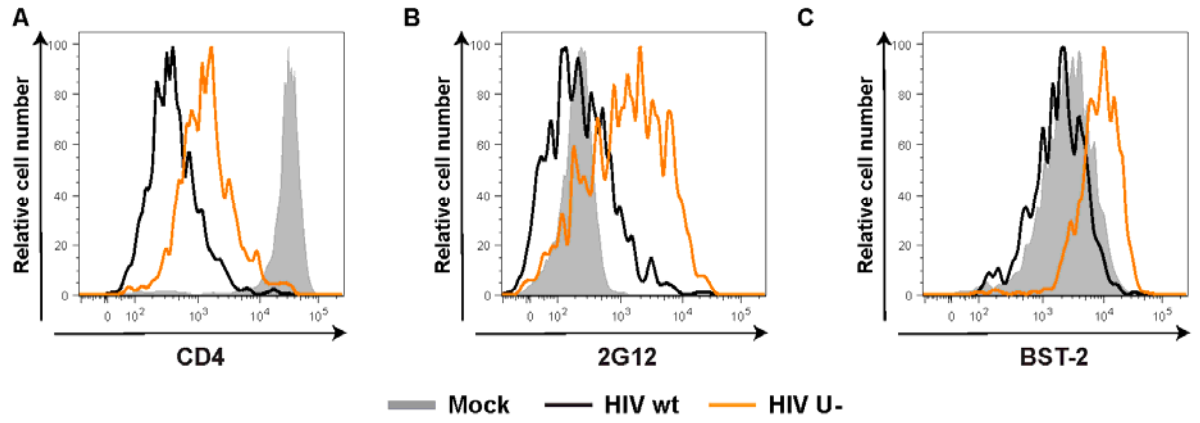

Figure 4.6S (Article 7 Figure S1) Cell-surface level of CD4, HIV-1 Env and BST-2 on HIV-1-infected primary CD4-T cells.
Primary CD4-T cells were mock infected or infected with NL4.3 GFP ADA viruses either wild-type (wt) or lacking Vpu (U-) and were stained 72 h post-infection for CD4, HIV-1-Env (2G12) or BST-2 and then fluorescently labeled with appropriate secondary Ab. Results shown are histograms depicting representative staining of infected (GFP+) or mock-infected cells.

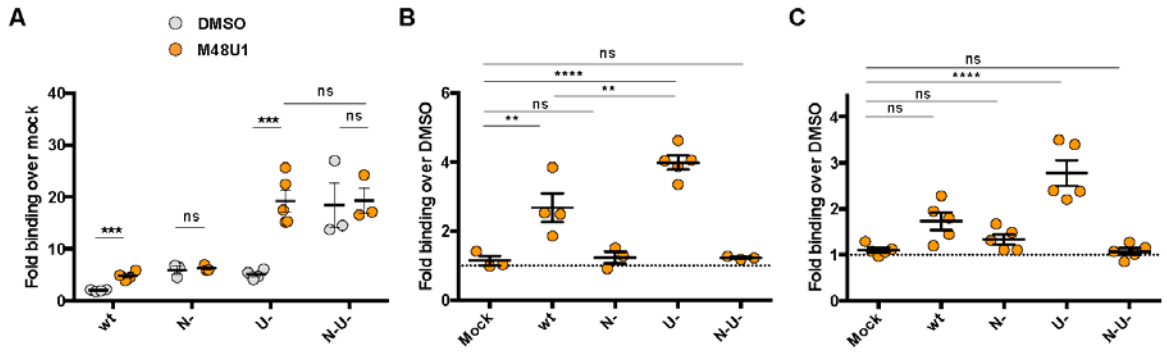

Figure 4.7S (Article 7 Figure S2) CD4-mimetic M48U1 enhances recognition of infected cells by HIV-1+ sera.

CEM.NKr cells infected with a panel NL4.3 GFP ADA viruses either wild-type (wt), lacking Nef (N-), Vpu (U-) or both Nef and Vpu (N-U-) were stained at 48 h post-infection with HIV+ sera (*A,B*) or (*C*) sera from 5 additional HIV-1-infected individuals in presence of M48U1 or the vehicle alone (DMSO). (*A*) Quantification as enhanced binding over mock or (*B-C*) as binding over the vehicle (DMSO) alone. Error bars indicate mean +/- SEM. Statistical significance was tested using (*A*) unpaired t-test (*B*) one-way ANOVAs or (*C*) paired one-way ANOVAs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

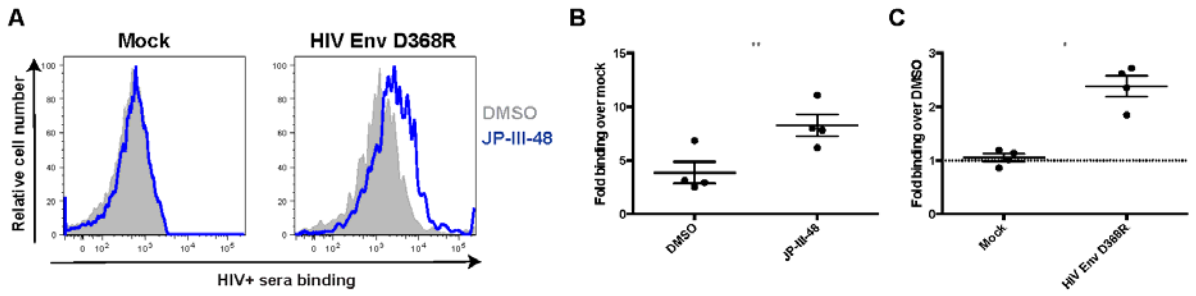

Figure 4.8S (Article 7 Figure S3) CD4-mimetic JP-III-48 enhances recognition of an HIV-1 envelope variant unable to efficiently engage with CD4.
Primary CD4-T cells were mock infected or infected with NL4.3 GFP ADA viruses expressing the D368R envelope (this CD4-binding site mutation is known to significantly reduce interaction with CD4) and were stained at 72 h post-infection with sera from four different HIV-1-infected individuals in presence of the CD4mc JP-III-48 or the vehicle alone (DMSO). Shown in (*A*) are histograms depicting representative staining of infected (GFP+) with one of the HIV+ sera. (*B*) Quantification as enhanced binding over mock or (*C*) as binding over the vehicle (DMSO) alone. Error bars indicate mean +/- SEM. Statistical significance was tested using a paired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

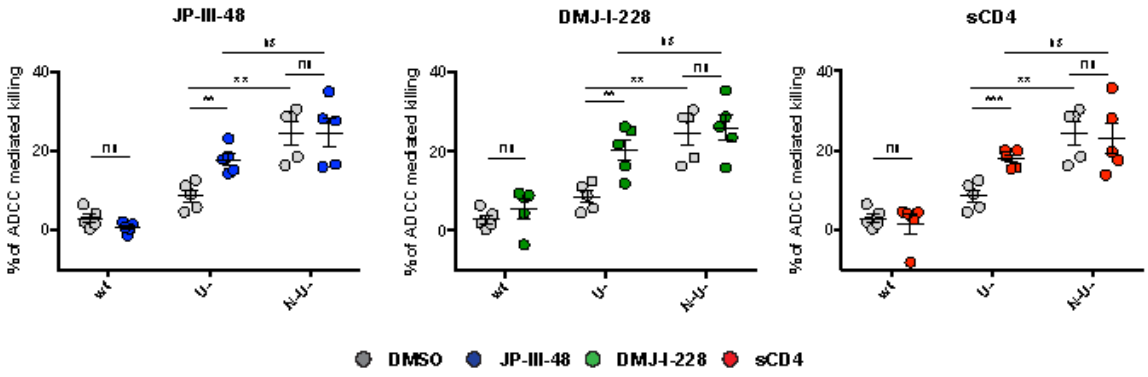

Figure 4.9S (Article 7 Figure S4) CD4-mimetics sensitize HIV-1-infected cells to ADCC killing mediated by HIV-1+ sera.
CEM.NKr cells infected with NL4.3 GFP ADA-based viruses, either wild-type (wt), lacking Vpu (U-) or both Nef and Vpu (N-U-), were used as target cells in our FACS-based ADCC assay in presence of CD4-mimetics or the vehicle (DMSO) using PBMCs from healthy donors. Error bars indicate mean +/- SEM. Statistical significance was tested using an unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

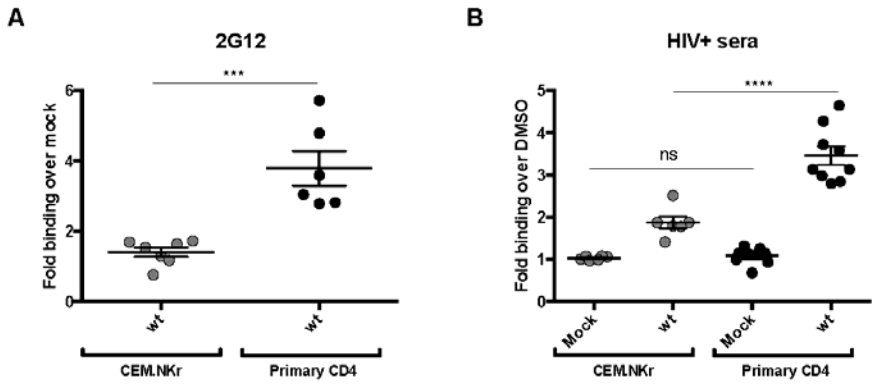

Figure 4.10S (Article 7 Figure S5) HIV-1 Env levels on the surface of primary CD4 T cells are higher than on CEM-NKr cells.

CEM.NKr cells or primary CD4+ T cells infected with NL4.3 GFP ADA wild-type (wt) were stained at 48-72 h post-infection with (*A)* anti-Env 2G12 or (*B)* with HIV+ sera in presence of the CD4-mimetic compound JP-III-48 or its vehicle alone (DMSO). Error bars indicate mean +/- SEM. Statistical significance was tested using an unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

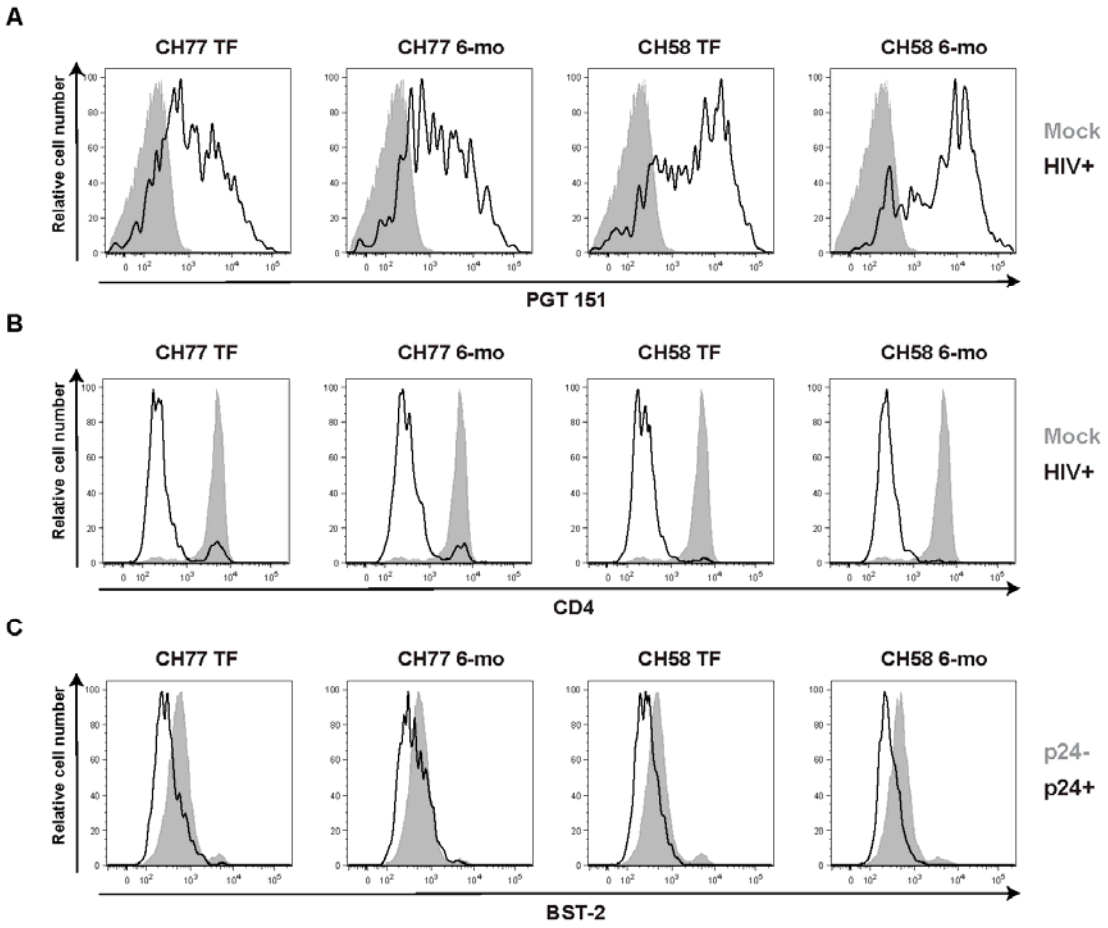

Figure 4.11S (Article 7 Figure S6) Cell-surface levels of CD4, HIV-1 Envelope and BST-2 on primary CD4+ T cells infected with HIV-1 primary isolates.
Primary CD4-T cells were mock infected or infected with transmitted/founder (T/F) viruses or their 6-month (6 Mo) counterpart. Cells were then stained at 72 h post-infection with (*A*) anti-Env PGT151 mAb (*B*), anti-CD4 mAbs or (*C*) anti-BST-2 mAbs and then fluorescently labeled with appropriate secondary Ab. All cells were stained intracellularly for HIV-1 p24 antigen. Results shown are histograms depicting representative staining of infected (p24+), uninfected bystander (p24-) or mock infected cells.

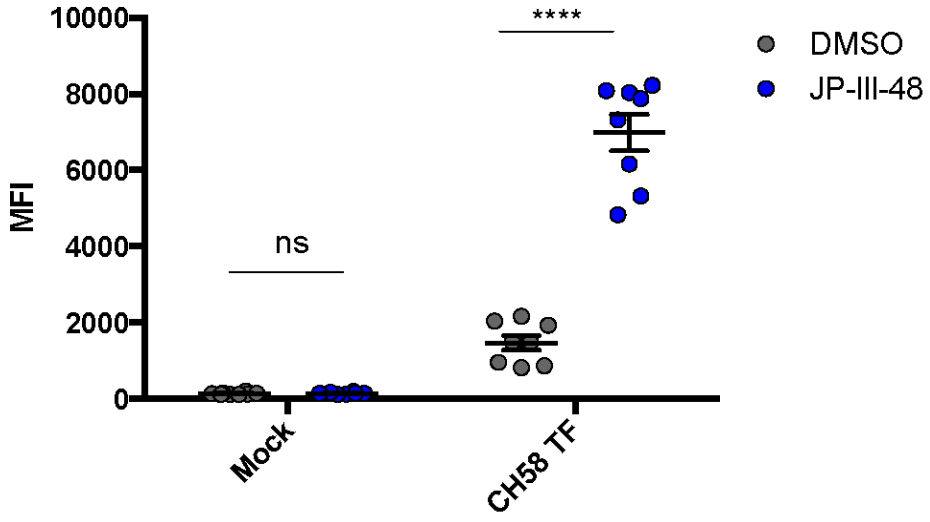

Figure 4.12S (Article 7 Figure S7) Small-CD4-mimetic JP-III-48 does not increase recognition of activated primary CD4 T cells from uninfected individuals by sera from HIV-1-infected individuals.

