# Protecting HIV-1-infected cells from ADCC: Role of Nef

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

# ABSTRACT

Despite the enormous efforts that are being made to develop new therapeutic strategies to fight HIV-1 infection, a better understanding of the elements contributing to HIV-1 virulence remains necessary to improve the effectiveness of these therapies. Emerging evidences suggest the importance role of Fc-mediated effector functions of anti-gp120 (glycoprotein-120) antibodies in the prevention and limitation of viral spread. This effector response was highlighted in the correlates of protection of the RV144 vaccine trial, the only trial that showed some levels of protection. However, the recognition of such antibodies relies on the necessity of CD4 and Envelope (Env) interaction that results in Env-conformational rearrangement and exposure of CD4-induced (CD4i) epitopes. The implication of the HIV-1 accessory protein Nef was suggested to be a major player in modulating the exposure of CD4i-epitopes and preventing the elimination of infected cells. Indeed, this multifunctional accessory protein is well characterized for its ability to regulate the surface expression of several receptors including CD4, MHC-I, CD28, and NKG2D ligands. Interestingly, the impairment of some Nef activities was described in elite controllers (EC), a rare subset of infected subjects who can control viral replication in the absence of antiretroviral treatment.

The work presented in this thesis, focuses on the relation between Nef's defective activities in EC, in modulating the surface levels of CD4 and NKG2D ligands, and antibody-dependent cellular cytotoxicity (ADCC) response. We demonstrated that the inability of Nef isolated from EC to fully remove CD4 from the surface of infected cells leads to Env-CD4 interaction, which ultimately render these cells susceptible to ADCC.

We also show that Nef's ability to reduce cell surface levels of NKG2D ligands also protects infected cells from ADCC. Cumulatively, our results suggest that in addition to the exposure of ADCC-mediating epitopes induced by the presence of CD4 at the cell surface, the accumulation of NKG2D activating ligands promotes NK cell cytotoxicity. Finally, during my PhD studies I also uncovered a new HIV-1 Env conformation (State 2A) that is vulnerable to antibody attack, rendering cells susceptible to ADCC. Importantly, this conformation is counteracted by Nef and might explain why its ability to downregulate CD4 from the cell surface is highly conserved and important for HIV-1 pathogenesis.

Altogether, the findings presented in this thesis emphasize the potential impact of ADCC in the development of new antiviral approaches while providing a better understanding of HIV-1 mechanisms of immune evasion.

**Keywords:** HIV-1,Env, Nef, CD4, NKG2D ligands, CD4i Abs, ADCC

# RÉSUMÉ

Malgré les énormes efforts pour développer de nouvelles stratégies thérapeutiques contre l'infection par le virus d'immunodéficience humaine (VIH-1), il n'existe à l'heure actuelle aucun vaccin efficace contre ce virus. La prévention de l'infection au VIH-1 exige de comprendre les mécanismes de virulence de ce virus. Il a été montré que les anticorps contre le VIH-1 possèdent une capacité à induire une réponse effectrice dépendante de leur portion Fc. Cette réponse effectrice joue un rôle important dans la prévention de l'infection, et aussi dans la protection observée dans le RV144, le seul essaie vaccinal anti-VIH à avoir démontré un certain degré de protection.

Cependant, les anticorps capables d'induire cette réponse contre le VIH-1 sont connus pour reconnaître les glycoprotéines de surface du virus (Env) dans une conformation dite « ouverte ». Cette conformation est adoptée lors de la liaison d'Env avec son récepteur cellulaire CD4 (épitopes CD4i).

La protéine accessoire Nef joue un rôle important dans la modulation de l'exposition des épitopes CD4i en empêchant l'élimination des cellules infectées. De plus, Nef intervient dans la diminution de l'expression membranaire des molécules impliquées dans les activités clés de la défense immunitaire, comme CD4, CMH-I, CD28 et des ligands de NKG2D. Il a été décrit que chez des patients particuliers et assez rare dits patients contrôleur d'élite (EC) la fonction de Nef est altérée. Ceci conduit à la suppression de la réplication de VIH-1 chez les patients EC en absence de traitement antiviral.

Les travaux de cette thèse se concentrent sur l'étude de la relation entre le rôle affecté de Nef chez les EC et la modulation de l'expression de CD4, les ligands de NKG2D ainsi que la réponse cytotoxique dépendante des anticorps (ADCC). Nous avons démontré que l'incapacité de Nef chez les EC à diminuer l'expression de CD4 sur les cellules infectées conduit à l'interaction de CD4 et Env, ce qui résulte dans l'exposition des épitopes CD4i et donc augmente la susceptibilité des cellules infectées à la réponse ADCC. Nous avons également montré que l'incapacité de Nef de réduire les niveaux des ligands de NKG2D permet l'activation des cellules NK et rende aussi les cellules infectées plus sensibles à l'ADCC. Finalement, mes études doctorales ont permis de découvrir et caractériser une nouvelle conformation d'Env qui est bien reconnue par les anticorps ciblant le domaine interne de la glycoprotéine gp120 et qui interviennent de manière efficace dans la réponse ADCC.

La modulation de la réponse ADCC par Nef représente un axe important de la perspective des réponses effectrices dépendante de Fc. Les études présentées dans cette thèse soulignent l'impact potentiel de l'ADCC sur le développement de nouvelles approches thérapeutiques contre l'infection au VIH-1.

**Mots-clés :** VIH-1, Nef, ADCC, CD4, NKG2D, Env, Fc, EC.

# PREFACE

This thesis was written in accordance with the guidelines of "Preparation of a thesis" from "Graduate and Postdoctoral Studies" of McGill University. I chose to present the study in a "Manuscript-based (Article-Based) thesis" format.

A general introduction about HIV-1 and ADCC responses is presented in Chapter I; Chapter II and Chapter III are based on published manuscripts; Chapter IV is based on a manuscript that has been submitted. I am the first author of all these manuscripts and the contributions of all authors are listed in "Contribution of Authors". Each chapter of Chapter II, III and IV is composed with its own introduction, materials and methods, results, discussion, and references. The references for the whole thesis are listed at the end.

In addition, I have also contributed to nine additional published manuscripts (2 as a 1st author) during my PhD which are not included in this thesis but are listed below:

1) SOSIP changes affect human immunodeficiency virus (HIV-1) envelope glycoprotein conformation and CD4 engagement.
   **Alsahafi N**, Anand SP, Castillo-Menendez L, Verly MM, Medjahed H, Prévost J, Herschhorn A, Richard J, Schön A, Melillo B, Freire E, Smith AB 3rd, Sodroski J, Finzi A. J Virol. 2018 Jul 18. pii: JVI.01080-18. doi: 10.1128/JVI.01080-18

2) Impact of HIV-1 Envelope Conformation on ADCC Responses.
   Richard J, Prévost J, **Alsahafi N**, Ding S, Finzi A. Trends Microbiol. 2018 Apr;26(4):253-265. doi: 10.1016/j.tim.2017.10.007. Epub 2017 Nov 20. Review.

3) Targeting the Late Stage of HIV-1 Entry for Antibody-Dependent Cellular Cytotoxicity: Structural Basis for Env Epitopes in the C11 Region.
   Tolbert WD, Gohain N, **Alsahafi N**, Van V, Orlandi C, Ding S, Martin L, Finzi A, Lewis GK, Ray K, Pazgier M Structure. 2017 Nov 7;25(11):1719-1731.e4. doi: 10.1016/j.str.2017.09.009. Epub 2017 Oct 19

4) Multiparametric characterization of rare HIV-infected cells using an RNA-flow FISH technique.

Baxter AE, Niessl J, Fromentin R, Richard J, Porichis F, Massanella M, Brassard N, **Alsahafi N**, Routy JP, Finzi A, Chomont N, Kaufmann DE. Nat Protoc. 2017 Oct;12(10):2029-2049. doi: 10.1038/nprot.2017.079. Epub 2017 Sep 7

5) Residues in the gp41 Ectodomain Regulate HIV-1 Envelope Glycoprotein Conformational Transitions Induced by gp120-Directed Inhibitors.

Pacheco B, **Alsahafi N**, Debbeche O, Prévost J, Ding S, Chapleau JP, Herschhorn A, Madani N, Princiotto A, Melillo B, Gu C, Zeng X, Mao Y, Smith AB 3rd, Sodroski J, Finzi A. J Virol. 2017 Feb 14;91(5). pii: e02219-16. doi: 10.1128/JVI.02219-16. Print 2017 Mar 1.

6) Co-receptor Binding Site Antibodies Enable CD4-Mimetics to Expose Conserved Anti-cluster A ADCC Epitopes on HIV-1 Envelope Glycoproteins.

Richard J, Pacheco B, Gohain N, Veillette M, Ding S, **Alsahafi N**, Tolbert WD, Prévost J, Chapleau JP, Coutu M, Jia M, Brassard N, Park J, Courter JR, Melillo B, Martin L, Tremblay C, Hahn BH, Kaufmann DE, Wu X, Smith AB 3rd, Sodroski J, Pazgier M, **Finzi** A. EBioMedicine. 2016 Oct;12:208-218. doi: 10.1016/j.ebiom.2016.09.004. Epub 2016 Sep 9.

7) Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals.

Baxter AE, Niessl J, Fromentin R, Richard J, Porichis F, Charlebois R, Massanella M, Brassard N, **Alsahafi N**, Delgado GG, Routy JP, Walker BD, Finzi A, Chomont N, Kaufmann DE.
Cell Host Microbe. 2016 Sep 14;20(3):368-380. doi: 10.1016/j.chom.2016.07.015. Epub 2016 Aug 18.

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

Richard J, Veillette M, Ding S, Zoubchenok D, **Alsahafi N**, Coutu M, Brassard N, Park J, Courter JR, Melillo B, Smith AB 3rd, Shaw GM, Hahn BH, Sodroski J, Kaufmann DE, Finzi A.
EBioMedicine. 2015 Dec 9;3:122-134. doi: 10.1016/j.ebiom.2015.12.004. eCollection 2016 Jan

9) Effects of the I559P gp41 change on the conformation and function of the human immunodeficiency virus (HIV-1) membrane envelope glycoprotein trimer.

**Alsahafi N**, Debbeche O, Sodroski J, Finzi A. PLoS One. 2015 Apr 7;10(4):e0122111. doi: 10.1371/journal.pone.0122111

# CONTRIBUTION OF AUTHORS

## CHAPTER II: NEF PROTEINS FROM HIV-1 ELITE CONTROLLERS ARE INEFFICIENT AT PREVENTING ADCC

**Nirmin Alsahafi**, Shilei Ding, Jonathan Richard, Tristan Markle, Nathalie Brassard, Bruce Walker, George K Lewis, Daniel E Kaufmann, Mark A. Brockman, and Andrés Finzi

Published in *The Journal of Virology*, February 2016

**Author Contribution:** NA conducted all the experiments, and analyzed the data. SD provided assistance with the experimental work and JR provided help with the model design. NB, and DEK provided access to PBMCs. TM, BW, and GKL provided reagents. AF, MAB, and NA prepared the manuscript.