Primary CD4-T cells were mock-infected or infected with transmitted/founder CH58 virus (CH58 T/F). Cells were then stained at 72 h post-infection with sera from HIV-1-infected individuals and then fluorescently labeled with an Alexa-Fluor-647-conjugated anti-human IgG secondary Ab. Cells were stained intracellularly for HIV-1 p24 antigen. Data shown represent the Median Fluorescent intensity (MFI) of cells incubated with the vehicle alone or JP-III-48. Error bars indicate mean +/- SEM. Statistical significance was tested using paired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Table S1. Properties of small-molecule CD4mc

	K_d, nm*	Viral neutralization (IC_{50}), μM† YU2	JRFL	ADCC, %‡
DMJ-I-228	250	23	87	9.3
JP-III-48	24	0.9	21	15.5

*K_d affinity between CD4mc and YU2 gp120 was measured as previously reported (1).
†IC_{50} was calculated as previously reported (2).
‡Mean of the percentage of ADCC-mediated killing obtained in presence of CD4mc for HIV-1-infected cells presented in Fig. 3.

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2. Madani N, et al. (2014) CD4-mimetic small molecules sensitize human immunodeficiency virus to vaccine-elicited antibodies. *J Virol* 88(12):6542–6555.

Table S2. Demographic, virologic, and immunologic characterization of viremic untreated HIV-1-infected individuals used to expand endogenously-infected CD4 T cells presented in Fig. 5

	Viremic untreated HIV-1-infected individuals			
Characteristics	Donor 1	Donor 2	Donor 3	Donor
Time after infection, mo	4	88	18	123
Age, y	38	49	25	45
Sex	Male	Male	Male	Male
Race	Caucasian	Caucasian	Hispanic	Caucasian
CD4 count, cells/mm^3	320	281	371	329
Viral load, RNA copies/mL	132,886	44,848	9,871	19,067

SMALL CD4 MIMETICS PREVENT HIV-1 UNINFECTED BYSTANDER CD4+ T CELL KILLING MEDIATED BY ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

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Résumé

L'infection au VIH-1 occasionne une déplétion progressive des cellules T CD4+. Malgré son rôle important dans la pathogénèse du VIH-1, le mécanisme précis responsable de la déplétion des cellules T CD4+ demeure à ce jour méconnu. Ici, nous démontrons l'observation que la réponse ADCC médie la mort de cellules non-infectées CD4+ dans un système de culture cellulaire du VIH-1. Alors que les cellules infectées sont protégées de la réponse ADCC via les protéines accessoires virales Nef et Vpu, les cellules non-infectées lient la gp120 soluble relâchée par les cellules infectées et sont efficacement reconnues par des anticorps capables de médier la réponse ADCC. Ceci suggère que la relâche de gp120 par les cellules infectées représente un mécanisme viral permettant de rediriger la réponse

immunitaire contre les cellules non infectées. De plus, nous démontrons que des petites molécules mimant CD4 peuvent empêcher la reconnaissance des cellules non-infectées tout en sensibilisant les cellules infectées à la réponse ADCC.

Highlights

- Gp120 shed from productively-infected cells binds to bystander CD4+ T cells
- Gp120-coated bystander cells are highly sensitivity to ADCC responses mediated by CD4-induced antibodies
- Small-molecule CD4-mimetics redirect CD4-induced antibodies to HIV-1-infected cells

Research in context

The hallmark of human immunodeficiency virus type 1 (HIV-1) infection is the progressive depletion of CD4+T cells. Using cultures of HIV-1-infected cells, we observed that a part of the machinery that the virus uses to infect cells (gp120) binds to uninfected cells. Antibodies elicited during the course of the infection against the gp120 can recognize uninfected cells and redirect an immune response to them that results in their elimination. Importantly, this phenomenon can be blocked with a small CD4-mimetic compound that abrogates the binding of gp120 to uninfected cells and redirects the immune system to infected cells.

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.

Graphical Abstract

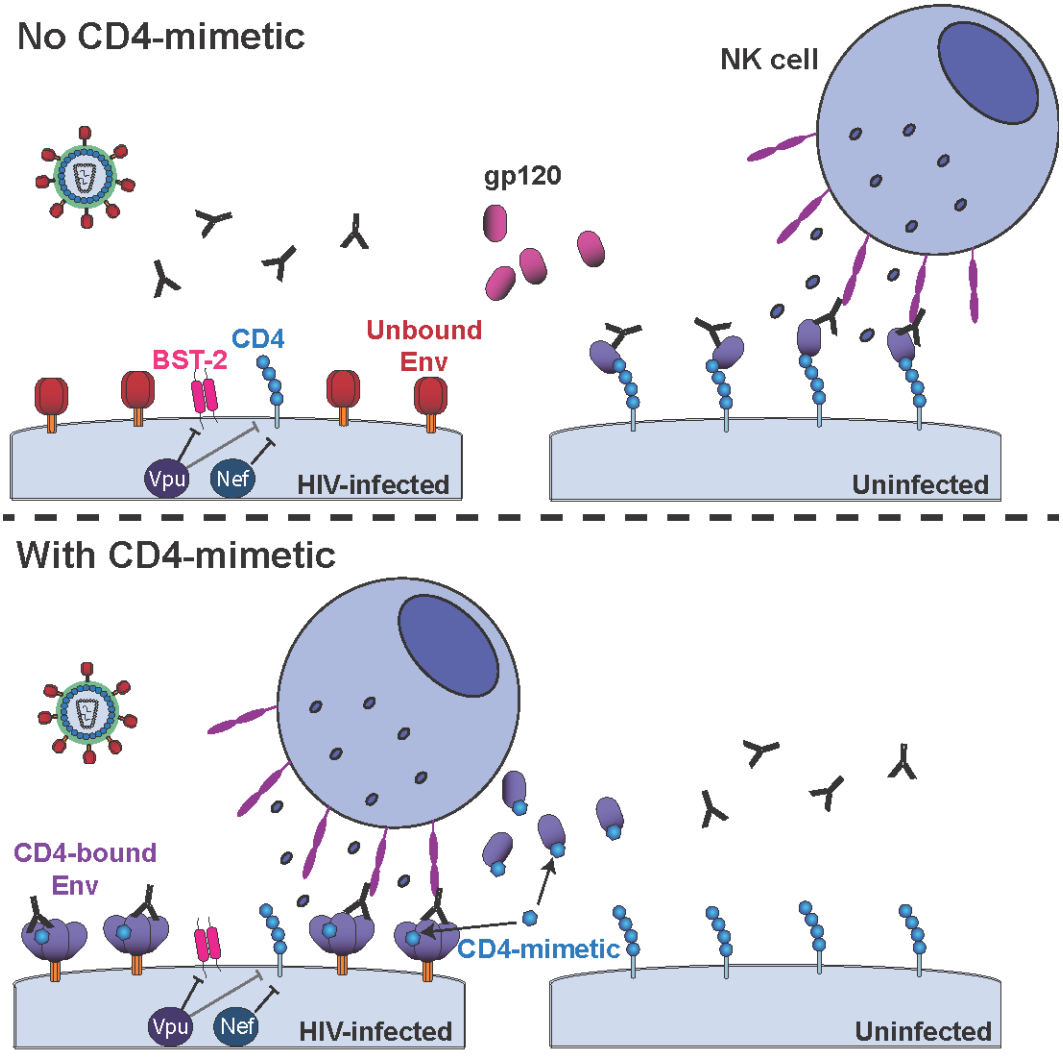


Figure 4.13 Résumé graphique représentant la reconnaissance et la lyse de cellules T CD4+ non-infectées.

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 an 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 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., 2015a, Veillette et al., 2014b) 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 elimination of HIV-1-infected cells. 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., 2015a, Veillette et al., 2014b, Veillette et al., 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.

MATERIAL AND METHODS

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).

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 hours 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).

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., 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). 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.

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).

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 (1x106 PBMC:1x105 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, Richard et al., 2015, Veillette et al., 2015a).

Co-culture assay

Activated primary CD4 T cells were stained with the eFluor-450 cell marker (1:500 dilution, eBiosciences) for 15 minutes 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 (Figure S1) (Richard et al.,

2015). Co-cultures were performed with or without transwells (3 □m 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.

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 * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Results

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., 2015a, Veillette et al., 2014b). 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 (Figure 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., 2015a, Veillette et al., 2014a). Interestingly, for the D368R variant, neither infected (GFP+) nor uninfected (GFP-) cells were recognized by A32 (Figure 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 (Figure 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, (Figure 1B), suggesting that bystander cell recognition by A32 depends on the amount of productive infection.

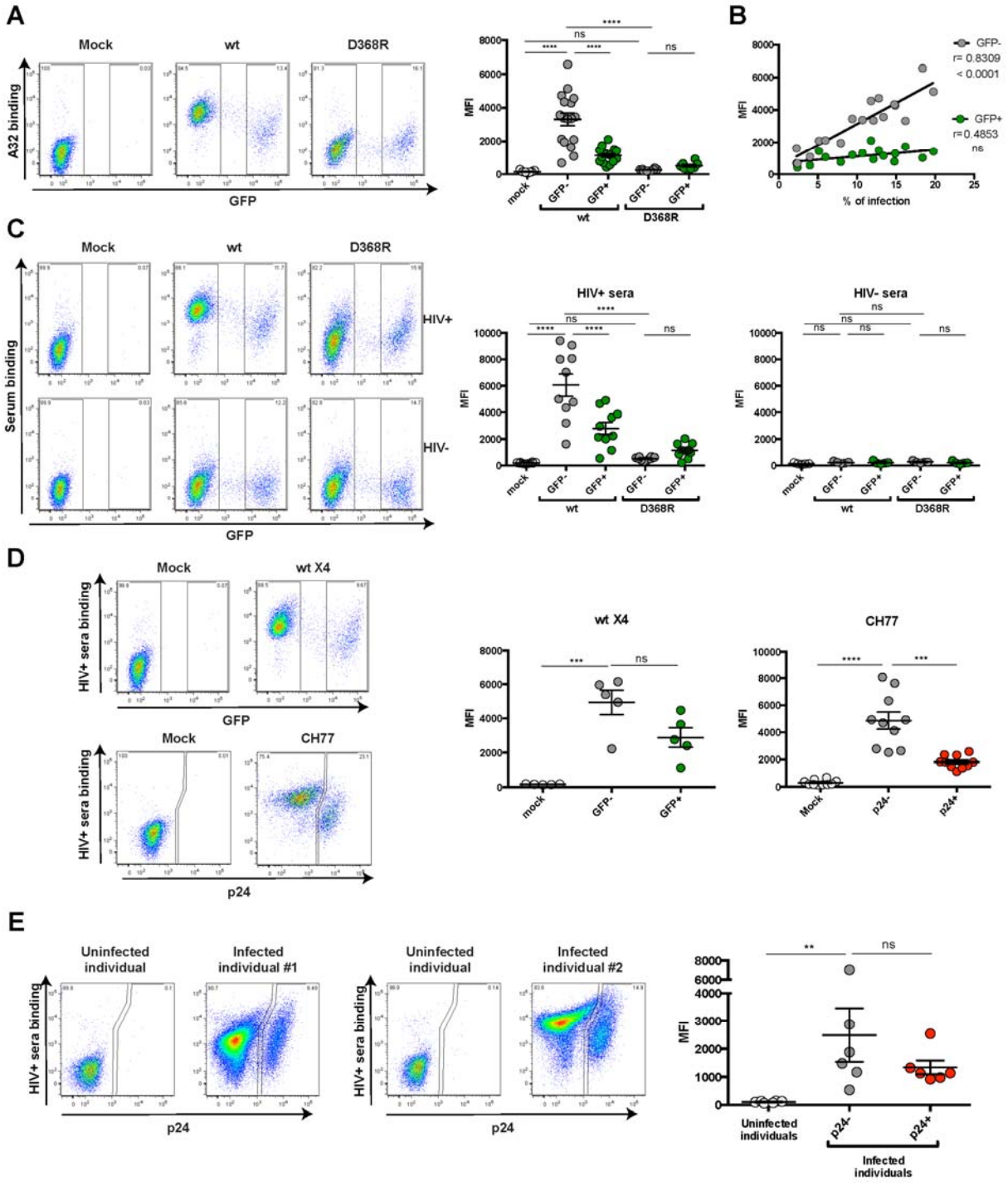


Figure 4.14 (Article 8 Figure 1) ADCC-mediating antibodies and sera from HIV-1-infected individuals efficiently recognize uninfected bystander CD4+ T cells.

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 grey) and infected (GFP+) CD4+ T cells (depicted in green) by the A32 mAb. (D) Cell-surface staining of primary CD4+ T cells infected with a 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 Intesnsity (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, (D) an unpaired *t* test, (E) a Mann-Whitney *t* test and (B) a Spearman rank correlation (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns: not significant).

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 (Figure 1C, upper panels). Sera from uninfected individuals did not recognize infected (GFP+) or uninfected (GFP-) cells (Figure 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 (Figure 1D and S3). In both cases, uninfected bystander cells were efficiently recognized by HIV+ sera (Figure 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 (Figure S4). Under these conditions, recognition of uninfected (p24-) cells isolated from HIV+ donors was significantly higher than cells from an uninfected individual (Figure 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 (Figure S5). Thus, independently of the viral strain or tropism, uninfected CD4 T cells present in an HIV-1 culture are efficiently recognized by ADCC-mediating Abs.

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 Figure 2, CD4i Abs A32 and C11 (an antibody that binds to the gp120 β-sandwich and N- and C-termini) (Robinson JE, 1992, Moore et al., 1994) recognized bystander (GFP-) significantly better than infected (GFP+) cells whereas the CD4-independent 2G12 antibody recognized both cell populations. 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 (Figure 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 (Figure 2 and S6). These results suggest that the majority of Env detected on bystander cells originates from shed gp120.

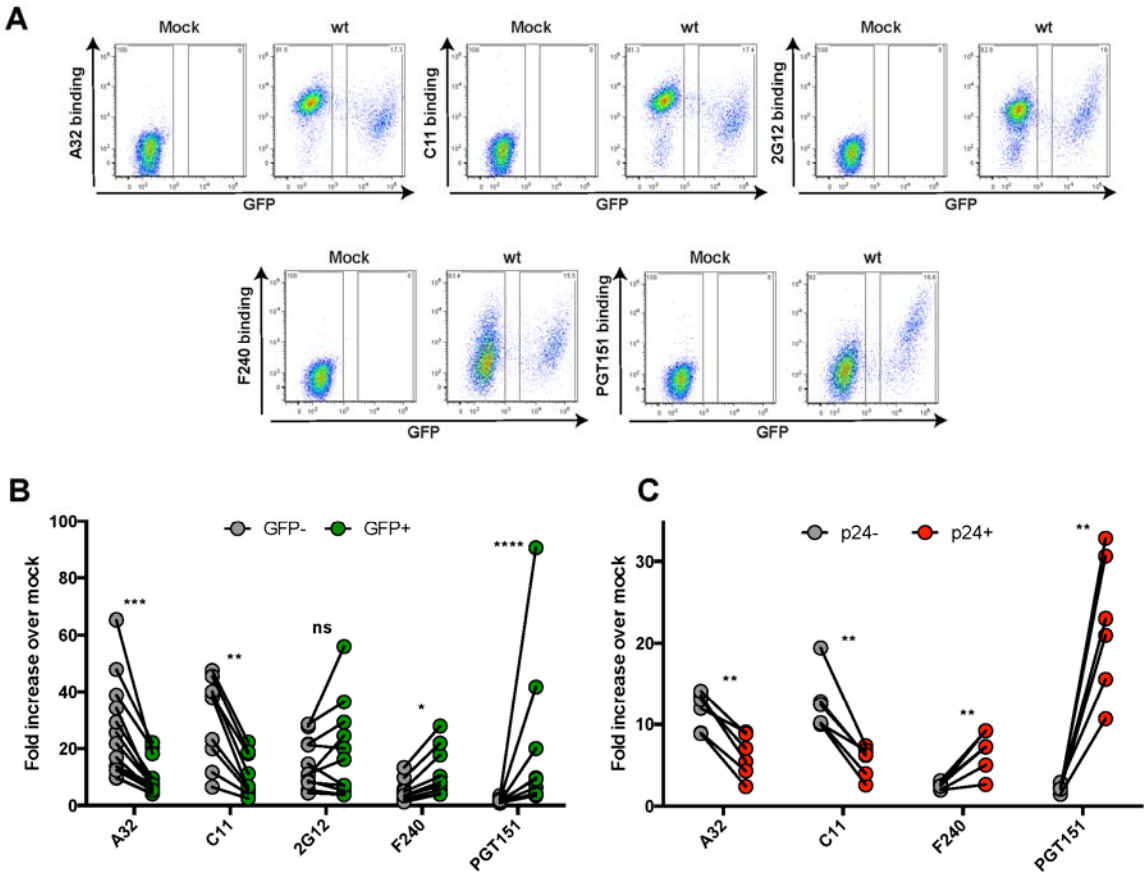
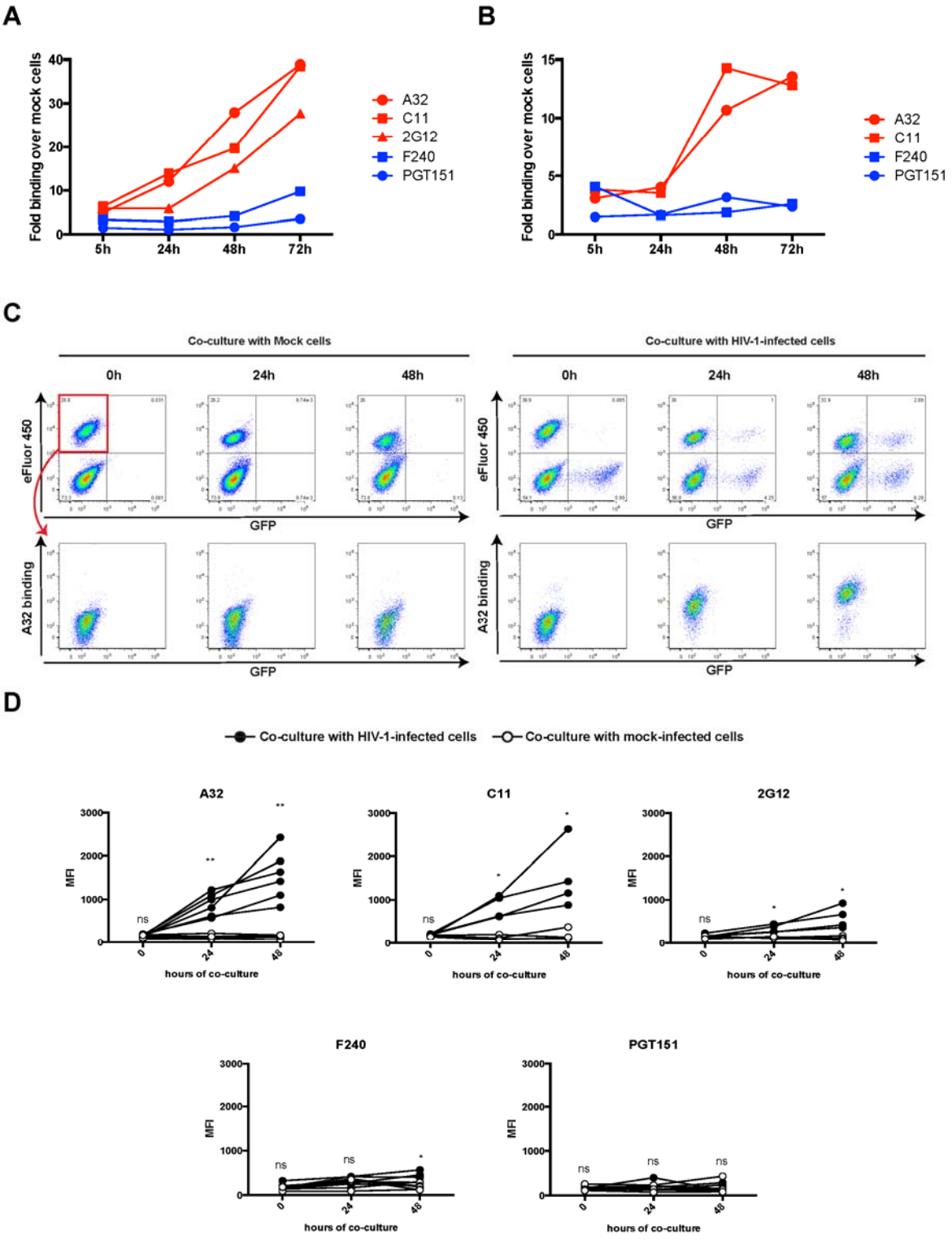


Figure 4.15 (Article 8 Figure 2) Shed gp120 represents the major source of Env present on the surface of uninfected bystander cells.
(A-B) Cell-surface staining of primary CD4+ T cells infected with the NL4-3 GFP ADA wt virus with anti-Env Abs (A32, C11, 2G12, F240 and PGT151). Shown in (A) are dot blots 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. Error bars indicate the mean +/- SEM. 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).

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 (Figure 3A and 3B). 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 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 (Figure 3C, top left squares), by anti-Env Abs was then evaluated by FACS (Figure 3D). Similar to the results in Figure 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 (Figure 3). As expected, no recognition of uninfected bystander cells was observed for cells co-cultured with mock-infected cells. In this assay, bystander cells were not directly infected; gp120 was detected on the bystander 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.


A
Fold binding over mock cells
40
30
20
10
0
5h
24h
48h
72h
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C11
2G12
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Fold binding over mock cells
15
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PGT151
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Co-culture with Mock cells
Co-culture with HIV-1-infected cells
0h
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Co-culture with HIV-1-infected cells
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A32
C11
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hours of co-culture

Figure 4.16 (Article 8 Figure 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-Env Abs (A32, C11, 2G12, F240 and PGT151) 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. Error bars indicate the mean +/- SEM. Statistical significance was tested using the Mann-Whitney *t* test (* $p<0.05$, ** $p<0.01$, ns: not significant).

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 48h. This system minimizes cell-to-cell contact and therefore any gp120 detected in eFluor-450-stained bystander cells must result from shed gp120 or particulate Env. As shown in Figure 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 (Figure 4), indicating that cell-to-cell contact and virological synapse formation is not necessary for gp120 coating of uninfected bystander cells.

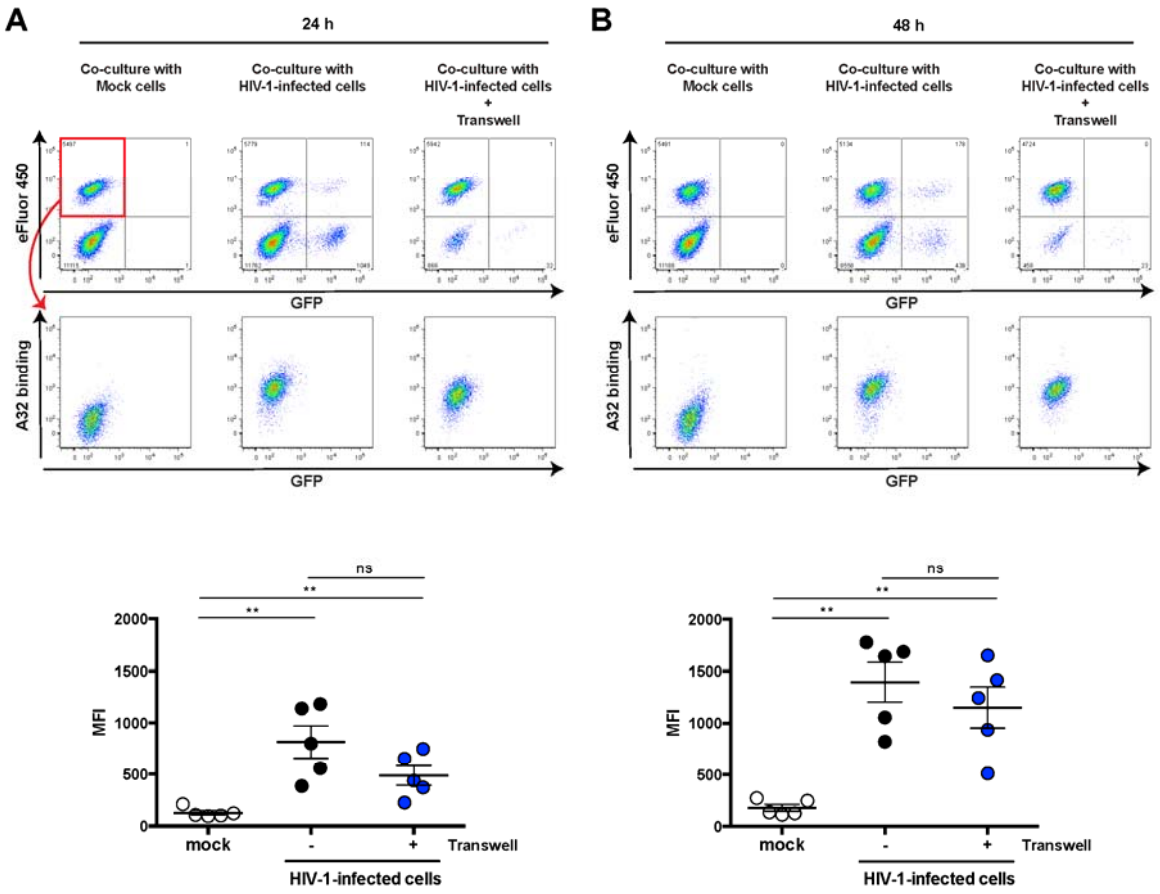

Figure 4.17 (Article 8 Figure 4) Cell-to-cell transmission is not required for the presence of gp120 on the surface of bystander cells.

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 the Mann-Whitney *t* test (* $p<0.05$, ** $p<0.01$, ns: not significant).

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 (Figure 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 (Figure 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 (Figure 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 (Figure 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.

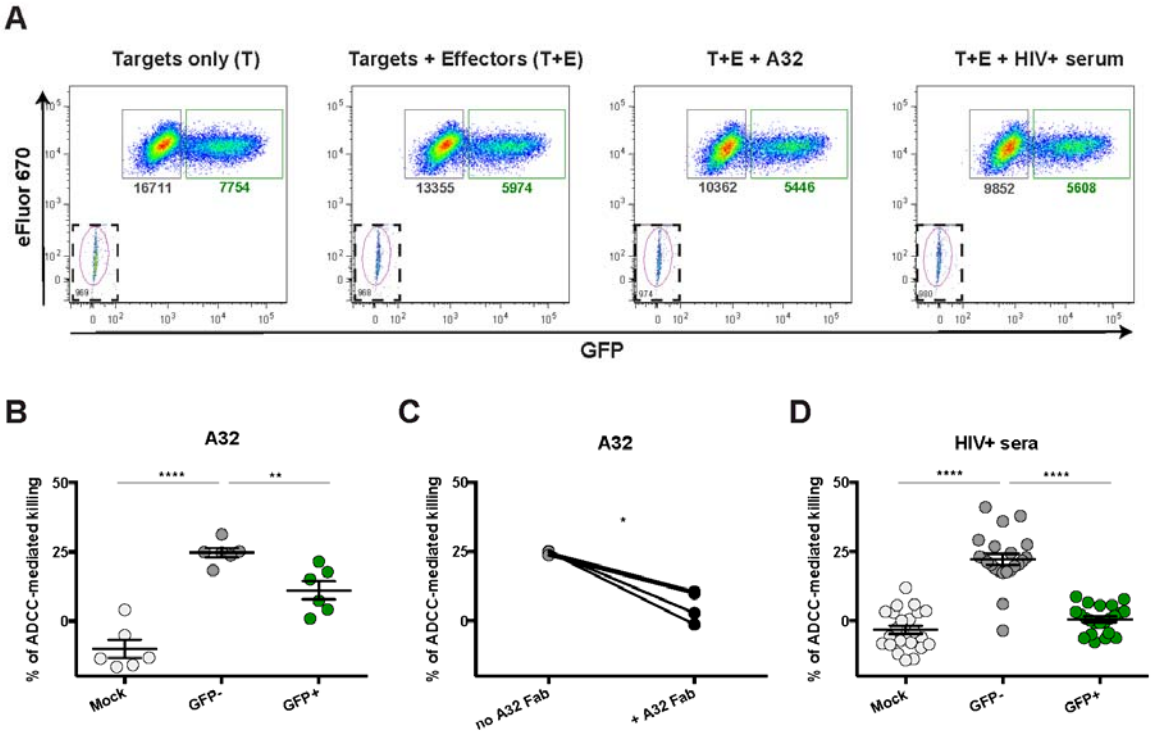

Figure 4.18 (Article 8 Figure 5) Uninfected bystander cells can be eliminated by ADCC.

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 (A,C) an unpaired *t* test or (B) the Mann-Whitney *t* test (* $p<0.05$, ** $p<0.01$, **** $p<0.0001$, ns: not significant).

CD4mc block recognition of bystander CD4+ T cells by ADCC-mediating antibodies

Since soluble gp120 binds uninfected bystander CD4+ T cells through CD4 interaction (Figure 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 (Figure 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 (Figure 6 A-B and S8). However, only JP-III-48 was able to expose CD4i epitopes recognized by HIV+ sera on infected cells (Figure 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.