## CHAPTER III: IMPAIRED DOWNREGULATION OF NKG2D LIGANDS BY NEF PROTEIN FROM ELITE CONTROLLERS SENSITIZE HIV-1-INFECTED CELLS TO ADCC

**Nirmin Alsahafi**, Jonathan Richard, Jérémie Prévost, Mathieu Coutu, Nathalie Brassard, Matthew S Parsons, Daniel E Kaufmann, Mark Brockman and Andrés Finzi

Published in *The Journal of Virology*, July 2017

**Author Contribution:** NA conducted all the experiments, and analyzed the data. JR provided help with the model design. JP and MC provided assistance with the generation of Abs and NB, and DEK provided access to PBMCs. AF, NA, JR, MB, and MSP prepared the manuscript.

## CHAPTER VI: HIV-1 ENVELOPE GLYCOPROTEINS SAMPLE A NEW CONFORMATION VULNERABLE TO ANTIBODY ATTACK

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Under review at *Cell Host & Microbe*

**Author Contribution:** NA and NB contributed equally to the work presented in this manuscript. NA, and NB designed, performed the experiments, and analyzed the data. JR provided assistance in statistical analysis and helped in performing *ex-vivo* experiments, SD, SA, and HM provided assistance with the experimental work. GGOD and DEK provided access to clinical samples. SK and ABS provided CD4ms. WM and JS provided valuable discussions. AF, NA, NB, and JM designed the research. AF and JM wrote the manuscript.

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# LIST OF ABBREVIATIONS

Ab: Antibody

ADAP: Adhesion and degranulation-promoting adapter protein

ADCC: Antibody-dependent cellular-cytotoxicity

AIDS: Acquired immunodeficiency syndrome

Ag: Antigen

AP-1: Adaptor Protein -1

AP-2: Adaptor Protein -2

APOBEC: Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide

ART: Anti-Retroviral Therapy

ATCC: American Type Culture Collection

ATP: Adhesion Triphosphate

BnAb: Broadly Neutralizing Antibody

CA: Capsid

CCR5: CC Chemokine Receptor 5

CD: Cluster of Differentiation

CD4i: Cluster of Differentiation 4-induced

CD4m: Compounds-mimicking the binding of CD4 to Env

CDC: Center for Disease Control and prevention

CDK9: Protein kinases-9 cyclin-dependent

cDNA: Complementary deoxyribonucleic acid

CDR: Complement Determining Regions

CMV: Cytomegalovirus

CP: Chronic Progressor of HIV-1 infection

CRF: Circulating Recombinant Forms

CTL: Cytotoxic T Lymphocyte

CTS: Central Termination Signal

CXCR4: C-X-C Chemokine Receptor 4

DC: Dendritic Cells

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleic Acid

dsDNA: Double stranded DNA

EC: Elite Controller of HIV-1 infection

Env: Envelope glycoprotein

ER: Endoplasmic Reticulum

ERAD: ER-Associated Degradation

ESCRT-I: Endosomal Sorting Complex 1 Required for Transport

ESCRT-III: Endosomal Sorting Complex 3 Required for Transport

Fc: Fragment Crystallizable

FcR: Fragment crystallizable Receptor

FDA: Food and drug Administration

Gag: Group-Specific Antigen

GPI: Glycosylphosphatidilinositol

HAART: Highly Active Antiretroviral Therapy

HR1: Helical heptad Repeat 1

HIV-1: Human Immunodeficiency Virus type-1

HIV-2: Human Immunodeficiency Virus type-2

HLA: Human Leukocyte Antigen

HSV: Herpes Simplex Virus

HTLV-III: Human T-lymphotropic virus III

Ig: Immunoglobulin

IFN: Interferon

IL: Interleukin

IN: Integrase

ITAM: Immunoreceptor Tyrosine-based Activation Motif

LEDGF: Lens-Epithelium-Derived Growth Factor

LPS: Lipopolysaccharide

LTR: Long Terminal Repeat

mAb: Monoclonal Antibody

MLV: Murine-Leukemia Virus

MHC: Major Histocompatibility Complex

MIP: Microphage Inflammatory protein

MPER: Membrane Proximal External Region

mRNA: Messenger RNA

MSM: Men having Sex with Men

NC: Nucleocapsid

Nef: Negative Regulatory Factor

NES: Nuclear Export Signal

NFAT: Nuclear Factor of Activated T cells

NK: Natural Killer

NKG2D: Natural Killer Group 2D

NKG2D-L: Natural Killer Group 2D Ligand

NLS: Nuclear Localization Signal

NRTI: Nucleoside Reverse-Transcriptase Inhibitor

NNRTI: Non-Nucleoside Reverse-Transcriptase Inhibitor

NF-$\kappa$B: Nuclear Factor-kappa B

ORF: Open Reading Frame

PAC2: Phosphofurin acidic cluster sorting protein 2

PI: Protease Inhibitor

PI3-k: Phosphoinositide kinase-

PIC: Pre-Integration Complex

PKF: Protein Kinase Family

Pol: Polymerase

PR: Protease

PVR: Polio Virus Receptor (CD155)

# ACKNOWLEDGMENT

*This thesis is dedicated to the memory of my Father, Mohammed Alsahafi. Papa, I miss you every day, but I am glad to know you saw this process through to its completion, offering the love and support to make it possible, as well as plenty of encouragement.*

The completion of my PhD-Thesis represents a critical step in my professional and personal development. In this sense, I would specially like to thank Dr. Andrés Finzi for welcoming me to his research team. His unequivocal support throughout my journey in his laboratory is appreciated at the highest point. I consider myself very fortunate to have had the opportunity to do my PhD under the supervision of a researcher as talented and dynamic as Dr. Finzi. His scientific vision has certainly inspired me and will continue to positively affect the rest of my academic and professional paths.

It is important to mention that the work presented in this thesis could not been done without the input of Dr. Finzi who I can't thank enough for everything.

I would like to express my gratitude to my co-supervisor, Dr. Martin Olivier, for his constant support, availability, and encouraging words.

Special thanks to Dr. Jonathan Richard, I really appreciated your critical thinking and your kind valuable discussions. Certainly, my PhD- studies would not have been the same without your presence and your valuable advice. Thank you Jonathan, I really enjoyed working with you.

Thank you to Dr. Shilei Ding for your great support, your good mood and your practical advice on science and life.

I would also like to specially thank Halima Medjahed her help in organizing the laboratory as well as her great sense of teamwork. I would also like to highlight her sense of camaraderie, which brings joy into the daily routine. I greatly appreciate the friendship we have both in the laboratory and outside. A big thank you to the other members of the laboratory, present or past, who have been able to support me and help me at various stages of my PhD: Laurie-Anne, Olfa, Maxime, Mathieu, Daria, Myriam, Jeremie, Sai Priya, and Gabrielle.

Without a doubt, the work presented here could not have been done without the help of various collaborators as well as the financial organizations. In that sense, a special thank you to the ministry of higher education of KSA and King Abdullah Scholarship for funding my doctoral studies.

Finally, a thousand of thanks to my family and friends for the support and patience they have shown during these long hours of work dedicated to my doctoral studies. Thank you Hisham for your presence, understanding, unconditional support and encouragement during all times. A special thank you to my Mom, Myriam, for her support, encouragement and unconditional love.

# CHAPTER I

# INTRODUCTION

The emergence of novel infectious diseases is often associated with pathogens evolving in new host species [1, 2]. The explosion and development of human society over the last hundred years have exposed us to transmission of numerous cross-species pathogens, creating a continuous threat to public health. Recent examples of health crises present in our daily lives include the viral outbreaks of Zika in South and North America, Ebola in West Africa and MERS-CoV (Middle East Respiratory Syndrome) in the Middle East. Among others, Human Immunodeficiency virus type-1 (HIV-1) is one of the best examples of such a virus emerging from non-human primates and with the ability to adapt and spread causing one of the most devastating pandemics in recent human history [3]. Thanks to the great global efforts to battle this virus, there have been significant impacts in reducing the spread of HIV-1 infection since the beginning of the epidemic. However, the most recent data from UNAIDS (United Nations on HIV/AIDS) [4] raises a worrisome alarm, revealing that more than 36 million people are currently living with HIV-1, and around 1.8 million more are still newly infected every year.

## 1.1 HIV-1 and acquired immunodeficiency syndrome (AIDS)

### 1.1.1 Viral Classification

HIV-1 belongs to the family of retroviruses (*Retroviridae*), which is comprised of a large and diverse group of viruses found in all vertebrates. This family of viruses replicates through an extraordinary and unique life cycle that sharply differentiates them from other viruses. The virion particles generally contain a genomic single-stranded

ribonucleic acid (ss-RNA) which uses virally encoded reverse transcriptase (RT) to allow the RNA conversion into double-stranded deoxyribonucleic acid (DNA). Indeed, the RNA-DNA conversion and the DNA integration into the host genome subsequent define retroviruses [5]. The genomic organization of retroviruses classify them into simple and complex viruses. Simple retroviruses contain three main genes: *gag* (*group-specific antigen*), *pol* (*polymerase*), and *Env* (*envelope*), while complex retroviruses are coded for additional regulatory and auxiliary genes. Although these three essential genes are sufficient to provide the structural and enzymatic components for viral replication, the presence of non-structural regulatory proteins is necessary for the survival of complex retroviruses, directing viral replication and pathogenicity [5-7]. These properties are found in several genera in the retrovirus family, including lentiviruses in which HIV-1, HIV-2 and simian-immunodeficiency virus (SIV) are categorized. Retroviruses, as their name in Latin implies, are slow viruses with a long incubation period that causes chronic infections and deadly diseases [5].

### 1.1.2 Origin of the virus

Ever since the identification of AIDS in the 1980s, the origin of the virus had been a subject of an extensive research and debate for years. AIDS was recognized in 1981 as a new disease that caused morbidity and mortality among previously healthy males in urban areas of the United States of America (USA), such as Los Angeles, San Francisco, and New York. The affected individuals showed severe impairment of their immune system as a result of generalized lymphadenopathy and opportunistic infections such as Pneumocystis jiroveci pneumonia, cytomegalovirus retinitis (CMV), and

cryptococcal meningitis. Patients were also observed to have greater susceptibility to develop unusual types of cancers such as non-Hodgkin's lymphomas or a Kaposi's sarcoma. Furthermore, these symptoms were associated with a profoundly depressed number of T-lymphocytes particularly a cluster of differentiation-4 (CD4 T cells) [8-10]. With the subsequent emergence of epidemiological data on AIDS, it became clear that the syndrome was not only affecting MSM (men who have sex with men) in the USA. Indeed, shortly after AIDS awareness, additional infections were observed among drug users [11], heterosexual couples in Africa [12, 13], patients receiving frequent blood transfusions [14, 15], and babies born to infected mothers [16]. These findings directed the medical and scientific community to hypothesize that this emerging epidemiological pattern was caused by an unidentified transmissible infectious agent through sexual contact or inoculation of blood or blood products with an affected individual [17].

Shortly after its initial description, the causative agent of AIDS was identified in the spring of 1983 by Luc Montagnier, Françoise Barré Sinoussi and their colleagues at the Pasteur Institute in France. The groups reported the isolation of a pathogen from the lymph nodes of a asymptomatic individual, presented with lymphadenopathy syndrome of unknown origin [18]. Additional characteristics of the isolated agent indicated its belonging to the retrovirus family, based on its replication in cell culture and electronic microscope (EM) features. Shortly after, subsequent isolation of a retrovirus from an AIDS patient was reported, that was named human T-cell leukemia virus type III (HTLV-III), and the first serologic evidence was obtained by Gallo and colleagues at the National Institute of Health (NIH) [19, 20]. The new isolated retrovirus was associated with AIDS

development in the USA, Europe, and Central Africa and is now known as Human Immunodeficiency virus or HIV (and subsequently HIV-1) [21]. A related yet immunologically distinct human retrovirus, known as HIV-2, was isolated in 1986 from individuals residing in several countries in West Africa such as Senegal, Ivory Coast, and Guinea [22].