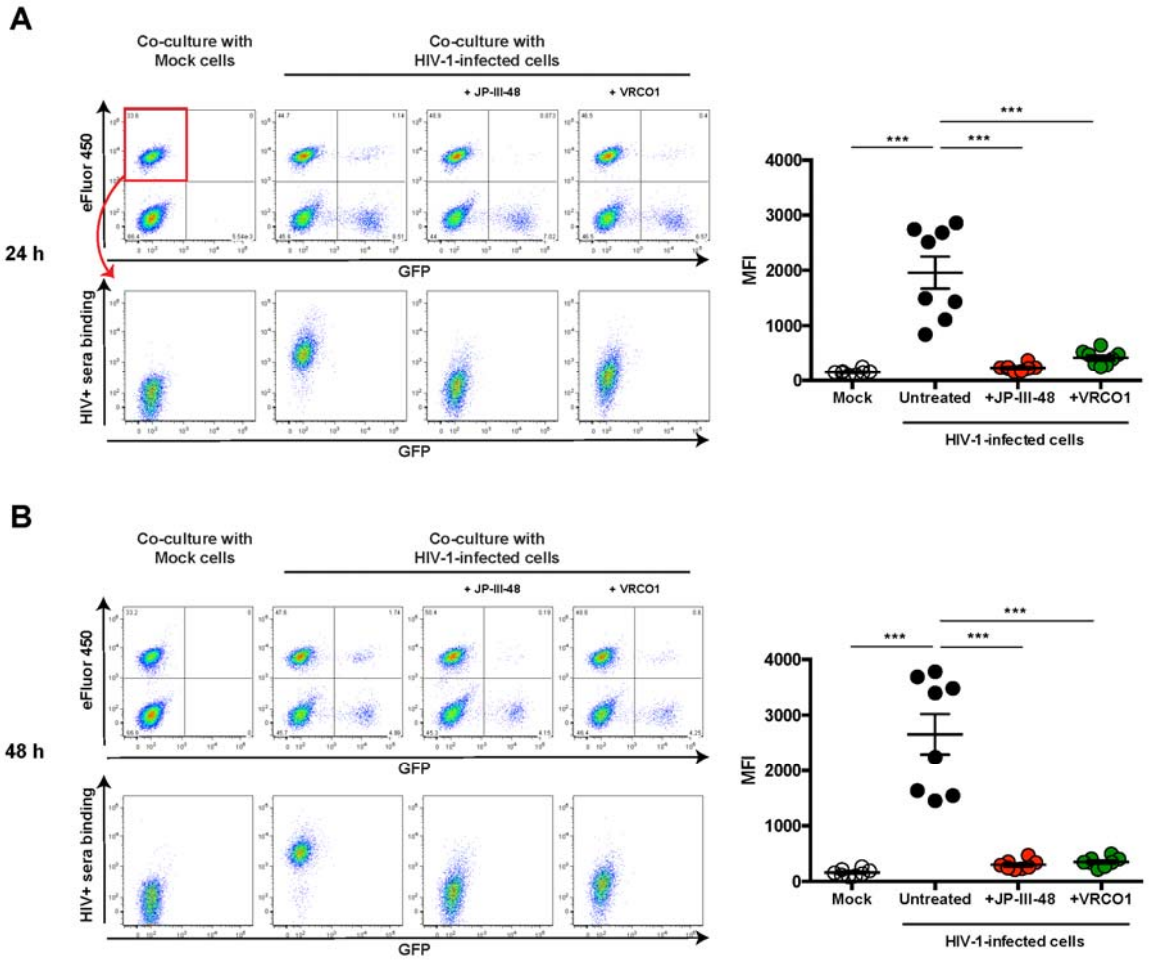
A
Co-culture with Mock cells
Co-culture with HIV-1-infected cells
+ JP-III-48
+ VRCO1
24 h
eFluor 450
GFP
HIV+ sera binding
GFP
MFI
***
***
***
Mock
Untreated
+JP-III-48
+VRCO1
HIV-1-infected cells
B
Co-culture with Mock cells
Co-culture with HIV-1-infected cells
+ JP-III-48
+ VRCO1
48 h
eFluor 450
GFP
HIV+ sera binding
GFP
MFI
***
***
***
Mock
Untreated
+JP-III-48
+VRCO1
HIV-1-infected cells
C
Relative cell number
HIV+ sera binding
HIV-1 wt
HIV-1 wt + JP-III-48
HIV-1 wt + VRCO1
MFI
ns
**
Untreated
+JP-III-48
+VRCO1
HIV-1-infected cells

Figure 4.19 (Article 8 Figure 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. Error bars indicate the mean +/- SEM. Statistical significance was tested using the Mann-Whitney *t* test (** $p<0.01$, *** $p<0.001$, ns: not significant).

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 results 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, Batraville et al., 2014, Baum et al., 1996, Bonsignori et al., 2012, Ferrari et al., 1994, Ferrari et al., 2011, Gooneratne et al., 2015, Hober et al., 1995, Lambotte et al., 2009, Lambotte et al., 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 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 CD56dimCD16+ 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 CD56dimCD16+ 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-coasted 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 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.

The authors have no conflicts of interest to report.

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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.

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Supplemental Experimental Procedures

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 or either goat anti-mouse Alexa Fluor-594, goat anti-human Alexa Fluor 647 or goat anti-human Alexa Fluor 594 mAbs (Invitrogen) were used as secondary Abs, and AquaVivid (Invitrogen) was used as 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 manufacturer 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., 2011; Fontaine et al., 2009) and the Canadian Cohort of HIV Infected Slow Progressors (International et al., 2010; Kamya et al., 2011; Peretz et al., 2007)], and research adhered to the ethical guidelines of CRCHUM and was reviewed and approved by the CRCHUM institutional review board (ethics committee).

Plasmids and site-directed mutagenesis

pNL43-ADA(Env)-GFP.IRES.Nef proviral vectors (*wt* or expressing the D368R Env variant) and the VSVG-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 for the HIV-1 transmitted founder (T/F) IMC CH77 was previously described (Bar

et al., 2012; Fenton-May et al., 2013; Ochsenbauer et al., 2012; Parrish et al., 2013)(Richard et al., 2015).

Supplemental Data

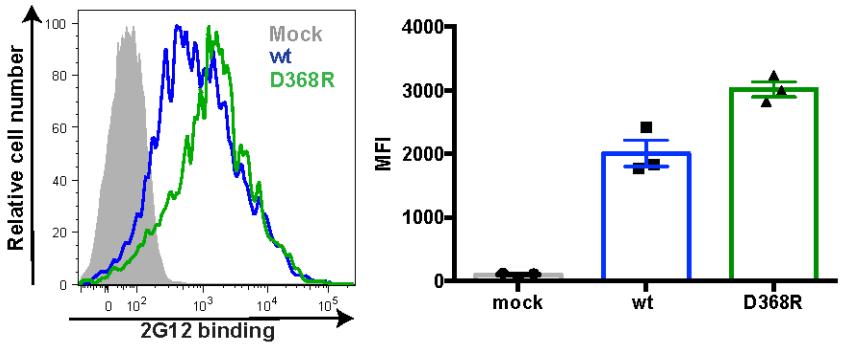

Figure 4.20S (Article 8 Figure S1) Cell-surface level of HIV-1 Env wt and D368R on HIV-1-infected primary CD4+ T cells.
Cell-surface staining of primary CD4+ T cells infected or not with NL4-3 GFP ADA based virus, either wild-type (wt) or expressing D368R Env with the CD4-independent anti-Env Abs 2G12. Shown in (left) are histograms depicting representative staining and (right) the Mean Fluorescence Intensity (MFI) obtained for three different donors. Error bars indicate mean +/- SEM.

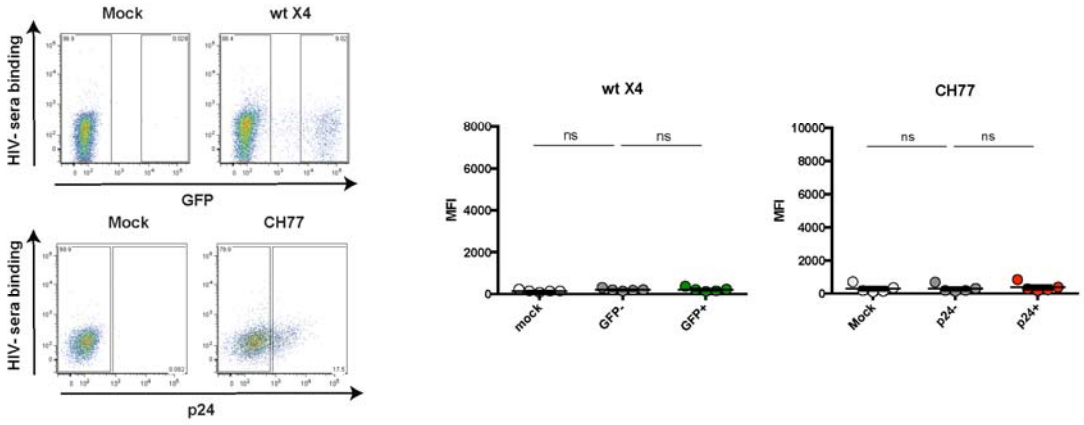

Figure 4.21S (Article 8 Figure S2) Detection of primary CD4+ T cells infected with X4-tropic and primary HIV-1 isolate virus by sera from uninfected individuals.

Cell-surface staining of primary CD4+ T cells infected or not with a X4-tropic virus (NL4-3 GFP) and a primary HIV-1 isolate (CH77) with sera form 5 uninfected individuals. Shown in (left) are dot blot depicting representative staining and (right) the Mean Fluorescence Intensity (MFI) for all tested sera. Error bars indicate mean +/- SEM. Statistical significances were tested using unpaired *t* test. (ns: not significant).

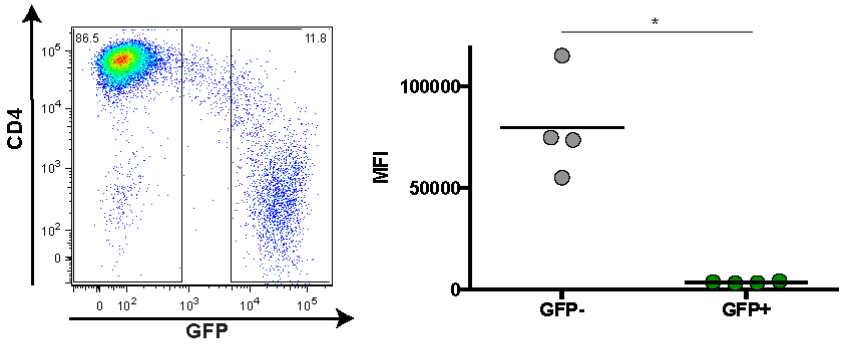

Figure 4.22 (Article 8 Figure S3) Level of cell-surface CD4 on uninfected bystander cells and infected cells.

Cell-surface staining of primary CD4+ T cells infected or not with NL4-3 GFP ADA based virus wild-type (wt) with an anti-CD4 mAbs. Shown in (left) is histogram depicting representative staining and (right) the Mean Fluorescence Intensity (MFI) obtained for four different donors. Error bars indicate mean +/- SEM. Statistical significance was tested using Mann-Whitney *t* test. (* $p<0.05$).

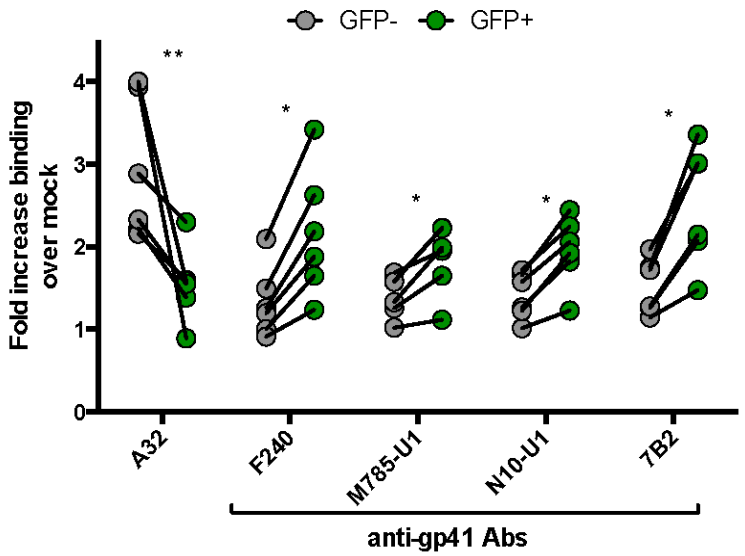

Figure 4.23 (Article 8 Figure S4) Detection of infected and uninfected bystander cells by ant-gp41 antibodies.

Cell-surface staining of CEM.NKr cells infected with NL4-3 ADA based virus wild-type (wt) with anti-gp120 Abs A32 and anti-gp41 Abs F240, M785-U1, M10-U1 and 7B2. Data presented as fold binding over mock cells obtained in 6 independent experiments. Error bars indicate mean +/- SEM. Statistical significances were tested using unpaired *t* test (* $p<0.05$, ** $p<0.01$).

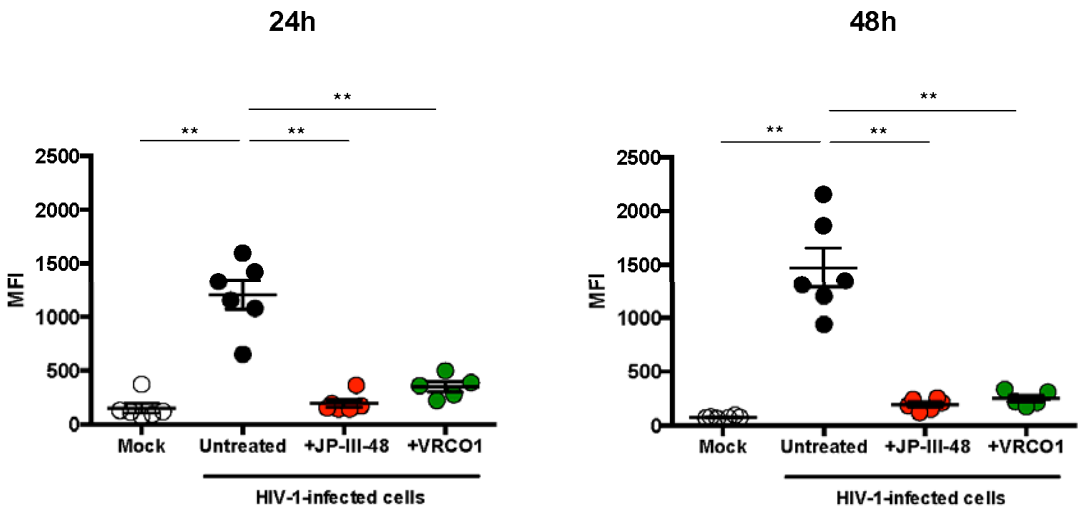

Figure 4.24 (Article 8 Figure S5) CD4-binding site ligands abrogates recognition of bystander CD4+ T cells by A32 Abs.

Uninfected cells, stained with the cellular dye eFluor 450, were co-cultured with unstained autologous mock 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 the A32 mAbs to recognize uninfected bystander cells, designated as eFluor-450+GFP- cells, was evaluated by FACS after (left) 24h and (right) 48h of co-culture. Show is the quantification of the results obtained with in at least 5 different donors. Error bars indicate mean +/- SEM. Statistical significances were tested using Mann-Whitney *t* test (** $p<0.01$).