The global genetic diversity of HIV-1 has helped scientists to trace the origin of the virus and imply the pre-existence of the virus among humans for decades before its isolation. High mutation and recombination rates during HIV-1 replication have created a high degree of sequence divergence, dividing HIV-1 into four groups: M (main), O (outlier), N (non-M and non-O), and P that showed various contributions to the epidemic [23]. Group M is responsible for the global pandemic, and is further subdivided into nine subtypes/ clades (A, B, C, D, F, G, H, J, and K) and numerous circulating recombinant forms (CRFs) [24]. The discovery of lentiviruses from nonhuman primates, termed simian immunodeficiency virus (SIVs), with a genomic structure similar to that of HIV-1 opened the possibility of zoonotic transmission to humans [3, 25]. An additional virus closely related to HIV-1 was isolated from two chimpanzee subspecies, *Pan troglodytes troglodytes* (*ptt*) and *Pan troglodytes schweinfurhii* [26, 27]. It was then confirmed that HIV originated from multiple zoonotic transfers of SIVcpz*Ptt* from monkeys to humans around the 1920s, and HIV-1 groups were derived from independent transmissions [28, 29]. HIV-1 groups M and O originate from the SIVcpz*Ptt*, while HIV-1 group N, and P are related to SIV from gorilla (SIVgor) [25, 28, 30, 31].

### 1.1.3 Viral Structure

The HIV-1 viral particles have a diameter ranging between 100-to-120 nM and are surrounded by a lipid bilayer that carries glycoprotein complexes, which are each composed of trimers of an external glycoprotein (gp120) non-covalently attached with a transmembrane spanning protein (gp41) [18, 32] (Figure 1.1). The inner surface of the viral membrane is lined with matrix protein (MA, p17), which forms the outer edge of the spherical shell and remains attached to the envelope. The centre of the virion is a condensed conical core composed of capsid (CA, p24) protein, enclosing the viral RNA. The p24 core carries two linear copies of positive sense single stranded RNA (ssRNA+), that are coated with nucleocapsid (NC, p7) proteins. The viral particles also contain all the enzymatic proteins essential for viral replication, which includes a reverse transcriptase (RT), an integrase (IN) and a protease (PR) [33-35]. The RT retrotranscribes the genomic ssRNA into double stranded DNA. This RT-dependent DNA synthesis is initiated by the binding of a cellular transfer RNA ($tRNA^{Lys3}$) primer, that is selectively packaged into the viral particles during assembly, to the primer binding site (PBS) within the viral RNA genome [36-38]. The HIV-1 virions also incorporate several accessory proteins that are believed to facilitate the early steps of infection, including the viral protein R (Vpr) [39], the viral infectivity factor (Vif) [40], and the negative regulatory factor (Nef) [41, 42]. During the process of budding, the virus incorporate various cellular proteins, that facilitate viral adhesion and infectivity, such as HLA class I and II proteins [43, 44], or the adhesion protein ICAM-1 [45].

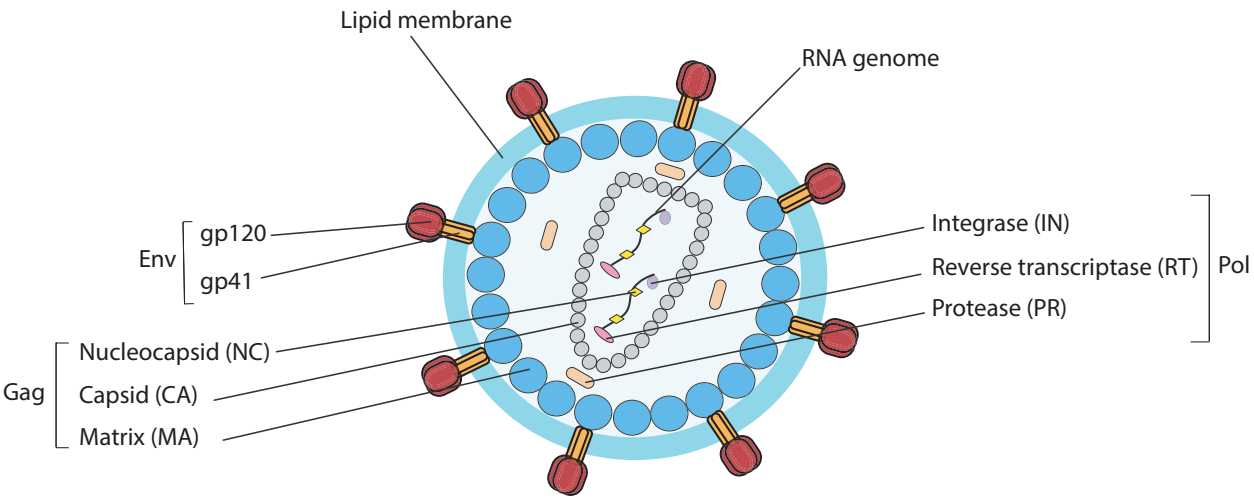


**Figure 1.1 Diagram of HIV-1 mature virion particle**
Schematic view of the mature HIV-1 particle detailing the localization of viral proteins and the virion structure.

### 1.1.4 Organization of HIV-1 Genome

As all lentiviruses, HIV-1 carries its genomic material in the form of a 9.6 kb ssRNA+ with nine open reading frames (ORF) coding for fifteen distinct viral proteins (Figure 1.2) [46, 47]. Three of these encode for *gag*, *pol*, and *env* polyproteins, which are essential components to all retroviruses, and are initially synthesized as polyproteins precursors that are cleaved into their different proteins by the viral PR or cellular proteases. The Gag precursor ($Pr55^{Gag}$) is cleaved by the viral PR into a series of products to form the main structural components of the virion core that are highly conserved among retroviruses and includes MA, CA, NC, and p6 [48, 49]. Cleavage of $Pr55^{Gag}$ also generates two short spacer peptides, SP1 and SP2 which are known to influence the sequential proteolytic processing of Gag for a proper viral assembly [50, 51]. The *pol* gene lacks an initiation codon and is expressed as a part of Gag-Pol (Pr160-Gag-Pol) precursor which is synthesized by a ribosomal frameshift. Cleavage of Pr160-Gag-Pol by the viral protease produces the viral enzymes, PR, RT, and IN [52, 53]. The

HIV-1 Env is synthesized as a glycoprotein precursor (gp160) that is proteolytically cleaved by host furin protease into the gp120 surface and the gp41 transmembrane subunits [54]. The viral genome also encodes for six additional proteins that have auxiliary and/or regulatory functions: Vif (*virus infectivity factor*), Vpr (*viral protein R*), Vpu (*viral protein U*), Nef (*negative regulatory factor*), Tat and Rev [47].

The viral DNA is flanked at both ends by a repeated sequence termed the long terminal repeats (LTRs) that contain binding sites for cellular transcription factors. These LTRs have major roles in the integration of viral DNA into the host genome, in the regulation of viral gene transcription, and in the viral RNA packaging [55, 56]. The LTRs are composed of three subregions that are distinguished as U3, R, and U5. Once the viral genome is integrated, the LTR on the 5' end serves as a promoter for gene transcription. The U3 region of LTR contains a DNA binding site for several cellular transcription factors that are required for viral genome transcription, such as NF-κB [57, 58]. The HIV-1 genome also contains non-coding RNAs (ncRNAs) that originate from both the virus as well as the host cells. The nRNAs are emerging as new players of the viral replication and latency [59]. For instance, the upregulation in the expression of miR-132 was shown to promote viral replication and reactivation of latently infected cells [60].

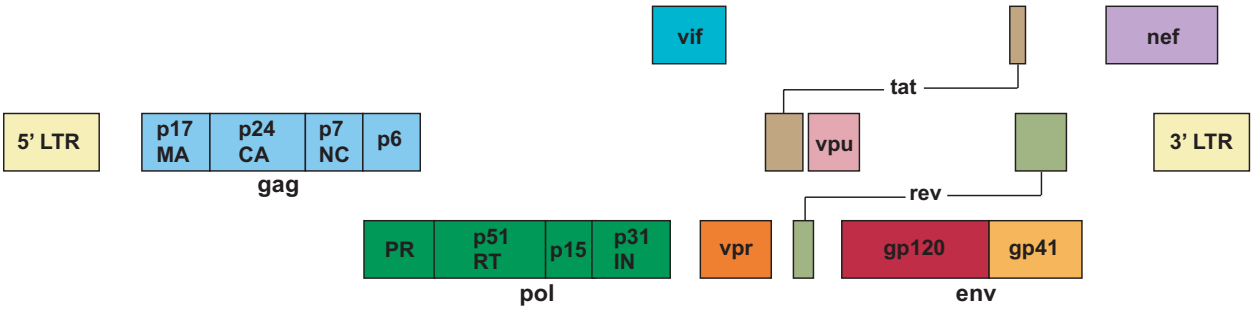


**Figure 1.2 HIV-1 genomic organization**
The diagram consists of the 5' end and 3' end of the long terminal repeats (LTR) and represents the order in which HIV-1 genes are arranged.

### 1.1.5 HIV-1 Replication Cycle

As other retroviruses, the HIV-1 life cycle proceeds in a series of events that can be divided into two distinct phases: “early” and “late”. The early phase ranges from cell binding to integration of the provirus DNA into the cell genome while the late phase starts with the expression of viral genes and continues through to the viral release and maturation of the progeny virions (Figure 1.3).

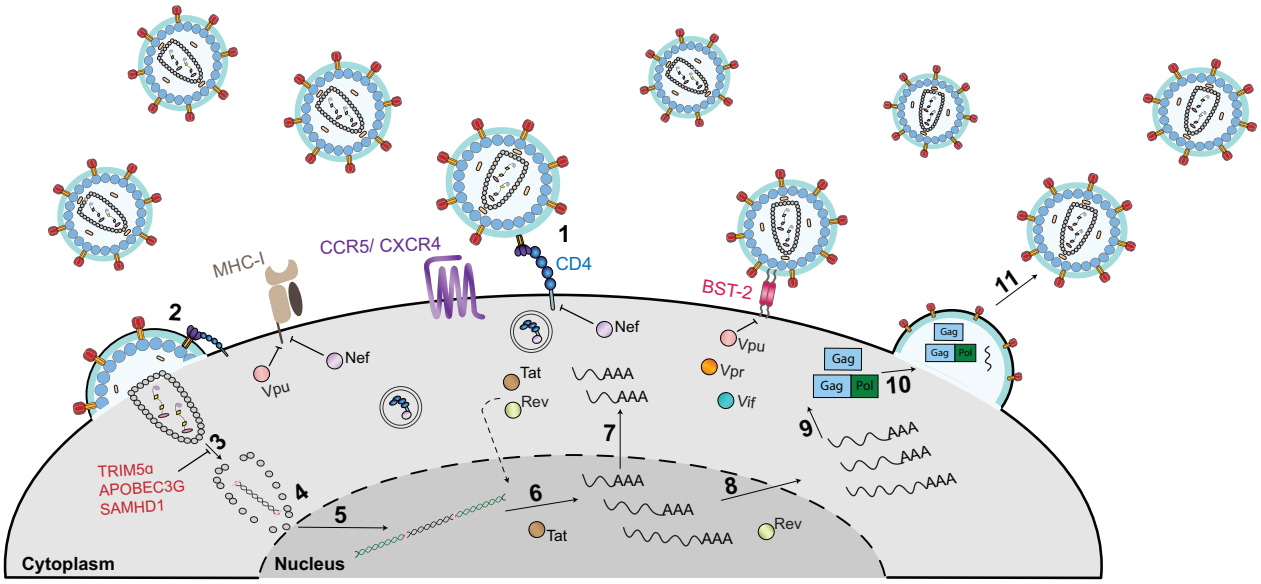


**Figure 1.3. HIV-1 Replication Cycle**
Schematic representation of the main stages of viral replication: (1) binding, (2) viral entry as a result of the viral and host membranes fusion, (3) decapsidation, reverse transcription, and translocation to the nucleus, (4) the finalization of reverse transcription and decapsidation, (5) integration, (6) transcription of viral mRNAs, (7) the export of mRNA and early translation, (8) the export of Rev dependent mRNAs, (9) the translation of viral proteins, (10) viral assembly, and (11) viral release and maturation.

#### 1.1.5.1 Viral Entry

The initial step of HIV-1 infection begins with the specific protein-protein interactions mediated by the surface glycoprotein gp120 of the mature particle and its CD4 receptor on the target cell [61]. The CD4 receptor is present on the surface of T helper (CD4+) cells, macrophages, dendritic cells, and astrocytes; however, its expression alone is not sufficient to render these cells permissive to HIV-infection [62-64]. These entry's obstacles were further explained by the discovery of the presence of the chemokine receptors CCR5 or CXCR4, which act as a co-receptor necessary for viral fusion [65, 66].

The fusion of the vial membrane with the host membrane requires a sequential multi-steps process. The initial step involves the interaction between gp120 and the CD4 receptor which induces conformational changes in the gp120, leading to the repositioning of the variable loop-3 (V3) is otherwise buried in the trimer [67-69]. This transition within the V3 loop mediates the interaction of gp120 with its co-receptor based on virus tropism. Thus, viruses using the CCR5 are denoted as R5 isolates; those preferentially using CXCR4 are referred as X4 tropics, and dual-tropic strains using both co-receptors are named R5X4 isolates [70]. After the formation of a gp120-CD4-coreceptor complex, gp41 exposes a hydrophobic region known as the fusion peptide, which allows the penetration into the target lipid bilayer, and the formation of the pre-hairpin intermediate, in which the heptad repeat 1 (HR1) coiled coil is assembled and exposed [71-74]. The subsequent interaction of this coiled coil with its complementary HR2 region results in the assembly of the six-helix bundle, bringing viral and target cell membranes together to mediate viral fusion [75, 76]. Fusion of the viral core allow the penetration of the viral core into cytosol [77].

#### 1.1.5.2 Post-entry early replication phase

Once inside the cell, the viral core undergoes a gradual and progressive disassembly “termed uncoating” which is essential for HIV-1 reverse transcription and nuclear import [78, 79]. During this multistep process the structural organization of the viral core is changed. The precise timing and cellular location of the uncoating process however are still subjects of debate. While some studies suggest that this process takes place in the cytoplasm, others believe that it occurs upon the viral core’s arrival to the

nuclear pore [79-81]. It was also suggested that the core uncoating begins within an hour of viral fusion and proper uncoating is linked to efficient reverse transcription [78, 82-84].

The retroviral genome is encoded into the virion as two copies of ssRNAs that is converted by the virion-packaged RT into a double-stranded DNA (dsDNA). A schematic of this process is depicted in Figure 1.4. Briefly, the RT functions as a heterodimer composed of 66 kDa (p66) and 51 kDa (p51) subunits that are derived from the $Pr160^{GagPol}$ precursor protein; the p51 is formed when the C-terminal of p66 is removed by PR [85, 86]. The p66 subunit possesses a DNA polymerase domain and an endonuclease (RNase H ) domain while the p51 subunit is only composed of the polymerase domain [87]. All three RT enzymatic activities are essential for the achievement of RNA-DNA conversion. Briefly, the minus-strand DNA synthesis is initiated at the 3′end by the cellular $tRNA_3^{Lys}$ that is selectivity incorporated into the virion, acting as a primer for reverse transcription (step1) [88, 89]. The DNA synthesis proceeds to the 5 'end of the genome while the RNaseH activity of RT digests the RNA of the newly formed RNA-DNA hybrid, thus freeing a single stranded DNA fragment (step2) (known as the minus-strand strong-stop DNA). This DNA fragment is then transferred to the 3′end of the genome, where it hybridizes via the repeated R region presented at both 5′and 3′ ends of the viral genome (step3). The ssDNA synthesis continues, accompanied by RNaseH-mediated RNA degradation of the resulting RNA-DNA hybrid (step4). However, fragments of RNA that are relatively resistant to removal by RNaseH, and contain a short polypurine tract (PPT) serve as primers for positive-DNA strand synthesis (step 5) [90]. The RNaseH of RT then removes the primer tRNA that was used for the plus-strand DNA

synthesis. This exposes the primer binding site (PBS) at the 3′ end of the positive polarity stranded DNA (step 6); thus, allowing its hybridization with the homologous region at the 3′ end of the negative polarity stranded DNA (known as second-strand transfer) (step 7). Synthesis of the positive-and negative-strands proceed to completion, with each of the strands of DNA serving as a template for the other one. The termination site of this synthesis is located near the centre of the genome and referred to as the central termination signal (CTS) [91]. However, the position of the central PPT upstream of the CTS leads to the displacement of nearly 100 nucleotides of the positive-strand DNA, resulting on the formation of a DNA *flap* that is believed to play a crucial role in the import of the viral DNA to the nucleus [92, 93].

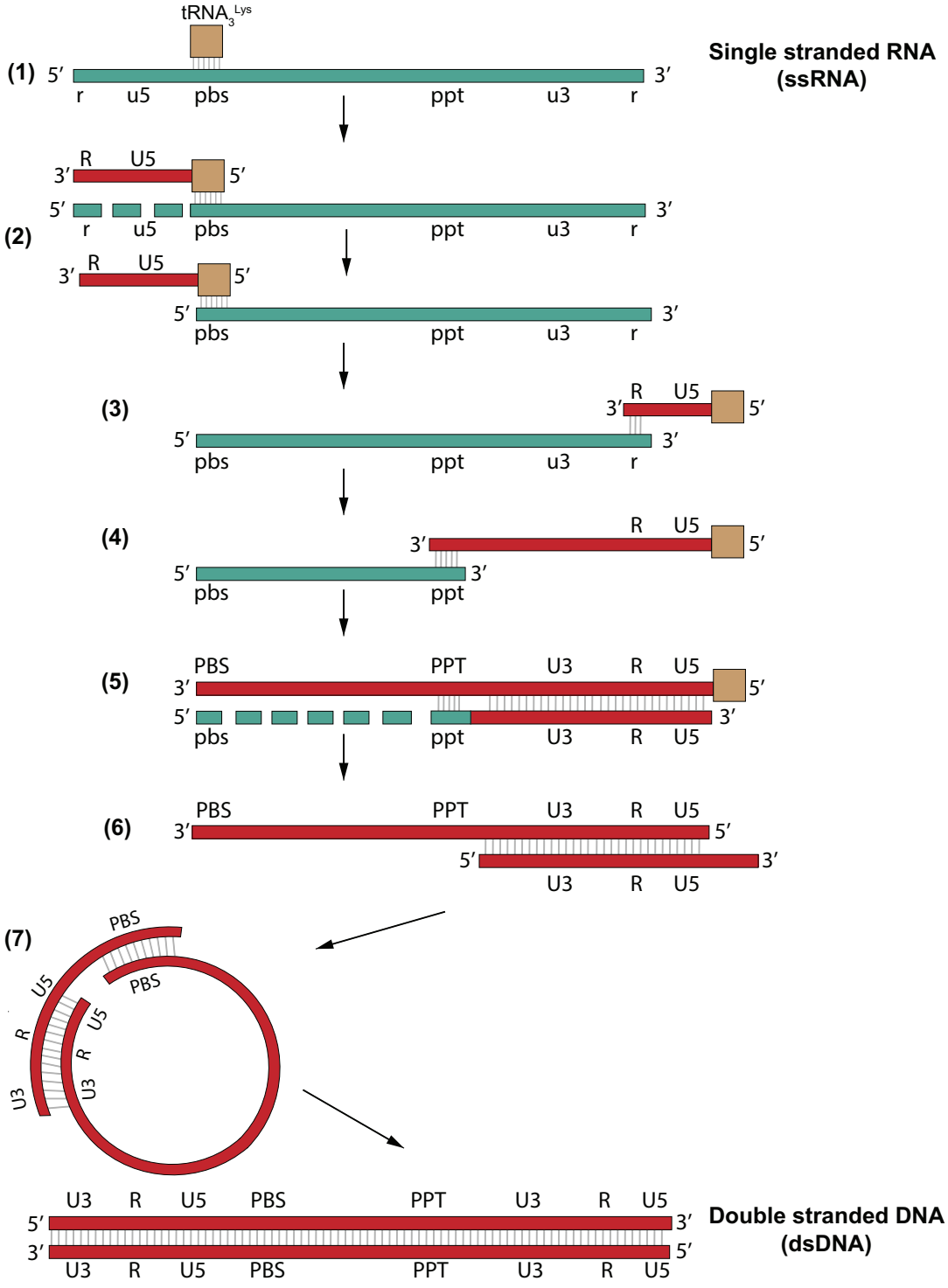


**Figure 1.4 Reverse transcription process of HIV RNA into dsDNA**. A schematic presentation of the main events of the reverse transcription of single-stranded RNA to the double-stranded DNA, as described in section 1.1.5.2. Adapted from [94].

During the events leading to its integration, the newly generated viral DNA remains associated with viral and cellular proteins in a large nucleoprotein complex that forms the pre-integration complex (PIC). The protein components of the PICs include viral NC, MA, RT, IN, and Vpr that are believed to contribute to its import into the nucleus [95-98]. However, the large size of the PIC, as estimated to be ~ 56 nM in diameter, limits its passive diffusion through the nuclear pores (~9 nM). This led to the probability that an active import of the PIC into the nucleus requires co-factors interplay between viral and cellular compartments. Several models have proposed the involvement of one or more of the viral proteins (MA, Vpr, and IN) or the DNA *flap* in the import of PIC, this remains controversial. Nevertheless, studies on an HIV-1 chimera-coding for MLV, which is incapable of infecting non-dividing cells, have revealed that both IN and CA components of PIC contribute to retain MLV's ability to infect nondividing cells [99, 100]. Another significant role of HIV-1 CA in this complex process is its ability to interact with host factors involved in promoting the genome transport that are essential for viral infectivity (TNPO3, NUP153, NUP358) [101-103]. Indeed, point mutations in the CA protein can disrupt these interactions with the nucleoproteins and the subsequent transfer to the nucleus [103, 104].