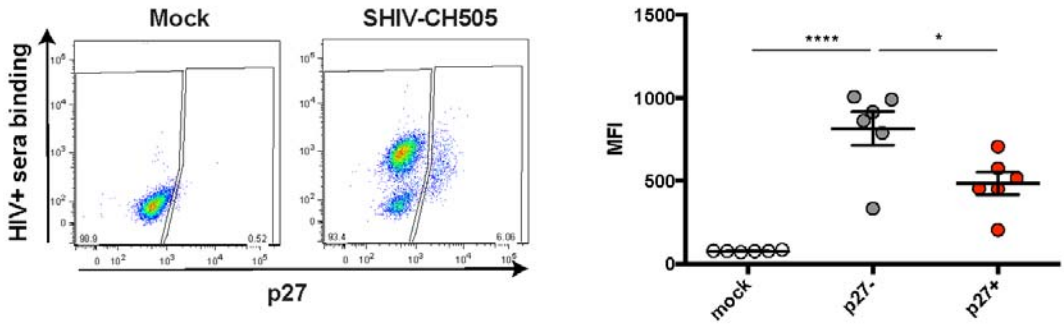

Figure 4.25 (Article 8 Figure S6) Recognition of uninfected bystander cells by sera from HIV-1-infected individuals in the context of SHIV-infected primary CD4+ T cells.

Cell-surface staining of primary CD4+ T cells infected with SHIV-CH505 with sera form 6 HIV-1-infected individuals. Shown in (left) are dot blot depicting representative staining and (right) Median Fluorescence Intensity (MFI) for all tested sera. Statistical significances were tested using unpaired *t* test (* $p<0.05$, **** $p<0.0001$).

Supplemental References

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CHAPITRE V

DISCUSSION ET PERSPECTIVES

5.1 Contributions de cet ouvrage

Les travaux présentés dans cet ouvrage apportent des nouvelles connaissances sur la biologie moléculaire et la pathogénèse de l'infection au VIH-1. Il a d'abord été présenté comment une technique d'ÉLISA à base cellulaire permet de mesurer les changements de conformation dans les glycoprotéines d'Env sous forme trimèrique en réponse à différents traitements ou expression d'autres protéines cellulaires. Ensuite, faisant face au besoin marqué d'avoir une méthodologie permettant d'évaluer de façon précise la réponse ADCC à la fois sur des cellules infectées que sur des cellules recouvertes de gp120 recombinante, une nouvelle technique de mesure de l'ADCC par cytométrie en flux a été mise au point. Ces techniques ont permis d'identifier comment l'interaction d'Env avec son récepteur CD4 engendre l'ouverture du trimère d'Env et permet l'exposition d'épitopes qui sont autrement occultés à l'intérieur du trimère. Nous avons également déterminé que ces épitopes, nommés épitopes CD4i, sont reconnus par un type d'anticorps hautement propice à induire une réponse ADCC et qui sont présents chez la grande majorité de personnes infectées par le VIH-1. Nos travaux démontrent cependant que le virus a évolué des mécanismes permettant de contrôler étroitement l'exposition de ces épitopes via le retrait de CD4 de la surface cellulaire par Nef et Vpu, ainsi que par les mécanismes intrinsèques régulant la conformation d'Env. Ceci est démontré par le fait que le retrait de Nef et Vpu occasionne une augmentation des niveaux d'Env et de CD4 à la surface de cellules infectées, résultant en une forte exposition d'épitopes CD4i et une susceptibilité accrue des cellules infectées à la réponse ADCC induite par des anticorps CD4i (Figure 5.1A). Puisque ces anticorps sont hautement prévalents chez les individus infectés, ils représentent une cible thérapeutique potentiellement exploitable. Nous avons introduit cette notion dans le chapitre 4 en démontrant que des petites molécules capables de mimer la liaison de CD4 à Env (CD4mc) occasionnent l'exposition des épitopes CD4i reconnus par des

anticorps présents chez les individus infectés et rend susceptible des cellules infectées par un virus sauvage à la réponse ADCC (Figure 5.1B).

De plus, nous avons démontré comment la gp120 soluble, produite par le détachement de gp120 à la surface des cellules infectées (Figure 5.1C), interagit avec le récepteur CD4 des cellules avoisinantes non-infectées et expose ainsi les épitopes CD4i (Figure 5.1D). Ces cellules non-infectées recouvertes de gp120 sont donc reconnues et éliminées par la réponse ADCC, phénomène qui pourrait contribuer à expliquer la déplétion massive de cellules T CD4+ non-infectées observée au cours de la pathogénèse du VIH-1. Nous avons également fait la démonstration que lorsque les CD4mc se lient à la gp120 soluble produite par les cellules infectées, ceux-ci sont capables de faire compétition pour la liaison au récepteur CD4 des cellules non-infectées, prévenant ainsi l'élimination de ces dernières par la réponse ADCC (Figure 5.1E).

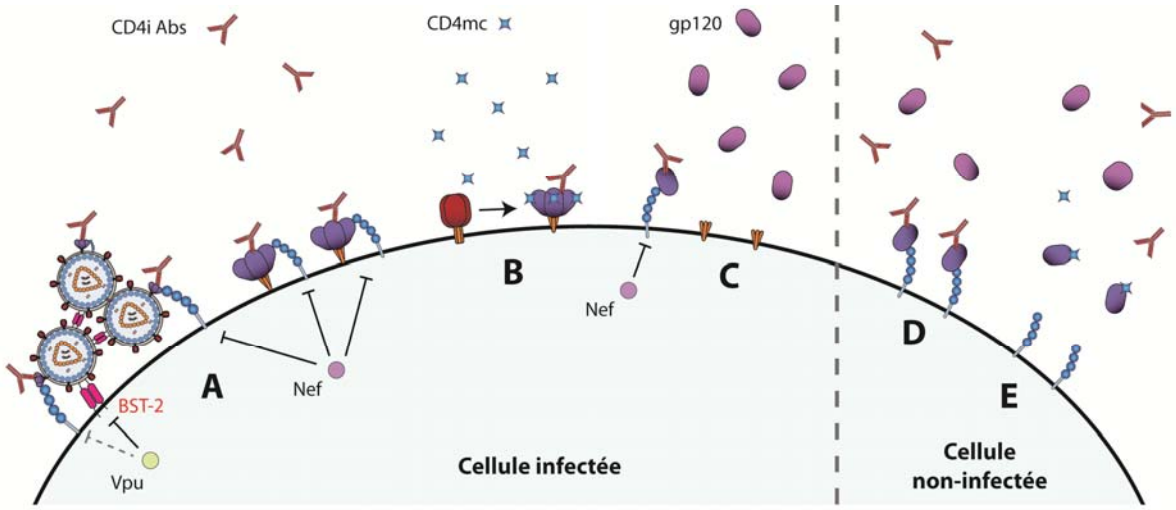

Figure 5.1 Contributions majeures de cette thèse
Représentation schématique des contributions majeures de cette thèse. (**A**) Le retrait de CD4 de la surface des cellules infectées par Nef et Vpu ainsi que l'inhibition du facteur de restriction Tétherine / BST-2 par Vpu empêchent l'accumulation d'Env et de CD4 et donc module l'interaction Env – CD4, prévenant l'exposition d'épitopes CD4i d'Env qui sont reconnus par des anticorps capables d'induire une réponse ADCC. (**B**) L'utilisation de petits composés mimant l'action de CD4 sur Env contre des cellules infectées par un virus exprimant Nef et Vpu permet de forcer l'exposition d'épitopes CD4i à leur surface, augmentant ainsi leur susceptibilité à la réponse ADCC des

anticorps CD4i. (**C**) Les cellules infectées relâchent des molécules de gp120 dans le milieu extracellulaire qui iront interagir avec CD4. Nef permet d'empêcher que cette gp120 soluble se lie à la surface des cellules infectées. (**D**) La gp120 relâchée par les cellules infectées interagit avec CD4 à la surface des cellules avoisinantes non-infectées, exposant ainsi les épitopes CD4i d'Env et induit la destruction des cellules non-infectées par la réponse ADCC. (**E**) Lorsque les CD4mc se lient à la gp120 soluble, ils compétitionnent la liaison de la protéine à CD4, empêchant ainsi le recouvrement des cellules avoisinantes non-infectées. L'utilisation des CD4mc protège donc les cellules avoisinantes non-infectées de l'élimination via la réponse ADCC contre les épitopes CD4i.

Pris ensemble, les travaux présentés dans cet ouvrage démontrent comment le VIH-1 contrôle étroitement l'exposition d'épitopes capables d'être reconnus par des anticorps incitant une réponse immunitaire cytotoxique. Or, ces observations semblent être diamétralement opposées à celles publiées par la communauté scientifique depuis près de 30 ans déjà, où il est fréquent d'observer des rapports indiquant que des cellules infectées au VIH-1 soient susceptibles à la réponse ADCC. En effet, les premières publications démontrant la présence d'anticorps capables d'éliciter une réponse ADCC contre le VIH-1 datent de la fin des années 1980 (441-443). Si le VIH-1 se défend d'une telle reconnaissance et réponse immune, tel que les travaux de cette thèse le décrivent, pourquoi est-il si fréquent d'observer une réponse ADCC contre des cellules infectées dans plusieurs publications de la littérature scientifique et qu'en sont les répercussions sur la biologie moléculaire du VIH-1 et les approches thérapeutiques envisageables?

5.2 Implications des connaissances apportées sur les diverses techniques mesurant les épitopes d'Env et la réponse ADCC

L'exposition d'épitopes d'Env induits par CD4 à la surface des cellules infectées au VIH-1 est au mieux très limitée. Or, comment peut-on expliquer que près de 30 ans de recherche sur le VIH-1 indique que les anticorps reconnaissant ces épitopes sont capables d'induire une réponse immune effectrice contre les cellules infectées? Une partie de la réponse réside dans les conséquences de la production de gp120 soluble, après sa dissociation du trimère d'Env (361-363), par les cellules infectées dans les systèmes *in vitro* utilisés pour mesurer ces réponses immunes. En effet, tel qu'observé dans le chapitre 4, les cellules non-

infectées avoisinantes lors de culture *in vitro* du VIH-1 se retrouvent couvertes de gp120 soluble via son interaction avec CD4, qui est hautement exprimé à la surface des cellules non-infectées. Ceci résulte en une forte exposition d'épitopes CD4i, une reconnaissance par des CD4i Abs et une susceptibilité accrue des cellules non-infectées à la réponse immune ADCC. Cette notion est critique à l'interprétation des différentes stratégies utilisées pour mesurer cette réponse *in vitro*, car elle implique que la susceptibilité des cellules infectées versus celles non-infectées soit différente, et ce au sein d'un même échantillon. Il n'est donc pas surprenant que, malgré les stratégies du VIH-1 pour prévenir cette reconnaissance immune contre les cellules infectées, des chercheurs aient identifié la présence de cette activité ADCC contre des cultures de VIH-1 dans des échantillons *in vitro*. Notamment, les premières observations de ce phénomène furent basées sur le marquage radioactif de lignées cellulaires chroniquement infectées (donc présentant à la fois des cellules infectées et non-infectées) ou de cellules recouvertes de gp120 recombinante (441-443). Ces deux stratégies, qui impliquent l'interaction de gp120 soluble sur des cellules exprimant CD4 à leur surface (dans un cas la gp120 est produite par les cellules infectées présentes dans la culture, dans l'autre cas, directement appliquée), offrent des cibles exposant ces épitopes CD4i d'Env. Cependant, lorsque la réponse ADCC est mesurée sur la population totale grâce à la relâche du marquage radioactif après la lyse des cellules, une réponse est observée sans toutefois pouvoir déterminer quelles cellules ont été tuées, ces techniques ne permettant pas de différencier les cellules infectées des non-infectées (dans le Chapitre 4 nous avons clairement démontré que ce sont ces dernières qui sont éliminées). Ceci résulte en une surestimation de la susceptibilité des cellules à la réponse ADCC et a mené plusieurs groupes à suggérer que ces anticorps sont capables d'induire une réponse ADCC contre les cellules infectées par des virus sauvages. Il faut souligner que dans au moins une occasion la réponse ADCC observée contre les cellules infectées était due au fait que les chercheurs ont utilisé sans s'en apercevoir des virus déficients en Nef et donc incapables d'enlever complètement CD4 de la surface des cellules (444, 445). Ce phénomène de sur-activation est probablement présent dans plusieurs autres techniques qui évaluent la réponse ADCC au niveau de la population totale ou qui ne peuvent distinguer les cellules infectées des cellules non-infectées avoisinantes couvertes de gp120, notamment celles qui sont basées sur la mesure de l'activation des cellules NK ou bien sur

l'observation de la présence de granzyme B dans les cellules cibles (voir la section 6 de l'article présenté dans le chapitre 1).

5.3 Implications sur la biologie et pathogénèse du VIH-1

5.3.1 *Génération des anticorps CD4i in vivo*

La réponse humorale requiert la présentation d'antigènes aux cellules B présentes dans les organes lymphoïdes secondaires. Plusieurs hypothèses ont été émises quant à la source des antigènes contre le VIH-1. Il est suggéré que les antigènes conformationels d'Env peuvent provenir de la reconnaissance du trimère d'Env à la surface des particules virales ou des cellules infectées (446, 447). Les particules virales présentent à la fois des formes fonctionnelles et non-fonctionnelles du trimère d'Env : la forme native, des formes non-clivées (gp160) ou encore simplement la portion transmembranaire gp41 laissée à la surface de la membrane après le détachement de la gp120 (447). Considérant que le VIH-1 présente des mécanismes afin de prévenir l'exposition d'épitopes CD4i à la surface des cellules infectées en retirant le récepteur CD4 de la surface cellulaire, il est très peu probable que la reconnaissance antigénique et la génération des anticorps CD4i s'effectuent en réponse à l'exposition directe d'épitopes CD4i à la surface des cellules infectées ou des particules virales. Or, les travaux présentés au chapitre 3 soulignent l'importance et la fréquence des anticorps CD4i lors d'infections naturelles au VIH-1. Ces observations s'inscrivent parmi d'autres publications démontrant la fréquence de ces anticorps CD4i (448) et surtout la description que plus de 47% des cellules B isolées chez des patients infectés présentent une affinité pour des antigènes CD4i (448, 449). Cependant, nos travaux peuvent être conciliés grâce aux observations que la gp120 se retrouve sous forme soluble dans le sérum des patients infectés (450-452). Bien que les travaux présentés au chapitre 4 de cet ouvrage démontrent que cette forme soluble de gp120 puisse se lier aux cellules exprimant CD4 et être reconnue par les anticorps capables d'induire une réponse ADCC, il est essentiel de reconnaître que cette forme soluble de gp120 est monomérique et donc expose naturellement des épitopes autrement occultés par les deux autres sous-unités de gp120 dans le contexte trimèrique. Il est donc fortement possible que cette région hautement conservée de la gp120, ses couches

topologiques internes, résulte en une génération massive d'anticorps CD4i grâce à sa forte présence sous forme soluble lors d'infection naturelle au VIH-1.

Puisque les petits composés capables de mimer l'action de CD4 sur Env peuvent également empêcher l'interaction de la gp120 avec les cellules exprimant le récepteur CD4 (voir article 2 du chapitre 4), il est possible que leur utilisation *in vivo* amène à une réduction de l'accumulation de gp120 soluble, résultant ainsi en une limitation de l'activation des cellules B spécifiques aux épitopes de gp120 CD4i. Un essai clinique actuel basé sur l'administration d'anticorps monoclonal VRC01 (453) offre la possibilité de vérifier cette hypothèse. En effet, il serait intéressant de quantifier la gp120 soluble et sous forme de complexes immuns avant et après l'administration de VRC01, puis de corréler une éventuelle diminution avec une diminution de la génération soutenue d'anticorps CD4i. D'une manière importante, l'observation de ce phénomène impliquerait que l'utilisation de CD4mc pour leur capacité à augmenter la sensibilité des cellules infectées aux anticorps CD4i pourrait nécessiter la surveillance des niveaux d'anticorps CD4i afin de conserver une action thérapeutique.

5.3.2 *La génération d'une réponse ADCC par les anticorps CD4i : fonctionnalité limitée à cette classe d'anticorps?*

Pris ensemble, nos travaux suggèrent que la grande majorité de la réponse ADCC contre les cellules infectées s'effectue grâce à des anticorps CD4i qui ciblent spécifiquement les couches topologiques 1 et 2 du domaine interne. Bien que d'autres classes d'anticorps soient capables de lier les molécules d'Env exposées à la surface des cellules infectées, cette reconnaissance ne semble pas induire une réponse ADCC (article 3 du chapitre 3). Néanmoins, compte tenu que la réponse ADCC s'effectue suite à la reconnaissance de la portion Fc des anticorps liés aux cellules cibles, il aurait été envisageable que la réponse ADCC puisse être le résultat de la liaison de différents types d'anticorps. L'apparente sélectivité pour les anticorps CD4i ciblant le domaine interne de la gp120 pour l'induction de la réponse ADCC, tel que démontré par les expériences de compétitions des anticorps des

sérums de patients par des mutants de gp120 soluble (voir les sections 2 et 3 du Chapitre 3), permet de soulever plusieurs hypothèses tant qu'à l'explication de ce phénomène.

Tout d'abord, il est possible que l'angle formé par l'anticorps lors de la reconnaissance du domaine interne de la gp120 à la surface de la cellule occasionne le positionnement distal de la portion Fc, facilitant ainsi la reconnaissance par le récepteur Fc présent à la surface des cellules effectrices. Néanmoins, la simple reconnaissance d'un anticorps qui a lié une cellule cible n'est pas suffisante pour induire l'activation de la cellule effectrice (454). Il est alors envisageable que les anticorps CD4i du domaine interne de la gp120 reconnaissent l'Env avec un angle d'approche permettant de concentrer plusieurs Fc vers le même sous-domaine, et que la trajectoire d'approche des autres types d'anticorps réduise leur capacité à induire l'agrégation des récepteurs Fc nécessaire à la réponse ADCC. Cette hypothèse est supportée par l'observation que deux anticorps, N5-i5 et 2.2c, présentent une différence dans leur capacité à induire une réponse ADCC de 75 fois alors qu'ils reconnaissent des épitopes similaires. Cette différence a été attribuée, au moins en partie, à l'angle d'approche des anticorps par la démonstration que l'échange de leurs régions V_H et V_L module la capacité des anticorps à induire une réponse ADCC (455).

Les anticorps ciblant la portion transmembranaire d'Env, la gp41, présentent en ce sens un élément positionnel ne favorisant pas la reconnaissance par les récepteurs Fc. Plusieurs épitopes de la gp41 se retrouvent dans le MPER, région près de la membrane cellulaire. Même si la portion Fc d'un anticorps liant le MPER puisse être relativement dirigée d'une manière distale à la membrane cellulaire, il est très peu probable que cette portion soit accessible aux récepteurs Fc qui ont une portée plutôt limitée (6.5 nm) et pourrait donc interférer avec d'autres protéines transmembranaires (456). Cependant, il est possible que le détachement de la gp120 expose d'une manière plus accessible les épitopes de gp41 à la surface des cellules infectées, ceux-ci pouvant alors induire une réponse ADCC dans des conditions très précises qui n'ont pas été retrouvées *in vitro* dans les travaux présentés dans cet ouvrage : forte quantité de gp41 n'étant plus obstruée par la portion gp120 couplée à un angle d'approche de l'anticorps anti-gp41 qui permet de positionner le Fc dans une région distale de la membrane cellulaire et accessible au récepteur Fc. La validation expérimentale de ces hypothèses

impliquerait l'étude directe de la liaison de récepteurs Fc aux anticorps gp41, mais pourrait également profiter de l'utilisation de mutants d'Env présentant un défaut d'association. De plus, il serait intéressant de déterminer si une construction peptidique présentant des épitopes de gp41 dans différents angles influencerait l'activité ADCC des anticorps contre ces épitopes.

5.3.3 *La réponse ADCC : rôle dans l'adaptation du VIH-1 à son hôte?*

Un point essentiel de la pathogénèse du VIH-1 est sa capacité à s'adapter et surmonter les défenses immunes de son hôte. L'identification d'une classe d'anticorps capable d'induire une réponse ADCC contre des épitopes CD4i d'Env du VIH-1 semble être un phénomène répandu à la fois lors d'infections naturelles et d'essais vaccinaux. Néanmoins, il est remarquable que ces épitopes représentent des régions hautement importantes dans la fonctionnalité de l'Env et donc qu'ils forment des régions hautement conservées de la protéine. L'agilité du VIH-1 pour limiter l'exposition de ces épitopes, via ses protéines accessoires Nef et Vpu, semble être suffisante pour que le virus soit résistant à cette réponse immune tout en n'ayant pas la perte du *fitness* viral des suites de mutations échappatoires dans les domaines hautement conservés d'Env. Néanmoins, il est important de souligner et de se rappeler les origines du VIH-1. Il est en effet suggéré que différents événements zoonotiques ont donné lieu à l'émergence des virus des groupes M, N, O et P. Ayant donc des origines différentes, la fonctionnalité de leurs diverses protéines accessoires pourrait être différente s'ils n'ont pas, ou leurs ancêtres, été confrontés à la même pression immune. Ceci a par ailleurs été illustré par la découverte que la protéine Vpu ait particulièrement contribuée au potentiel de dissémination des différents groupes du VIH-1 (457). Il est connu que le Vpu du VIH groupe M participe au retrait du récepteur CD4 de la surface de la cellule et, d'une manière plus importante, contrecarre tétherine. Cependant, le VIS_{cpz}, le plus récent précurseur du VIH-1, utilise la protéine virale Nef plutôt que Vpu pour empêcher l'activité de ce facteur de restriction. Cette activité anti-tétherine de Nef n'est toutefois pas observée contre son orthologue humain et suggère donc que l'évolution d'une activité anti-tétherine dans l'une des protéines accessoires du VIH lors de la zoonose est primordiale pour la propagation du virus dans son nouvel hôte. De plus, il semblerait que les protéines Vpu des groupes moins répandus (N et O), soient moins bien adaptées à leur hôte comparativement au groupe M : le groupe O

ne contrecarre que très légèrement tétherine alors que le groupe N ne retire pas CD4 de la surface cellulaire grâce à Vpu. Ces observations mènent à formuler l'hypothèse que les isolats primaires d'autres groupes que M puissent être désadaptés à se protéger de la réponse ADCC induite par les anticorps CD4i puisque cette protection est procurée par l'action conjointe de Nef et Vpu. Néanmoins, il serait envisageable que certains isolats de d'autres groupes aient pu évoluer une stratégie échappatoire différente, avec une contribution des protéines accessoires Nef et Vpu pouvant varier selon l'évolution. Des données préliminaires (voir Figure Annexe 1) suggèrent cependant que la réponse ADCC optimale des anticorps CD4i s'observe lorsque Nef et Vpu sont retirés simultanément d'isolats primaires des groupes N et O. Ces données préliminaires ne peuvent cependant répondre par elles-mêmes à la question de la susceptibilité accrue à la réponse ADCC des souches sauvages de ces virus, élément de réponse allant nécessiter la comparaison directe de la susceptibilité des souches sauvages de ces groupes. Néanmoins, l'apparente conservation du phénomène d'augmentation de la réponse ADCC lors du retrait de Nef et Vpu chez ces virus suggère que cette stratégie virale d'évasion immunitaire est un trait conservé lors de différentes zoonoses vers l'humain et qu'il serait alors possible que ce phénomène puisse être observé chez des virus plus anciens dans l'arbre phylogénétique du VIH et des VIS. L'élucidation du moment d'apparition et du degré de conservation de ce mécanisme d'évasion immunitaire chez les lentivirus de primates permettrait de mieux cerner l'ampleur et l'importance de la réponse ADCC dans l'évolution et l'adaptation de ces virus.

5.3.4 *Rôle de la présence de gp120 soluble dans la dysfonction immunitaire*

L'importance de la gp120 dans diverses facettes de la pathogénèse du VIH-1 a fait l'objet de plusieurs publications. Notamment, il a été suggéré qu'elle peut induire directement la mort apoptotique des cellules T CD4+ qu'elle recouvre, ou bien mener à un état de sur-activation immunitaire suite à sa liaison à CD4 ou CXCR4 (458). Cette dysfonction s'observe principalement des suites de la sécrétion de diverses cytokines en réponse à la stimulation et l'activation de cellules CD4+ par la gp120 soluble (452, 459). Puisque les travaux présentés dans cet ouvrage démontrent une forte susceptibilité des cellules non-infectées à l'induction d'une réponse ADCC suite à la reconnaissance par des anticorps CD4i, il serait envisageable

que le VIH utilise ces anticorps afin de pousser le système immun de l'hôte à s'activer contre de multiples cibles, favorisant ainsi sa sur-activation et son éventuelle dysfonction. Notamment, il est connu que la stimulation des cellules NK via leur récepteur Fc induit son retrait de leur surface et donc un état d'anergie pour la cellule effectrice (460). Ceci est confirmé par différents groupes qui ont observé une capacité diminuée des cellules effectrices à induire une réponse ADCC lors de l'infection chronique (461-464). Le détachement de la gp120 participerait donc à la protection des cellules infectées à la fois par la diminution d'épitopes potentiels mais également par la diminution de la capacité de l'hôte à répondre adéquatement à cet antigène, en le forçant à éliminer des cellules recouvertes de gp120 mais non-infectées. Le virus aurait donc trouvé un mécanisme permettant de tourner la réponse immune contre des épitopes hautement conservés vers l'hôte, favorisant ainsi sa prolifération dans ce contexte.

Encore une fois, il semblerait que les approches *in vivo* utilisant des composés (mAbs ou CD4mc) capables d'empêcher la liaison de la gp120 à son récepteur CD4 sur des cellules non infectées pourraient potentiellement aider à contrer cette dysfonction immunitaire.

5.3.5 *Redirection de la réponse immune contre des protéines d'enveloppe virales exposées : stratégie virale répandue?*

Il apparaît maintenant évident que le VIH-1 a évolué des mécanismes permettant à la fois d'échapper à la réponse immune contre des épitopes conservés tout en redirigeant cette activité contre des cellules non-infectées. Ces cellules, étant beaucoup plus nombreuses, sont alors source potentielle de la sur-activation du système immunitaire tel qu'observé dans les infections virales chroniques. Une question se pose alors : est-ce une stratégie que seuls les lentivirus ont évoluée? Après tout, plusieurs virus se doivent d'avoir des épitopes conservés au sein de leur machinerie de fusion. Se pourrait-il que d'autres virus soient susceptibles à une réponse ADCC des suites de l'exposition de ces épitopes conservés? Il est intéressant de constater que d'autres virus utilisent le détachement de leurs glycoprotéines de surface et qu'elles peuvent ainsi recouvrir les cellules non-infectées avoisinantes. C'est notamment le cas du virus d'Ébola, dont les cellules infectées relâchent l'ectodomaine de leur glycoprotéine

suite à un clivage par l'enzyme de conversion du TNF-α (465). La stratégie de ce virus semble jouer un rôle dans la sur-activation immune et dans l'induction de la tempête de cytokines qui est observée dans la pathogénèse *in vivo* de ce virus (466). De plus, il a récemment été proposé que la gp d'Ébola à la surface des cellules infectées nuit à la fonction de d'autres protéines de surface et mène à une cytotoxicité contre les cellules infectées (467). Ébola, grâce à la relâche de ces gp hors des cellules infectées, aurait donc développé un système permettant de balancer la nécessité et les effets néfastes de la présence de gp à la surface cellulaire. Il serait intéressant de déterminer si des anticorps peuvent également reconnaître les cellules non-infectées au cours de l'infection à EBoV et si cette reconnaissance a des répercussions sur la viabilité de ces cellules ou sur l'état d'activation du système immunitaire. Ce n'est ici qu'un exemple de virus, mais il est possible que plusieurs autres utilisent des stratégies similaires et l'élucidation de ces mécanismes viraux pourrait permettre de mieux cerner l'ampleur et la prévalence de ce type de stratégie chez différentes familles de virus.

5.4 Implications thérapeutiques

5.4.1 *Design d'approches vaccinales pour prophylaxie*

Alors qu'un vaccin prophylactique contre le VIH/SIDA se fait toujours attendre, toutes connaissances scientifiques pouvant avancer les efforts mis de l'avant pour sa conception sont les bienvenues. Actuellement, les efforts se concentrent sur le design et l'utilisation d'antigènes pouvant induire la production d'anticorps neutralisants ciblant une vaste gamme de souches virales afin de procurer une immunité adéquate face à la diversité antigénique du VIH-1. Cependant, ces anticorps sont difficiles à induire car ils présentent habituellement des niveaux élevés de mutations somatiques et nécessitent donc une stimulation antigénique évolutive soutenue (468). De plus, il a été suggéré que les cellules B parentales de la lignée germinale des anticorps neutralisants à large spectre ne sont que très faiblement stimulées par les antigènes reconnus par la forme mature des anticorps (468). Néanmoins, il est intéressant de constater que les anticorps non-neutralisants ciblant la conformation ouverte d'Env sont très souvent induits dans les essais vaccinaux chez l'humain et les primates non-humains (406, 469, 470). Or, les travaux présentés ici suggèrent que ces anticorps n'ont qu'une action très

limitée *in vivo* en raison des mécanismes protecteurs déployés par le VIH-1. Il serait souhaitable de déterminer si l'induction d'une conformation ouverte d'Env par les CD4mc pourrait rendre susceptible des isolats primaires du VIH-1 à cette réponse humorale vaccinale, facilitant ainsi l'établissement d'une immunité protectrice chez des individus à haut risque d'infection.