Once the linear double stranded viral DNA is inside the nucleus, its integration reaction into the host chromosome is carried out by IN, which requires the presence of a ubiquitous nuclear protein LEDGF/p75 (lens epithelium-derived growth factor) to increase its binding to DNA [105]. The integration process is initiated when the viral IN clips off two nucleotides from the 3' termini of both strands of viral DNA, thus generating a

molecule of double-stranded DNA with a 3′-recessed end. Additionally, IN catalyzes the cleavage of cellular DNA; allowing the 3′-recessed ends of viral DNA to covalently join the cleaved end of the cellular genome in a reaction known as a strand transfer. The integration process comes to completion as the cellular repair machinery fills the gaps between the viral and the host target DNA [96, 106, 107].

#### 1.1.5.3 Post-entry late replication phase

Following integration, the viral integrated DNA is referred to as “provirus” that behaves like a cellular gene. This provirus then serves as a template for the viral RNA synthesis that ultimately encodes for all structural, regulatory, and accessory proteins. It was well demonstrated that HIV-1 transcription depends on varied and complex interactions between its regulatory elements in the LTR and inducible host transcription factors that include SP1 (specificity protein-1), NF-kB (nuclear factor-kappa-light-chain-enhancer of activated B cells), AP1 (activator protein-1), and NFAT (nuclear factor of activated T cell) [57, 108-110]. The transcription process initiates through the recruitment of RNA polymerase II (pol II RNA), which binds to the HIV-1 promoter. The transcription factors, SP1 and NF-kB are located approximately 25 nucleotides upstream of the transcriptional initiation site and their removal abolishes viral replication [111]. In HIV-1, SP1 plays an important role in the initiation of basal transcriptional levels of HIV LTR [112], which ultimately results in the generation of a distinctly complex pattern of spliced RNA transcripts; giving rise to the Tat, Rev, and Nef proteins [113, 114].

The Tat protein is a key trans-activator of HIV-1 gene transcription that stimulate the expression of all genes linked to the viral LTR by enhancing the processivity of pol II RNA [115-117]. It consists of 101 amino acids encoded by two exon-RNA that rely on its binding to specific sequences located within the 5′ end LTR, known as TAR (Transactivation Response Element) to mediate its functions. The Tat-TAR interaction leads to the recruitment of cyclin T1 protein, cyclin-dependent protein kinase-9 (Cdk9), and the transcription elongation factor b (pTEF-b), thereby catalyzing the C-terminal phosphorylation of the Pol-II RNA, leading to increased its activity [115-119]. The mutagenesis of the *tat* gene severely impairs proviral transcription, therefore affecting viral replication [117, 118].

In the early stages of viral gene expression, only the completely spliced mRNAs are exported to the cytosol, these code for Tat, Rev and Nef proteins. Subsequently, the Rev protein binds to a specific sequence located in all singly-spliced and unspliced viral RNAs termed Rev Responsive Element (RRE) to mediate their nuclear export to the cytoplasm. The viral Rev protein consists of 116 amino acids encoded by two exons, both of which are necessary for its interaction with RRE [119]. Rev contains a leucine-rich nuclear export signal (NES) that allows Rev to shuttle between the nucleus and cytoplasm. Briefly, the binding of Rev-RRE recruits the cellular chromosome maintenance gene-1 (CRM-1) and RanGTP proteins (Ran guanosine triphosphate) through its NES; thus, forming a complex that interacts with the nuclear pore machinery allowing their release into the cytoplasm [120-123]. The exported mRNAs are then be translated into the various viral proteins or the genomic RNA is encapsidated into assembling viral

particles. Notably, the optimal ratio generated of the structural proteins encoded by the *gag* gene and the enzymatic proteins included in the Gag-pol precursor (estimated as 20:1, conserved among retroviruses) is ensured by a frameshifting event at the end of the translation of the Gag precursor [124]. This Gag/Gag-Pol ratio is important for the generation of infectious viral particles and the stability of the virion RNA dimer [125-127].

Viral assembly takes place at the plasma membrane of infected cells [128-131]. The multi-domain Gag polyprotein is responsible for all necessary events of viral assembly and budding that includes encapsidation of the viral genome and recruitment of the necessary cellular factors for viral budding. Gag precursor represents the structural base of the viral particle and itself alone is sufficient for the formation of non-infectious virus-like particles (VLPs). The production of infectious viral particles, however, requires the presence of the Gag-Pol precursor, the incorporation of Env, and the packaging of the viral genomic RNA [132, 133]. The MA subdomain of Gag/Gag-Pol polyprotein has a myristoylated domain essential for Gag localization and binding to the plasma membrane [134-136]. The NC subdomain of Gag also interacts with the genomic RNA to initiate Gag multimerization and forms the NC-RNA complexes that are critical for Gag dimerization, an essential step for the formation of VLP [137-141]. The process by which the HIV-1 Env is incorporated into the virus particle remains unclear, but several models have been invoked to explain it. These strategies involve: (1) the accumulation of Env glycoprotein at the plasma membrane, resulting in its passive incorporation into the VLPs. (2) The direct interaction of the MA domain of Gag with the cytoplasmic domain (CT) of the gp41 subunit of Env could allow its incorporation. (3) The co-recruitment of Gag and Env to the

same site of the plasma membrane; thus, enhancing Env incorporation. (4) The existence of a host cell adaptor protein (AP-1 and AP-2) that allows indirect Gag-Env binding and incorporation [142-145].

The latest step of viral assembly involves the budding and release of viral particles in a process that is orchestrated by the sub-domain p6 of Gag. Mutational studies have mapped a conserved Pro-Ala-Pro (PTAP) motif of P6 domain that is required for recruiting the ESCRT machinery (endosomal sorting complex required for transport), which is made up of numerous multi-protein complexes and accessory factors to mediate membrane budding and scission processes in the producing cell [146-148]. The newly released viral particle undergoes a maturation process to become infectious [149]. This process occurs during or shortly after viral release and is initiated by PR domain of the Gag-Pol precursor. The viral PR sequentially cleaves Gag and Gag-Pol polyprotein precursors to generate the mature forms of Gag and Pol proteins, consisting of MA, CA, NC,P6, PR, RT, and IN [150]. The specificity and efficiency with which the viral PR cleaves its target sites dictates a processing cascade that is required to produce a fully infectious virus particle [50, 151, 152]. The viral PR-mediated Gag and Gag-Pol processing results in major changes in virion morphology. In the immature particles, Gag and Gag-Pol polyproteins are assembled in a radical manner that is reassembled to form the conical capsid shape feature of retroviruses in mature particles [153-155].

### 1.1.6 HIV-1 Accessory Proteins

#### 1.1.6.1 Nef

Nef is a small myristoylated protein of 27-34 kDa that is highly conserved among all primate lentiviruses. Nef is expressed early during the viral replication cycle and is described in two forms; a membrane myristoylated form and a non-myristoylated cytoplasmic form both of which are critical for its multiple roles in viral infection. It is an important player in the viral replication cycle and during disease progression to AIDS [156, 157]. Nef is extremely important for viral replication *in vivo* as shown by patients infected with viruses lacking or harboring a defective form of Nef, which shows no or very slow rates of disease progression (called long-term-non-progressors-LTNPs) [157-160]. A number of functions have been associated with HIV-1 Nef that include its ability to down-modulate a wide array of cell surface molecules such as CD4, major histocompatibility complex class-I (MHC-I), CD8, CD3 T cell receptor complex, costimulatory CD28 molecule, NKG2D ligands, and others [161, 162]. Nef also increases viral infectivity and replication, it is known to alter cellular signal transduction and activation. Many of these functions are linked to a general disruption of the endosomal trafficking pathways that are induced by Nef [163, 164]. These immunoregulatory effects of Nef will be addressed in detail in section (**1.1.11**).

Nef also plays a positive role in viral spread through its effects on antigen presenting cells (APCs). Nef increases the expression of a dendritic cell-specific lectin receptor (DC-SIGN), which mediates capture of infectious viral particles transmit them to permissive CD4+ T cells. This ability of Nef to upregulate the surface expression of DC-

SIGN is thus believed to facilitate viral transmission [165-167]. In macrophages, Nef triggers the secretion of the CC-chemokines macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β), which leads to the recruitment and activation of resting target cells at the site of viral replication [168-170].

The mechanisms by which Nef increases viral infectivity have been a subject of intensive research. Several possibilities were raised to explain the role of Nef in viral infectivity in a CD4-independent manner, including the involvement of dynamin 2 (Dyn2), a key regulator of vesicular trafficking, and the alteration of the early endosomal compartments and their recycling [171, 172]. In the last few years, new findings have emerged revealing the ability of Nef to interfere with cellular serine incorporator 3 and 5 proteins (SERINC3 and SERINC5) preventing their incorporation into virus particles, which otherwise would block the viral post-entry replicative cycle [173, 174]. Finally, Nef interacts with cellular signal transduction pathways and can influence infected cell activation states. The effect of Nef on cell activation is in part related to its interaction with Ser/Thr kinase p21- activated kinase 2 (PAK2), which reportedly occurs in lipid rafts and results in recruitment of Nef to the immunologic synapse [175-177]. The Nef-PAK2 co-localization leads to up-regulation of the expression of transcription factor nuclear factor of activated T cell (NFAT), and ultimately results in cell activation and viral genome transcription [178].

#### 1.1.6.2 Vif

Vif is a cytoplasmic 23 kDa protein that is expressed late in the viral replication cycle and is known to have a critical role in the production of infectious virions. Vif can enhance HIV-1 replication in certain cell types that include T-cells, macrophages, and transformed T-cell lines [47, 179, 180]. This cell type specificity of Vif effect on the productivity of infectious viruses led to the identification of a cellular restriction factor known as APOBC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide like 3G) that plays a role in Vif's Function. APOBEC3G is a member of the APOBEC family of editing enzymes that can mutate polynucleotides by deaminating cytidine (C) bases to uridine (U) in the negative ssDNA [181-183]. In the absence of Vif, APOBEC3G is incorporated into the newly produced virions [184-186], and ultimately leads to the accumulation of a G to A hypermutation in the positive polarity strand [187-189]. In addition to its ability to induce hypermutation, APOBEC3G can directly impact the reverse transcription process by inhibiting the elongation of HIV-1 reverse transcripts [190]. HIV-1 Vif effectively antagonizes this antiviral effect of APOBEC3G through its ability to recruit a cullin-RING-ligase and the induction of polyubiquitylation and degradation of APOBEC3G using the ubiquitin-proteasome machinery [191, 192].

#### 1.1.6.3 Vpr

Vpr is a 14 kDa accessory protein that is incorporated into viral particles through its direct interaction with the p6 domain of Gag and is believed to be critical for the early steps of the viral life cycle [193-196]. Vpr is especially important for its ability to regulate the induction of cell cycle arrest in the G2 phase of infected proliferating cells [197, 198].

The packaging of Vpr protein into both infectious and non-infectious viral particles is responsible and sufficient to mediate cell cycle arrest [199]. However, the mechanism underlying Vpr-mediated-G2 arrest remains controversial and would requires activation of the ATR (ataxia-telangiectasia and Rad3-related) pathway to promote the DNA-damage pathway [200, 201]. Studies have documented that the interaction of Vpr with the Vpr-binding protein (VprBP) allows recruitment of the CLU4A E3 ubiquitin ligase complex (Clu4A/ DDB1/VprBP), causing polyubiquitination and degradation of mini-chromosome maintenance-10 proteins (MCM10 protein), a DNA replication factor. This Vpr-mediated depletion of MCM10 protein has been suggested to be related to ATR activation and induction of G2/M arrest [202-205]. In addition, HIV-1 Vpr can selectively induce the cell surface expression of specific activating NKG2D ligands, ULBP-2, in infected or non-infected cells; thus, promoting efficient recognition and NK cells mediated lysis [206].

Several other activities of Vpr have also been a subject of increased research such as, the nuclear translocation of the PIC, transactivation of the HIV-1 LTR, modulation of gene expression, induction of apoptosis, fidelity of reverse transcription, and suppression of immune activation [207]. The involvement of Vpr in the nuclear translocation of the PIC was among the first identified functions of this protein [208]. This phenomenon is facilitated through the interaction of Vpr with importin-α, permitting the localization of viral nucleic acids into the nucleus of non-dividing cells [209-211]. Vpr also has the ability to transactivate the HIV-1 LTR as well as other cellular promoters [212]. This increase in transcription is partially linked to the non or nearly integrated viral DNA

[213, 214]. Several studies have shown that HIV-1 Vpr has the ability to induce apoptosis during HIV-1 infection in both host cells and bystander uninfected cells. This apoptosis process seems to be dependent on the ATR activation pathway and linked to the Vpr-mediated G2/M arrest [215-217].

#### 1.1.6.4 Vpu

Vpu is 16 kDa type 1 transmembrane protein that is a distinguishable feature of HIV-1 lineage of primate of lentiviruses. It is absent from HIV-2 and rarely expressed in some SIVs [27, 218, 219]. Vpu is an oligomeric multifunctional protein composed of an N-terminal hydrophobic membrane anchor followed by a hydrophilic cytoplasmic C-terminal domain [220, 221]. The cytosolic domain of Vpu comprises two α-helical regions that are interconnected by a flexible loop containing a highly conserved pair of serine residues, which are constitutively phosphorylated at position 52 and 56 by casein kinase-2 (CK-II). The phosphorylation of Vpu's residues appear to be a major determinant of its biological activities [222-225]. Among the several functions attributed to Vpu, two are seen as major: CD4 downregulation and counteracting the host restriction factor BST-2 (Bone marrow stromal cell, CD317) otherwise known as tetherin [226-229].

In contrast to Nef, Vpu-mediated CD4 downregulation occurs during the transport of CD4 to the plasma membrane. Vpu targets newly synthesized CD4 for degradation using the ER-associated degradation (ERAD) pathway [230-233]. This degradation of CD4 is especially important since it liberates the Env gp160 precursor from interacting with CD4, thereby enabling Env to traffic to the cell surface.

A critical phenotype attributed to Vpu is its positive impact on viral release. HIV-1 isolates lacking a functional *vpu* displayed a pronounced impairment in the release of viral particles [219, 234]. In the absence of Vpu protein, the restriction factor BST-2/ tetherin promotes the accumulation of viral particles budded at the surface of infected cell [227, 228]. BST-2 is a type II transmembrane protein that consists of two membrane anchors: an N-terminus transmembrane domain and a C-terminus linked to a glycophosphatidylinsitol (GPI) anchor, allowing it to anchor both in the cell and viral membrane. However, the mechanistic details of Vpu-mediated BST-2 antagonism is still poorly understood, but it is thought that Vpu could displace BST-2 from the viral assembly site by promoting its sequestration, endosomal, and lysosomal degradation as a result of their transmembrane domain interactions [235-240]. It has been shown that this Vpu/BST-2 interaction results in a proteasomal degradation in a β-transduction repeat containing E3-ubiquitin protein ligase (β-TrCP2) dependent manner [241]. While some studies suggest that Vpu antagonizes/targets the newly synthesized BST-2, others believe that Vpu might be targeting both the newly synthesized and the recycling BST-2 toward the plasma membrane (PM) [242-244]. BST-2 or Tetherin protein can be expressed in two distinct isoforms (known as long and short forms), and Vpu preferentially targets the long form of the protein [245, 246]. Vpu-mediated BST-2 antagonism also prevents the induction of the NF-κB pathway and thereby, limits the activation of innate immune responses [247].

Finally, Vpu also interferes with the early innate immune responses by decreasing the host surface expression of CD1d, NTB-A ligands and HLA-C molecule; thus, affecting the recognition and elimination of infected cells by NK cells or CTLs [248-250]. These activities of Vpu supports its virulence role in viral pathogenicity and disease progression.

### 1.1.7 HIV-1 Envelope glycoproteins

The mature HIV-1 envelope glycoproteins (Env-gp160) are essential for virus infection. The HIV-1 Env glycoproteins are responsible for the initial binding linking the viral particle with the host cell and represent the only viral antigen located at the surface of infected cells [145]. The polyprotein gp160 precursor is synthesized on the rough endoplasmic reticulum (RER) from a singly-spliced *vpu/env* bicistronic mRNA that is co-translationally glycosylated and directed into the lumen of the ER. This mRNA also encodes an endoplasmic reticulum signal peptide that serves to target Env to the RER membrane, and is cotranslationally removed via signal peptidases within the ER [251, 252]. Shortly after synthesis, gp160 monomers oligomerize predominantly into trimers, although forms of dimers and tetramers could also be observed [253-256]. This formation of an oligomer structure is believed to be required for Env transport from the ER to the Golgi complex, since a mutated Env product that does not form oligomers fails to be transported from the ER [257, 258]. In the Golgi apparatus, the gp160 is proteolytically cleaved at a highly conserved Lys/Arg-X-Lys/Arg motif (where X is any amino acid) [259, 260] by a cellular furin or furin-like proteases to yield the surface gp120 and the

transmembrane gp41 subunits (Figure 1.5) [54, 261, 262]. Following cleavage, gp120 glycoprotein remains noncovalently associated with the gp41 subunit [263-266].

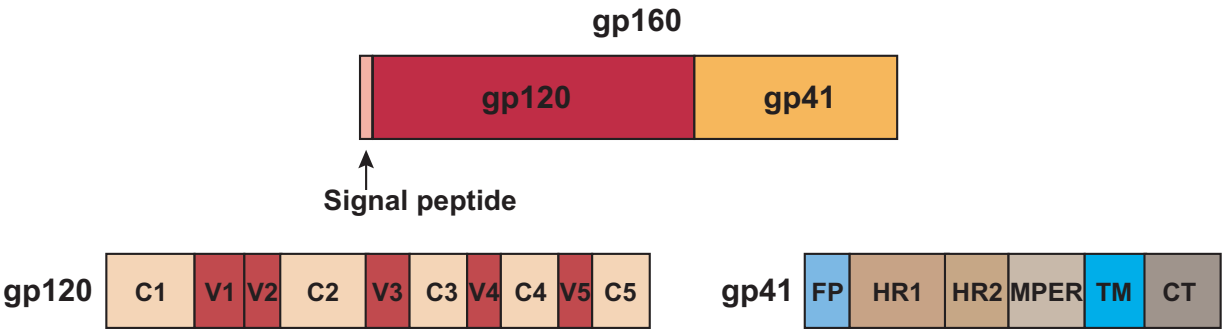


**Figure 1.5 HIV-1 Envelope glycoprotein: gp160 components.**
A schematic representation of the HIV-1 Env gp160 organization, illustrating the gp120 and gp41 subunits linked to the signal peptide. The gp120 glycoprotein encodes five constant domains (C1-C5) and five variable regions (V1-V5) while the gp41 is composed of the N-terminal fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), membrane-proximal region (MPER), transmembrane domain (TM), and cytoplasmic tail (CT).

Extensive work has been carried out to determine the structural organization of the native HIV-1 Env. Researchers have faced several challenges to characterize the structural arrangement of Env glycoproteins, including its heavy glycosylation, high flexibility, the propensity of gp120 to dissociate from the gp41 (gp120 shedding), and the attachment of gp41 to viral or cellular membranes [267]. The first insights into HIV-1 Env organization were reported in an X-ray crystal structure of an engineered monomeric gp120 core bound to two domains of soluble CD4 (sCD4) construct and a Fab fragment of the monoclonal 17b antibody to limit its flexibility [268]. This structure unveiled the architecture of the gp120 core and the molecular interfaces involved in its binding to CD4 and co-receptor antibody. The knowledge generated from this structure has been used

to improve the potency and the breadth of small CD4 mimetic molecules (CD4mc) and to design a probe that proved useful in the isolation of several broadly neutralizing antibodies (bNAbs), such as the CD4-binding site VRC01 antibody [269-271]. Since then, several crystal structures of monomeric gp120 cores, both in the free form or in complex with various ligands have been determined. These structures have presented a similar overall conformation, with a clear separation between the outer and the inner domains of the gp120 portion and helped delineate its glycan shield [272-277].

The early use of cryo-electronic microscopy (Cryo-EM) approaches provided low resolution structural information of the unliganded Env-trimer [278-281]. The Env-trimer's metastability has created a significant challenge in the purification of native Env complexes. Therefore, a soluble gp140 glycoprotein that is cleaved at residue 664 of gp41 to prevent its anchor to the cell membrane was created to overcome this challenge [282-284]. This cleaved HIV-1 Env trimer has been stabilized by introducing an artificial disulfide bond bridging the gp120 and gp41 subunit through adding two cysteines at positions 501 of gp120 and 605 of gp41, termed SOS. Additionally, an isoleucine to proline mutation at position 559 of gp41, termed IP, was introduced to further stabilize Env trimer association to create the soluble gp140 SOSIP [282]. Although this SOSIP construct and its structural studies [282, 284] represented a real breakthrough for the field, my doctoral work showed that the introduction of the I559P modification within the gp41 HR1 region affects the conformational rearrangement of the protein [285]. We also found that the effect of the SOSIP mutations was not only limited to the disruption of Env antigenic profile, but also to its ability to engage with CD4 leading to a negative

cooperativity induced by both SOS and IP changes. This results in abnormal Env-conformation transitions, thus SOSIP-bearing Env samples a conformation that differs from the native unliganded one [286]. Recently, double-electron-electron resonance (DEER) spectroscopy measurement has uncovered multiple conformations in SOSIP Env [287]. These findings emphasize the necessity to acquire further structural information on the unliganded native Env conformation, as this represents the key target for bNAbs.

#### 1.1.7.1 The gp120

The gp120 is heavily glycosylated; approximately half of its mass is covered with oligosaccharide side chains that effectively mask Env from immune responses [288-290]. The gp120 is composed of interspersed conserved (C1 to C5) and variable (V1 to V5) domains (as shown in Figure 1.5) [291, 292]. The conserved domains in the gp120, in particular C1, C3, and C4, mediate the interaction with CD4 receptor. Within the gp120 conserved regions, flexible topological layers (layer 1, 2, and 3) are also responsible for the association with gp41 and play an essential role in controlling and orchestrating the CD4-triggered conformational transitions [263, 265, 268, 293-295].