Malgré nos observations démontrant les mécanismes par lesquels le VIH-1 échappe à la réponse ADCC, il a été suggéré que cette réponse avait joué un rôle protecteur lors de l'essai vaccinal RV144. À priori, les travaux présentés ici viennent à l'encontre de ces observations puisque si le virus se protège adéquatement de la réponse ADCC, il n'y a que de très faibles chances que cette réponse immunitaire puisse jouer un rôle protecteur *in vivo*. Cependant, il est essentiel de souligner que l'essai vaccinal RV144 s'est déroulé en Thaïlande, où une majorité des souches circulantes appartiennent à la forme circulante recombinante CRF01_AE (471, 472). L'analyse de la séquence de ces virus révèle des caractéristiques intéressantes et inhabituelles pour l'Env du groupe M. En effet, ils présentent habituellement un petit acide aminé (sérine) en position 375 d'Env, alors qu'un plus grand (histidine) est observé chez presque tous les isolats du CRF01_AE (473). Cet acide aminé joue un rôle important dans la conformation d'Env puisqu'il se retrouve dans ce qui est nommé la cavité Phe43, site où l'acide aminé Phe43 de CD4 interagit avec Env. Il est connu que la mutation du résidu en 375 par un acide aminé plus grand pousse Env à adopter une conformation plus ouverte, probablement en simulant la présence de Phe43 du récepteur dans cette cavité d'Env (362, 474). Compte tenu que les isolats du CRF01_AE présentent une histidine plutôt qu'une sérine à cette position, il est envisageable que ces isolats présentent intrinsèquement une conformation plus ouverte et soient ainsi plus susceptibles à la réponse ADCC. Cette hypothèse est supportée par des données préliminaires (voir Figure Annexe 2) qui démontrent que le fait de muter la position 375 par des résidus plus gros (histidine et tryptophane) est suffisant pour induire une meilleure reconnaissance des cellules infectées (par un virus exprimant un Env de clade B) par des anticorps monoclonaux CD4i et du sérum de patients infectés. Cette possibilité pourrait avoir des répercutions majeures sur le design d'approches vaccinales car il serait alors envisageable qu'une approche ayant eu un certain succès contre

des virus CRF01_AE puisse ne pas avoir d'efficacité contre des isolats de d'autres clades à moins qu'une composante visant à ouvrir Env tel que les CD4mc ne soit incluse (475).

5.4.2 *Design d'approches thérapeutiques*

Une autre implication de nos travaux concerne l'utilisation d'anticorps CD4i contre Env dans des approches thérapeutiques plutôt que prophylaxiques. La possibilité de cibler les réservoirs dormants du VIH-1 pourrait être une stratégie de choix pour aider à éradiquer le virus chez les individus infectés. Pour ce faire, une approche nommée *shock and kill* (réactivation et élimination des cellules infectées) vise à réactiver le virus latent et à induire sa destruction par le système immunitaire tout en empêchant l'infection *de novo* d'autres cellules. Différentes stratégies ont été mises de l'avant afin de réactiver la latence du virus, mais la description de celles-ci est hors du cadre de cet ouvrage (voir (476, 477) pour revues de la littérature sur le sujet). Les travaux du chapitre 4 ont mis la table pour l'utilisation de la réponse ADCC dans l'axe « kill » ou 'élimination' de cette stratégie. La réactivation *in vitro* de l'infection dans des cellules de patients infectés a démontré qu'il est possible d'induire une reconnaissance accrue des cellules infectées grâce à l'utilisation de molécules capables de contrer les stratégies défensives du VIH-1 contre la réponse ADCC induite par les anticorps CD4i. De par leur mécanisme d'action, les CD4mc ont le potentiel d'avoir un effet bénéfique via un ensemble d'effets antiviraux : une activité antivirale directe (475, 478, 479), la potentialisation de l'activité neutralisante d'anticorps CD4i (475, 480), la sensibilisation des cellules infectées à la réponse ADCC (voir section 1 du Chapitre 4), et une diminution de la susceptibilité à l'ADCC des cellules non-infectées avoisinantes en réduisant leur recouvrement par la gp120 soluble (voir section 2 du Chapitre 4). Il est critique de garder en tête que le VIH-1 a une forte capacité adaptative et que des souches virales qui présentent un résidu plus grand en position 375 d'Env sont incapables d'être liées par les CD4mc et sont donc résistantes à leurs effets antiviraux (473). L'aisance avec laquelle le virus pourrait réussir à échapper à la pression des CD4mc dans un contexte thérapeutique est difficile à évaluer compte tenu de ses différents mécanismes d'action. Afin d'éviter que cela ne se produise, la combinaison avec d'autres régimes antiviraux pourrait être nécessaire. De plus, à ce jour il n'existe aucune donnée concernant la toxicité et la tolérance *in vivo* des CD4mc.

D'autres groupes ont récemment suggéré que des molécules à doubles affinités (DART, *dual-affinity re-targeting*) permettant de cibler la réponse des cellules T cytotoxiques (CTL) pourraient être utilisées contre le VIH-1. Ces molécules sont essentiellement formées de deux fragments d'anticorps, l'un ciblant Env et l'autre liant la molécule CD3 sur les CTLs afin d'induire leur activation contre la cellule reconnue (481, 482). L'analyse des publications de ces groupes dans le contexte des travaux présentés ici est intéressante. Tout d'abord, les auteurs démontrent que divers anticorps contre Env sont capables d'induire une réponse cytotoxique contre des cellules infectées avec des isolats primaires, incluant des anticorps CD4i tels qu'A32. Or, nos travaux suggèrent que les épitopes CD4i ne sont presque pas exposés à la surface des cellules infectées par les virus exprimant les protéines virales Nef et Vpu. Comment se fait-il que les DARTs utilisant des anticorps CD4i tels que A32 puissent avoir une efficacité contre les isolats sauvages? Il est ici important de reconnaître qu'une certaine quantité d'épitopes CD4i est présente à la surface des cellules infectées, probablement des suites de l'interaction d'Env avec de faibles niveaux du récepteur CD4 toujours présents à la surface cellulaire malgré l'action combinée de Nef et Vpu ou via la présence du précurseur non-clivé d'Env à la surface qui expose certains épitopes CD4i en absence de CD4 (483). Cette reconnaissance de base par des anticorps CD4i ne semble toutefois pas suffisante pour induire une réponse ADCC *in vitro* contre les cellules infectées. Il est possible que la redirection des cellules CTLs grâce aux DARTs requiert des niveaux plus faibles d'exposition antigénique, ce qui expliquerait leur activité lorsqu'ils sont composés d'anticorps CD4i. Il serait intéressant de quantifier les ratios minimaux et optimaux des antigènes de surface et des anticorps (ou DARTs) pour l'induction des réponses ADCC et CTL. Une autre possibilité est que l'induction des épitopes CD4i s'effectue suite à l'interaction d'Env avec la surface d'une cellule non-infectée avoisinante. De plus, il est suggéré que les épitopes CD4i sont exposés lors de l'entrée virale et persistent à la surface cellulaire pendant un certain temps après celle-ci (484). Il est donc possible que cette exposition transitoire des épitopes CD4i soit suffisante pour l'induction d'une réponse immune, plus particulièrement avec des approches la facilitant telles que les DARTs. De plus, compte tenu que nous démontrons que des épitopes d'Env sont exposés à la surface des cellules non-infectées qui sont recouvertes de molécules de gp120 liées à CD4 (et donc reconnues par les anticorps CD4i), il serait intéressant de déterminer si

les DARTs utilisant des anticorps CD4i peuvent occasionner une réponse cytotoxique contre ces cellules avoisinantes non-infectées, hypothèse qui n'a pas été étudiée par les auteurs de ces articles (481, 482).

5.4.3 *La conformation d'Env : cibler Env, Nef et Vpu*

Nos travaux démontrent clairement le potentiel thérapeutique de cibler la conformation d'Env dans le but de la pousser vers sa conformation liée à CD4. Cependant, ils ne répondent pas clairement à la question de savoir quelle serait la meilleure approche dans un tel contexte. Serait-il préférable de cibler directement Env, comme en utilisant des CD4mc, ou bien est-ce que cibler les fonctions des protéines accessoires Nef et Vpu représente une meilleure alternative ? Cette section vise à discuter du mérite et de la faisabilité de chacune de ces approches.

Tout d'abord, il est clair que les CD4mc ont le potentiel d'agir sur la conformation d'Env. Néanmoins, la section précédente a suggéré quelques limitations pour leur utilisation *in vivo*. L'une de ces limitations réside dans la capacité du VIH-1 à muter et à échapper à l'action des molécules antivirales. Or, les mutations permettant la résistance aux CD4mc identifiées jusqu'à maintenant se situent dans la cavité Phe43, en position 375 d'Env. Par ailleurs, il a été démontré que des mutations dans le domaine interne de la gp120 peuvent aussi affecter, indirectement, les changements de conformation d'Env (362, 485) et pourraient possiblement empêcher le changement de conformation induit par la liaison des CD4mc. Les mutations en position 375 présentent cependant un effet opposé : ils occasionnent le comblement de la cavité Phe43. Nos données préliminaires suggèrent par ailleurs que la présence d'un résidu allant bloquer l'interaction des CD4mc avec Env occasionne des changements de conformations qui ouvrent Env à la reconnaissance par des anticorps CD4i (voir Figure Annexe 2). Ces observations suggèrent donc que d'échapper aux CD4mc en mutant la cavité Phe43 risque de poser une problématique pour la réplication virale en augmentant sa susceptibilité aux anticorps CD4i hautement prévalents.

Qu'en est-il des stratégies visant la dysfonction des protéines accessoires virales Nef et Vpu? Non seulement des suites de leur implication dans la protection des cellules infectées contre la réponse ADCC, mais également car ces protéines sont essentielles dans la régulation du système immunitaire par le VIH-1, plusieurs groupes s'efforcent activement à identifier des molécules étant capables d'empêcher ou de limiter leurs activités biologiques. Compte tenu de ses multiples fonctions, Nef représente une cible de choix. Nef ne présente cependant pas d'activité catalytique intrinsèque mais agit plutôt grâce à son interaction avec des partenaires de l'hôte, les tests à haut débit afin d'identifier des inhibiteurs en sont donc compliqués. Bloquer la formation de dimères de Nef pourrait interférer avec sa capacité à retirer CD4 de la surface cellulaire (486). Des molécules ont déjà été identifiées en ce sens, mais il demeure à confirmer si leur utilisation puisse mener à une augmentation de l'exposition d'épitopes CD4i (487).

Une autre stratégie afin d'inhiber l'activité de Nef est l'utilisation d'anticorps à simple domaine. Ceux-ci, dérivés de lamas immunisés avec la protéine Nef recombinante, peuvent interférer spécifiquement avec les fonctions biologiques de Nef d'isolats distincts du VIH-1 (Neffin) (488, 489). Bien qu'intéressante, cette stratégie requiert une approche non traditionnelle, telle que la thérapie génique, pour son utilisation. Son mécanisme d'action a cependant été récemment élucidé. L'anticorps lierait un épitope de surface près du C-terminal de Nef, allant ainsi empêcher son interaction avec ses partenaires de l'hôte et donc ses activités biologiques. Puisqu'un tel anticorps est difficile à utiliser d'une manière thérapeutique, il est valide de se questionner à savoir si de petites molécules seraient suffisantes pour aller cibler l'épitope reconnu par Neffin et possiblement exercer les mêmes effets biologiques contre la diversité de Nef présente dans la population infectée. Ces nouvelles connaissances pourraient venir complémenter les efforts de criblage *in silico* déjà utilisés afin d'identifier des inhibiteurs de Nef en précisant l'épitope cible pouvant avoir un effet biologique (489).

Quoi qu'il en soit, Nef représente une cible de choix pour la mise en place de stratégies antivirales. Ceci est par ailleurs illustré lorsque la protéine Nef est comparée entre des isolats viraux d'individus progresseurs versus des non-progresseurs à long terme. Cette comparaison

permet d'observer que les Nefs des non-progresseurs présentent une activité réduite pour le retrait de CD4 de la surface cellulaire (490-492). Une autre étude de notre groupe complémente cette observation en démontrant que cette augmentation des niveaux de CD4 chez les contrôleurs virologiques bien que minime est suffisante pour l'exposition accrue d'épitopes CD4i d'Env et donc d'une augmentation de la susceptibilité des cellules infectées à la réponse ADCC (492).

Ces observations suggèrent qu'il est possible que la capacité de Nef à empêcher l'interaction Env – CD4 puisse influencer les paramètres de progression de la pathogénicité. Or, il est toujours incertain si l'atténuation de la fonction de Nef est intrinsèque à la souche virale ayant fondé l'infection chez un contrôleur virologique ou bien si elle découle de mutations des suites d'une autre réponse immune, par exemple la réponse CTL (490). Néanmoins, le rôle de Nef dans la progression de la maladie est apparent dans une cohorte d'individus infectés avec une souche du virus n'encodant pas Nef : ils présentent des caractéristiques de progresseurs lents (493). Il est possible d'imaginer que l'infection par ces souches virales pourrait mener à une plus grande exposition d'épitopes CD4i à la surface des cellules infectées. Compte tenu de la capacité du VIH à s'adapter, il est également possible que ces souches aient amélioré d'autres aspects de leur génome afin de mieux se défendre contre la réponse ADCC. Il serait intéressant de vérifier si une adaptation de Vpu ou d'Env peut être observée chez les virus infectant ces individus au fil du temps afin de compenser pour la perte d'efficacité de Nef à retirer CD4 à la surface de la cellule infectée.

En ce qui a trait à l'inhibition de Vpu dans l'optique d'augmenter la susceptibilité des cellules infectées à la réponse ADCC associée aux anticorps CD4i, Vpu semble être à première vue une cible intéressante puisque son inhibition pourrait donner lieu à une augmentation des niveaux de CD4 et d'Env à la surface cellulaire, deux déterminants que les travaux présentés ici ont démontré comme étant majeurs dans le contrôle de la réponse ADCC. Or, une étude récente rapporte qu'un inhibiteur du complexe β-TrCP/cullin1, le MLN4924, est capable d'induire une diminution de la capacité de Vpu à retirer CD4 de la surface cellulaire mais que cette activité est limitée dans son ampleur et ne résulte ainsi pas en une augmentation de la susceptibilité des cellules infectées à la réponse ADCC (494). Cette inefficacité serait

principalement due à l'incapacité de MLN4924 à inhiber l'activité anti-tétherine de Vpu et ainsi d'augmenter les niveaux de surface d'Env. Or, nous avons démontré que l'exposition d'épitopes CD4i par des CD4mc, sans augmentation des niveaux d'Env à la surface cellulaire, est suffisante pour l'induction d'une réponse ADCC contre les cellules infectées. Néanmoins, l'activité anti-CD4 de Vpu semble être limitée et il est envisageable que l'inhibition de cette activité ne surmonte pas la forte action de Nef contre CD4. Prises ensembles, ces observations et les nôtres suggèrent que de cibler l'activité anti-CD4 de Vpu ne serait pas suffisant et qu'il est donc préférable de cibler Nef ou Env ou les deux ensemble.