Structural information of the inner domain of gp120 either in conjunction with CD4 or several antibodies, defined the presence of N- and C-terminal domains that maintain the gp120-gp41 interaction [294]. These N- and C terminals are linked by a β-sandwich that seems to contribute to the gp41 association [263, 266]. The inner domain of the gp120 is home for highly conserved epitopes with the majority of them targeted by non-neutralizing antibodies [296]. The inner domain epitopes are exposed upon the

interaction of gp120 with CD4, but the viral entry process appears to be too advanced to allow viral neutralization by antibodies targeting these epitopes [297]. The antibodies targeting the gp120 inner domain are known as anti-cluster A Abs and belong to the larger family of CD4-induced (CD4i) antibodies

#### 1.1.7.2 The gp41

The transmembrane portion (gp41) of the HIV-1 Env spike complex is organized into three major domains: an extracellular N-terminal ectodomain, a transmembrane domain (TM), and a C-terminal cytoplasmic tail (CT).

Firstly, the extracellular domain can be subdivided into the following five functional regions: the fusion peptide (FP) [298-300], the N-terminal heptad repeat (known as the HR1 region), the loop region, the C-terminal heptad repeat (known also as the HR2 region) [75, 76, 301], and Trp-rich domain termed the membrane-proximal external region (MPER) [302, 303] (as shown in figure 1.5). The HR1 and HR2 domains are linked by a disulfide bridge in the hydrophilic loop, and their interaction is able to assist the fusion process. The fusion peptide is normally buried in the trimer and exposed as a result of the binding of gp120 to CD4 and coreceptor, which induces changes in the gp41 structure causing its penetration into the membrane of the target cell [71, 304]. The relative long half-life of the gp41 ectodomain makes it a potential target for viral entry inhibitors and for drug development, such as the antiviral peptide T20 (Enfuvirtide). T-20 is a 36-amino acid peptide that binds to the pre-hairpin intermediate and allows the inhibition of membrane fusion [305, 306]. Notably, the MPER region of the gp41

ectodomain is one of the main targets for a broad spectrum of neutralizing antibodies [307].

The gp41 transmembrane domain or TM comprises around 25 highly-conserved residues that anchor Env in the cellular lipid bilayer [308]. The cytoplasmic domain or CT of gp41 is a long domain that consists of approximately 150 amino acids and is found near the internal cytoplasmic membrane. This long feature of the cytoplasmic tail is common among lentiviruses and is thought to play an important role during viral replication and control of immune responses during viral invasion [309-311]. Indeed, this CT is believed to influence multiple properties of the gp120-gp41 complex that involve Env incorporation into virions, viral infectivity, Env-surface expression, shedding of gp120, and the Env-fusion process [312-316]. The cytoplasmic tail could also influence Env-internalization; therefore, limiting its exposure and recognition by immune responses such as Antibody-dependent cellular cytotoxicity (ADCC) [317, 318].

#### 1.1.7.3 Neutralizing Antibodies

Anti-Env antibodies could be divided into two types based on their immune activities, either neutralizing or non-neutralizing- mediating antibodies. The initial anti-Env antibody response appears approximately two weeks after infection [319, 320], targeting epitopes within the gp120 and gp41 subunits that lack neutralizing activity. However, neutralizing antibodies activity in the serum of HIV-1 infected individuals become detectable a few months post-viral exposure [321], and target the autologous virus, which evolves continuously to evade their action [322-324].

The detection of cross-reactive neutralizing antibodies becomes possible in the blood much later than those with autologous activity, approximately 2.5 years post-infection [327, 328]. Only 10-20% of HIV infected individuals are able to develop these antibodies that are able to neutralize diverse heterologous viruses [327, 329, 330]. These Abs target different sites on the Env trimer, including the CD4 binding site, the V1/ V2 domain, the V3 loop glycans, the gp41, and the interface between gp120 and gp41 [271, 331]. Table 1.1 provides an example of numerous broadly neutralizing antibodies for each category of Env vulnerability sites.

**Table 1.1: Characteristics of some bNAbs**

| Antibody Name | Neutralization Breadth (%) | $IC_{80}$ ($\mu$g per ml)* |
|---|---|---|
| **CD4-binding site (CD4BS)** | | |
| b12 | 33% | 2.70 |
| VRC01 | 87% | 0.98 |
| 8ANC131 | 57% | 4.02 |
| CH103 | 34% | 8.00 |
| **V1/V2 gp120** | | |
| PG9 | 70% | 0.31 |
| PGT145 | 60% | 0.31 |
| **V3 Glycan** | | |
| 2G12 | 18% | 4.85 |
| PGT121 | 53% | 0.08 |
| PGT128 | 56% | 0.11 |
| **MPER gp41** | | |
| 2F5 | 48% | 9.42 |
| 4E10 | 88% | 8.94 |
| 10E8 | 97% | 2.05 |
| **Interface between gp41 and gp120** | | |
| PGT151 | 66% | 0.024 |
| 35O22 | 62% | 0.20 |

*$IC_{80}$: the inhibitory concentration of 80%.
Modified from Kwong, Mascola, and Nabel., Nature Review Immunology, 2013 [325], and Falkowska., et al., 2014 [326].

### 1.1.8 HIV-1 vaccines

Soon after the identification of HIV as the causative agent of AIDS in the early eighties, the US Secretary of Health and Human Services declared that an HIV vaccine will be available for testing in two years. More than thirty years later, this vaccine is still a far-fetched goal [332]. Nevertheless, the efforts of developing an effective vaccine has continued since the late 1980s and more than 100 vaccine trials have been clinically tested at least in phase I. To date, studies of only six clinical efficacy trials have been completed, and the RV144 study was the only to demonstrate modest efficacy [333]. These trials and their results are summarized in Table 1.2.

#### 1.1.8.1 Vaccine trials

Diverse vaccine approaches against HIV-1 have been used and could be divided into three categories: (1) the induction and maintenance of a protective humoral response that is based on the generation of bNAbs or effective immune defences, (2) the stimulation of CD8 or cytotoxic cellular responses, (3) the combination of both strategies [334]. These three approaches were based for the design of a wave of vaccines and viral vectors for an antigenic expression of portions of the HIV-1 genome, including Env (recombinant gp120), Gag, Pol, and Nef. The early waves of vaccine trials aimed to evaluate a recombinant form of Env (AIDSVAX or VaxGen) and targeted the induction and secretion of BNAbs [335, 336]. The failure of the VaxGen trials led to the examination of another approach aiming to activate CTL response, and thus the development of an immunogen based on an adenovirus type 5 (Ad5) vector expressing HIV-1 clade B Gag, Pol, and Nef (HVTN 502 or the STEP trial). This study demonstrated that the use of an

Ad5 viral vector could elicit a cellular-mediated immune response against HIV-1 acquisition, yet did not prevent infection [337, 338]. Nevertheless, this vaccine resulted in risk of HIV infection in men who were Ad5-serpositive and uncircumcised [339].

#### 1.1.8.2 The RV144 trial

In October 2009, the results of the HIV-1 vaccine trial conducted in Thailand, the RV144, were released showing a modest impact with 31.2% efficacy in preventing HIV infection [333]. The trial tested the protection efficacy using a prime-boost combination of two vaccine regimens. These include the ALVAC vaccine, which is a canarypox based vector vaccine delivered at four doses (0, 1, 3, and 6 months) and the AIDSVAX B/E vaccine, which consists of gp120 protein in a regimen delivered at two doses (3 and 6 months). ALVAC-HIV encodes the *gagpol* gene and the transmembrane domain of gp41 of clade B (LAI strain) and gp120 of 92th023 from the CRF01_AE clade. The AIDSVAX B/E carried out the monomeric recombinant gp120 from CM244 (CRF01_AE) as well as MN of clade B. The efficacy of the vaccine calculated 12 months post-vaccination was 60% but declined over time to 31.2% as a result of decreased immune responses [333, 340, 341]. Despite this positive efficacy in preventing infection, the vaccine had no effect in terms of disease progression in infected vaccine recipients [342]. The comparison of the immune responses observed two weeks post the last vaccination identified two main protection correlates. The immune correlates revealed that V1V2 IgG antibodies may have contributed to the protection against HIV-1 infection while the presence of IgA recognizing Env antibodies may correlate with a high risk of infection [343-345]. The presence of IgA in particular appeared to facilitate the risk of

infection through decreasing the ability of protective immune responses, including ADCC or the other activity of Abs against HIV-1 Env [346-349]. However, these studies failed to identify neutralizing antibodies as a potential correlation of protection, thus directing the attention of scientists toward the role of non-neutralizing Abs [350].

**Table 1.2: HIV-1 vaccine efficacy trials**

| Trial Name | Immunogen component | Region(s) | Immune response | Efficacy | References |
|---|---|---|---|---|---|
| **VAX004** (1998-2003) | Recombinant gp120 B/B | The US & Europe | Antibody | No | [351] |
| **VAX003** (1999-2003) | Recombinant gp120 B/E | Thailand | Antibody | No | [346] |
| **STEP** (2004-2007) | MRKAd5/ HIV-1 clade B Gag /Pol/ Nef | Australia, the Caribbean, & North and South America | T cell response | No Increased infection in recipients | [352] |
| **Phambili** (January 2007-September 2007) | MRKAd5/ HIV-1 clade B Gag /Pol/ Nef | South Africa | T cell response | No Increased infection in male recipients | [353] |
| **RV144** (2003-2009) | ALVAC (canarypox vector), AIDSVAX B/E vaccine & gp120 from CRF01-clade A/E | Thailand | T cell and antibody responses | 31.2 % efficacy in preventing HIV infection | [343, 346, 354] |
| **HVTN 505** (2011-2013) | VRC DNA prime/ Ad5 boost | The US | T cell and antibody responses | No | **[355, 356]** |

### 1.1.9 New Strategies to fight HIV-1

An effective HIV-1 vaccine remains a major public health priority with 1.8 million people newly infected with HIV in 2017 [357]. Elicitation of bNAbs has been a challenging goal for HIV-1 vaccine design. Besides their ability to neutralize the virus, antibodies produced upon infection could initiate multiple immune responses combining humoral and cellular immune axes. However, one of the unsolved goals to date is the generation of an immunogen that would elicit BnAbs [358-360]. BNAbs are clinically rare as only 10-30% of infected individuals can develop them after 2 to 4 years post-infection [327, 361, 362]. Characterization of bNAbs CH103 isolated from an African donor who has developed a serological breath and potency revealed the presence of extensive epitope evolution and somatic maturation of the antibody [363]. The HIV-1 bNabs have a long heavy chain and high levels of somatic mutations; for example, the heavy chain genes of newly generated bNAbs frequently carry over 80 $V_H$ mutations whereas the human IgG Abs typically carry 10-20 $V_H$ gene somatic mutations [364-368]. Therefore, multiple approaches are being pursued to generate bNAbs by immunization: (1) designing a sequence of immunogens that could mimic bNAb epitopes by using native-like Env trimers or the creation of bNAb epitope scaffolds [369], (2) the utilization of immunogens that have the ability to bind to germline precursors of bNAbs and stimulate various stages of antibody clonal lineage, known as B cell lineage vaccine [360, 370].

CD4 mimetic compounds (CD4mc) are small molecules that have the ability to bind to the well conserved Ph-43 cavity within gp120 and induce conformational changes in Env. Studies have demonstrated that CD4mc can sensitize viruses to neutralization

and ADCC by CD4i Abs which normally arise in HIV-1-infected individuals and Env vaccine recipients [371-374]. A recent study has shown that CD4mc protect gp120-immunized rhesus macaques from multiple high-dose intrarectal challenges of simian-human immunodeficiency virus (SHIV) [375]. Since it has been shown that CD4mc can act on many levels to protect against HIV-1 infection: blockade of the CD4 binding site [375, 376], "forces" Env to expose vulnerable epitopes that can then mediate viral neutralization [371, 372, 377], but also ADCC [373, 375]. CD4mc could represent a unique candidate for pre-exposure prophylaxis.