Il semblerait que viser Env offre une alternative plus simple et directe d'augmenter la réponse ADCC mais la possibilité de résistance apporte un risque à l'utilisation *in vivo* de cette stratégie. Viser Nef semble être également intéressant alors que son inhibition occasionne l'augmentation la plus marquée des niveaux de CD4. Vpu, pour sa part, représente une approche plutôt limitée car son influence sur les niveaux cellulaires de CD4 est réduite en présence d'un Nef fonctionnel. Malgré une augmentation drastique des niveaux d'Env à la surface des cellules infectées si tétherine n'est pas inhibée par Vpu, si CD4 n'est pas présent la réponse ADCC ne sera pas augmentée. Néanmoins, l'inhibition des protéines accessoires représente une stratégie globalement intéressante puisqu'elle ne se limite pas seulement à l'augmentation de la réponse ADCC, mais pourrait bel et bien jouer d'autres importants rôles *in vivo*.

CONCLUSION

Les travaux présentés dans cette thèse ont démontré comment des anticorps, induits contre une conformation adoptée par les glycoprotéines d'Env lors de leur interaction avec CD4, induisent une réponse ADCC lorsque les épitopes qu'ils reconnaissent sont exposés. Nous avons élucidé comment le virus régule cette interaction Env – CD4 par l'action de ses protéines accessoires Nef et Vpu, qui limitent à la fois les quantités de CD4 et d'Env à la surface des cellules infectées. Nos travaux démontrent également comment le virus redirige la pression immune par ces anticorps vers les cellules non-infectées en détachant la glycoprotéine d'Env de la surface de la cellule infectée. Celle-ci peut ainsi interagir avec le CD4 des cellules avoisinantes, ces dernières étant reconnues par les anticorps CD4i alors que les cellules infectées en sont protégées par l'action de Nef et Vpu. Cependant, l'utilisation de petits composés mimant la liaison de CD4 à Env force l'adoption de la conformation ouverte d'Env à la surface des cellules infectées, augmentant ainsi leur susceptibilité à la réponse ADCC. De plus, puisque ces composés peuvent lier la protéine d'Env soluble et faire compétition pour son interaction avec CD4, ils empêchent la reconnaissance et l'élimination des cellules avoisinantes par la réponse effectrice des anticorps CD4i.

Prises ensembles, ces observations suggèrent que la réponse ADCC dirigée contre les épitopes CD4i d'Env est un élément critique de la réponse immune duquel le virus doit se protéger. Ainsi, les résultats présentés dans cet ouvrage constituent une avancée majeure des connaissances scientifiques dans l'interaction du VIH-1 avec les réponses effectrices des anticorps. L'identification d'un mécanisme d'évasion de la défense immune qui est hautement conservé offre une cible de choix pour le design d'approches thérapeutiques futures.

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ANNEXE I

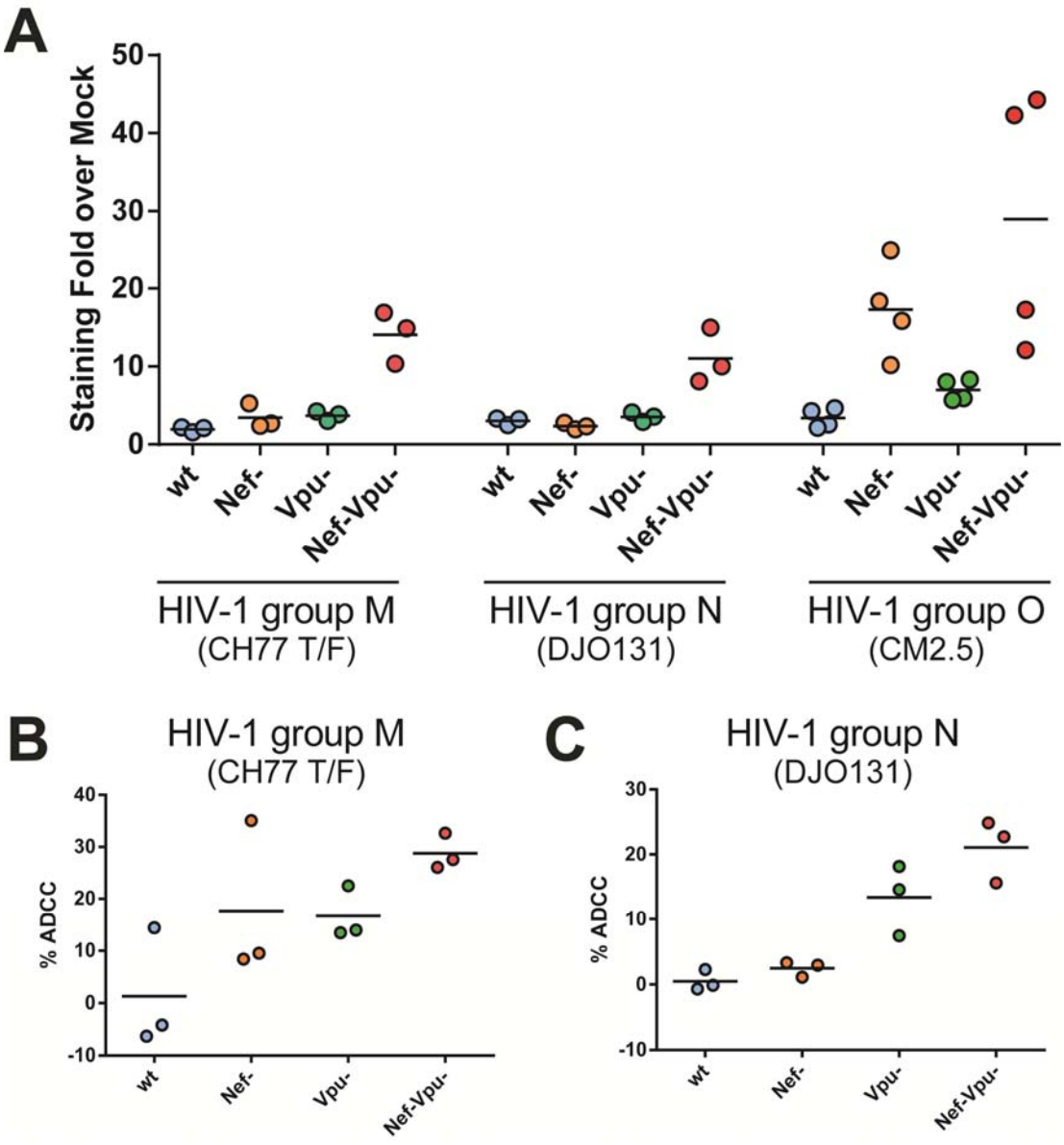

Figure Annexe 1 Les protéines Nef et Vpu d'isolats primaires des groupes M, N et O préviennent l'exposition d'épitopes CD4i d'Env et donc réduisent la susceptibilité des cellules infectées à la réponse ADCC.

Des CEM.NKr (A) ou cellules T CD4+ primaires (B-C) ont été infectées par des isolats primaires des groupes M, N ou O présentant ou non des délétions pour leurs protéines accessoires Nef et Vpu. (A) Mesure de la reconnaissance des cellules infectées par les anticorps présents dans le sérum d'individus infectées. (B-C) Mesure de la susceptibilité des cellules

infectées à la réponse ADCC des anticorps présents dans le sérum d'individus infectés.

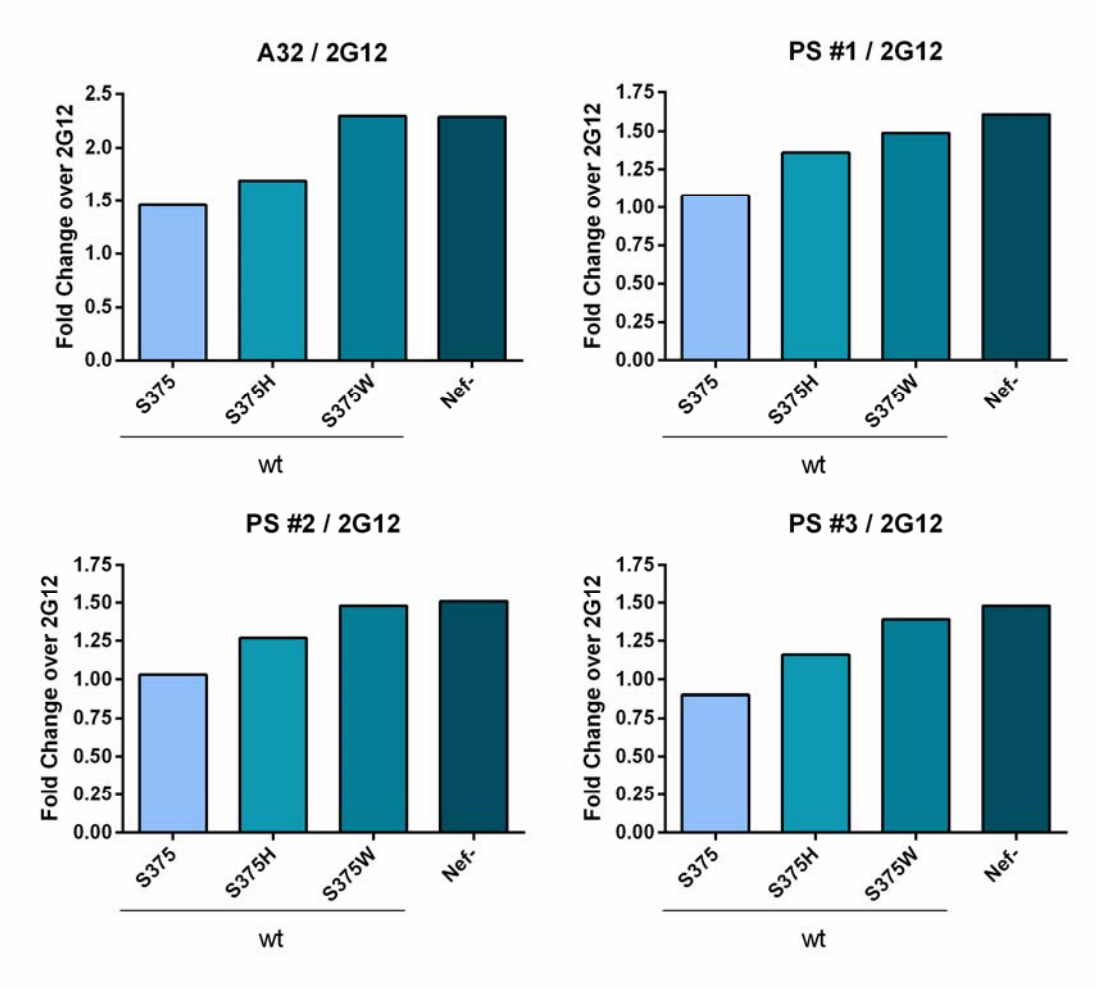

Figure Annexe 2 Modulation de l'exposition des épitopes CD4i d'Env lors du comblement de la cavité Phe43

Des cellules CEM.NKr ont été infectées par un virus NL4.3 ADA.GFP sauvage ou présentant un Env muté en position 375 (S375H / W) ainsi qu'avec le virus contrôle NL4.3 ADA.GFP ΔNef. 48 heures post-infection, l'exposition des épitopes CD4i d'Env à la surface des cellules infectées à été mesurée par marquage de surface par l'anticorps monoclonal CD4i A32 ou trois sérums d'individus infectés au VIH-1. Les valeurs obtenues sont normalisées selon la reconnaissance des cellules par l'anticorps 2G12, qui reconnaît Env d'une manière indépendante de sa conformation.

ANNEXE II

UNCOVERING HIV-1-INFECTED CELLS

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Cet éditorial représente un résumé des travaux présentés dans cet ouvrage. Les trois auteurs ont participé à la rédaction et préparation du manuscrit.

It has been known for more than 30 years that Human Immunodeficiency Virus 1 (HIV-1) infection drives a very potent B cell response resulting in the production of anti-HIV-1 antibodies targeting several viral proteins, particularly its envelope glycoproteins (Env) which are exposed at the surface of viral particles and infected cells [1]. Enthusiasm by the generation of these anti-Env antibodies was short-lived since the majority were found to be unable to efficiently neutralize viral particles and do not appear to control viral replication. This class of antibodies were therefore called non-neutralizing antibodies. We recently reported that these antibodies, elicited in the majority of HIV-1-infected individuals, do have the potential to eliminate HIV-1-infected cells by an immune mechanism called antibody-dependent cellular cytotoxicity activity (ADCC). However, HIV-1 developed a highly-sophisticated strategy to avoid it [2, 3]. We found that these ADCC-mediating antibodies present in sera [2, 4], breast milk [4] and cervicovaginal lavages [4, 5] of HIV-1-infected individuals preferentially target Env in its CD4-bound conformation. In other words, they only recognize epitopes exposed upon interaction with the viral receptor CD4. In order to avoid ADCC responses, HIV-1 accessory proteins Nef and Vpu decrease the overall amount of Env (via Vpu-mediated BST-2 downregulation) and CD4 at the cell surface [3]. In our view the presence of antibodies with the capacity to eliminate HIV-1-

infected cells by Fc-mediated effector functions, including ADCC, represents one of the driving forces for HIV-1-mediated CD4-downregulation. Henceforth, the vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, likely limiting the exposure of CD4-induced (CD4i) Env epitopes at the surface of HIV-1-infected cells; thus, protecting them from ADCCmediated killing.

Can we exploit this information to fight HIV back? As we discussed, the majority of infected individuals do possess antibodies with the potential to eliminate infected cells but the virus "knows" this and avoids exposure of the epitopes being targeted (Env CD4i epitopes). We could hypothesize that strategies aimed at preventing Nef and Vpu-mediated CD4 / BST2 downregulation or designed to "push" Env towards its CD4-bound conformation could potentially increase the susceptibility of HIV-1-infected cells to ADCC and other Fc-mediated effector functions. In a proof-of-concept approach we tested the later possibility by using small-CD4 mimetics and observed that they can indeed force Env to sample the CD4-bound conformation and therefore increase the susceptibility of HIV-1-infected cells to ADCC [4]. In light of these promising results we think that other properties of nonneutralizing antibodies should be highlighted; we believe that non-neutralizing antibodies should be better studied by the HIV-1 scientific community. Unfortunately, their inability to neutralize viral particles did not make them very attractive to HIV-1 researchers so far. Of note, under certain circumstances, such as in the presence of CD4- mimetics, these "non-neutralizing" antibodies can actually neutralize primary viruses [6]. We strongly believe that there is more to an antibody than its neutralization capacity. Through their Fc portion, antibodies can mediate several immunological responses (ADCC, antibodymediated complement activation, antibody-mediated cellular phagocytosis (ADCP), antibody-dependent cellmediated virus inhibition (ADCVI), transcytosis inhibition or opsonization) that could be beneficial in fighting viral infections including HIV-1. We think that these important properties should be emphasized and therefore we propose to call them non-neutralizing effector function competent (nNeFC) antibodies.

While we still do not know whether strategies aimed at exposing Env epitopes recognized by nNeFC antibodies will translate into clinical benefits for HIV-1 infected individuals, data generated so far certainly underscores the importance of studying these

antibodies in more detail. In the future, nNeFC antibodies through their Fc-effector function might play an important role in the design of new strategies aimed at specifically eliminating HIV-1-infected cells.

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