The modest success achieved with the RV144 trail in Thailand [340, 378] has brought a new attention to the potential role of non-NAbs, especially those with potent ADCC activity [379, 380]. This highlights the potential utility of gp120 V2 region as a target site to block viral acquisition. In this regard, the RV144 follow up on clinical trial RV305 and HVTN studies were designed to enhance the elicitation of V2-specific Ab responses [381, 382].

Over the past 20 years, several efficacy therapeutic HIV vaccine trails have been evaluated during acute and chronic phases of infection and all resulted in minimal success. However, therapeutic vaccines even if partially effective are believed to be valuable for infected individuals by serving as an intensive treatment with ART to increase immune responses and limit the establishment of the viral reservoir [383, 384]. Multiple strategies were used to design therapeutic HIV vaccines based on peptides or recombinant Env proteins [385-388], such as HIV p24-like peptide [389, 390] and a tat

protein [391-393] and both were able to improve antiviral immunity and resulted in delayed disease progression. Other delivery options of bNAbs also involve a gene transfer. This includes the use of Adeno-associated virus (AAV) vector to enable direct expression of bNAbs in non-human primate [394], and a passive bNAbs transfer, which consists of the direct administration of antibodies into the host. The administration of VRC01 along with 10E8 and PG9 to rhesus macaques showed a complete protection against SHIV [395]. The use of VRC01 in phase I clinical trials revealed its safety profile and ability to suppress viral replication in HIV infected individuals [396, 397]. Additionally, 3BNC117 is a potent and broad neutralizing antibody that targets the CD4 binding site and was shown to prevent infection in animal models as well as supress viremia in HIV-1 infected patients [398-400]. The infusion of 3BNC117 was shown to significantly decrease viral loads in HIV-1 recipients [401]. The passive administration of bNAbs could be beneficial as a proof of concept in term of immediate protections, but this strategy might be challenging due to its costly production.

### 1.1.10 HIV-1 pathogenesis

HIV-1 is commonly transmitted in humans through direct contact with an infected individual's body fluids. These fluids include blood, semen, vaginal fluids, and breast milk. Most cases of transmission worldwide are reportedly caused by practicing unprotected sex, but also can result of contaminated needles use among drugs users, transfusion of contaminated blood, or vertical transmission from mother to child during birth or breastfeeding. These transmissions in most cases (60 to 80%) are carried out by a transmitted founder isolates (TF) [402, 403].

HIV-1 targets numerous cell types including CD4+ T cells, monocytes/ macrophages, dendritic cells, and microglial cells of the central nervous system [404]. To ensure survival, HIV-1 targets the main axes of the immune system and can be clinically characterized by a progressive loss of CD4 T cells and a persistent immune activation [405-407]. This induces a gradual impairment of cellular immunity, which leads to increased susceptibility to opportunistic infections that might ultimately cause clinical complications and death of infected patients [407].

#### 1.1.10.1 Phases

At least three distinct phases of HIV-1 infection have been described: the acute phase (primary infection), the chronic phase (asymptomatic), and the AIDS phase.

The primary stage of infection includes the first days or weeks following exposure of the host to the virus. The virus at this stage is usually undetectable in the plasma of newly infected individuals; this is also called the eclipse phase that can generally last from 7 to 21 days, with unspecified flu-like symptoms [402, 408-410]. After mucosal infection, the virus replicates in mucosal and submucosal surfaces, and rapidly spreads to lymphoid tissues. The acute phase of HIV-1 infection is also associated with high titers of viral load (approximately $10^6$ to $10^7$ RNA copies/mL); thus, allowing the dissemination of the virus through the host and depletion of CD4 T cells [411, 412]. This loss of CD4 T cells is not only noticed at the peripheral level, but CD4+ T cells are massively depleted within the gut associated lymphoid tissue (GALT) [413-415], where

around 30 to 60% of cells are productively infected and eliminated as shown in a model of SIV infected macaques [416, 417]. The analysis of SIV infected macaques revealed that the initially infected memory cells were not all activated, but surprisingly resting cells that are capable of supporting viral production [416]. The end of this early phase of HIV-1 infection is marked with an immunological control of the virus that involves reduced viral load in the peripheral blood and partial restoration of CD4 T cells count. However, the subsequent importance in reducing the initial high level of viremia on the efficacy of the HIV-1 specific immune response to control viral replication is still an open debate [418, 419]. Indeed, it is within this stage of infection that a latent reservoir is established [420, 421], mostly within central memory and transitional memory CD4+ T cells [422]. These cells are not considered productively infected, which makes them unsusceptible to standard antiretroviral treatment or control the immune system [420]. This reservoir ensures viral survival for years through the cellular ability to persist by homeostatic proliferation [422]. Therefore, it is believed to cause one of the major challenges that faces the eradication of HIV-1 in patients.

While the acute phase of HIV-1 infection could be characterized by intense viral replication and significant immune activation, the chronic stage is known as the asymptomatic phase can vary in length from 8 to 12 years [423, 424]. During this time, persistent viral replication continues, creating the necessary antigenic stimulation for immune activation and cellular depletion. Although most infected individuals remain asymptomatic during much of this stage, the ongoing viral replication results in a

continuous decrease in CD4 T cells with an average rate of 25 to 60 cells/$mm^3$ per year that is combined with a high scale of viral heterogeneity and escape [425-428].

If left untreated, HIV-1 infection eventually reaches its last stage, in which, the viral load is rapidly raised with a simultaneous drop in CD4+ T cell count causing the disruption of the immune system. This last stage is termed AIDS, and is defined when the CD4+ levels drop below 200 cells/$mm^3$. The massive depletion of CD4+ at this stage correlates with high risk of opportunistic infections, thus, presenting the most harmful complications such as the dissemination of Mycobacterium avium infection or the reactivation of CMV infection [429, 430]. The disruption of the immune system in HIV-1 infected individuals could also influence the development of lymphoid cancer, such as Kaposi's sarcoma and Hodgkin's lymphoma [431].

Nevertheless, the outcome of HIV-1 exposure has been shown to be different among infected individuals. While some rare individuals are able to control infection and remain asymptomatic for years, others progress rabidly towards AIDS. Each of these various groups represent a potential lesson toward understanding the underlying biological mechanisms against HIV-1 infection [432, 433].

#### 1.1.10.2 Treatment

Antiretroviral combinational therapy has resulted in a profound decrease in the rate of AIDS-related mortality by more than 80% in developed countries [434-436]. To date, more than thirty antiretroviral drugs and drug combinations have been approved to

use in six distinct classes for HIV-1 drugs. These classes of drugs target multiple steps of viral replication and include: nucleoside/nucleotide RT inhibitors (NRTIs), non-nucleoside inhibitors (NNRTIs), integrase inhibitors, protease inhibitors, fusion inhibitors, and coreceptor antagonists. The first drug approved in 1987 by the USA Federal Drugs Administration (FDA) was zidovudine, known also as azidothymidine (AZT), which belongs to the NRTIs group that lacks a 3' – OH group and depends on a thymidine kinase for its initial phosphorylation [437, 438]. RT is also targeted by NNRTIs that occupy a potential binding pocket within RT that induces conformational changes to essentially inactivate it. The NNRTIs are preferred agents to be used for the first-line regimens and for protection of mother to child transmission [439, 440].

The process of entry and viral fusion is also one of the important targets for antiviral molecules. Although the use of the soluble form of CD4 (sCD4) for treatment have showed limited efficacy for viral isolate neutralization, the development of small molecule inhibitors that could block the gp120 and CD4 interaction showed high potential [441, 442]. Potent inhibition of the viral replication has been demonstrated in clinical studies by the use of a small molecule chemokine receptor antagonist, termed a maraviroc. This drug prevents the binding of gp120 to CCR5 [443, 444]. Similarly, potent efficacy was clinically demonstrated for the fusion inhibitor enfuvirtide (T-20), a sequence of 36-amino acid oligopeptide that blocks the formation of a six-helix bundle by binding to the HR1 loop [445, 446]. A humanized anti-CD4 monoclonal antibody, ibalizumab, has also showed a strong anti-HIV-1 activity in infected patients through binding to domain 2 of the extracellular portion of CD4 and acting as a post-attachment inhibitor of viral entry

[447, 448]. Some viral proteins such as PR and IN are also used as targets for the development of highly effective antiretroviral compounds [449-452]. Extensive studies have also focused on potential targets for drug development and involve the viral accessory proteins Nef, Vif, Vpu, and Vpr.

### 1.1.11 HIV-1 Nef

#### 1.1.11.1 Structural features

HIV-1 Nef consists of 206 amino acids and this protein has the ability to associate with cellular membranes [453]. Initially, it was identified as an open reading frame that partially overlaps with the viral 3′ LTR that is abundantly expressed in the early phase of HIV infection; representing three quarters of the early viral mRNAs load of the cell [454, 455]. While a full-length structure of Nef is too flexible for structural analysis, fragments of the protein has been characterized (Figure 1.5). The structural features of Nef have been solved using X-ray crystallography and nuclear magnetic resonance (NMR) analyses, both alone or in complex with the third Src-homology (SH3) domain [456-459]. These structural studies revealed that HIV-1 Nef acquires a genetically diverse and structurally flexible N-terminal myristoyl group and a poly-basic patch responsible for membrane association [460, 461]. The N-terminal is followed by a conserved folded core domain that consists of a type II polyproline (PPII) helix that has the main binding site (PxxPx sequence) for the Src-family kinases [462-464]. The Nef core is also followed by several helical regions; two α helices, a four-stranded anti-parallel β sheet, and two additional α helices. These regions of the Nef core domain form an homodimerization interface complexes with Src family kinases (SH3 and SH2) regulatory domains [457,

465]. This dimerization form of Nef protein is critical for most of its functions and presents a hot-spot for small molecule inhibitors against Nef [466, 467]. Finally, the C-terminal region of Nef protein is composed of the acidic cluster (D174-E178) that is required to colocalize Nef with cellular trafficking proteins. Nef also has a flexible loop located near the C-terminal end of the core that carries a dileucine based internalization motif ($E/D^{160}$ xxx$LL^{165}$) and makes direct interaction with the AP-2 trafficking adaptor protein, an essential interaction for Nef-mediated CD4-downregulation [468, 469].

Analysis by hydrogen exchange (HX) mass spectrometry (MS) of membrane associated protein revealed that the HIV-1 Nef protein undergoes conformational rearrangement based on the density of lipid packing. At high packing density, Nef protein maintains a compact conformation and remains un-attached to the lipids while at lower packing density, the protein is able to insert its N-terminal arm causing the displacement of the core domain and conformational changes of the C-terminal loop [470]. It is believed that Nef undergoes a transition from a closed (solution) conformation to an open (membrane-associated) conformation that positions the core domain; this, enabling the membrane-associated Nef to interact with host proteins [471-474]. This conformational transition upon insertion of the N-terminal arm and subsequent displacement of the core domain from lipid membrane allow the exposure of several critical motifs of Nef that are essential for interaction with host proteins [460, 475].

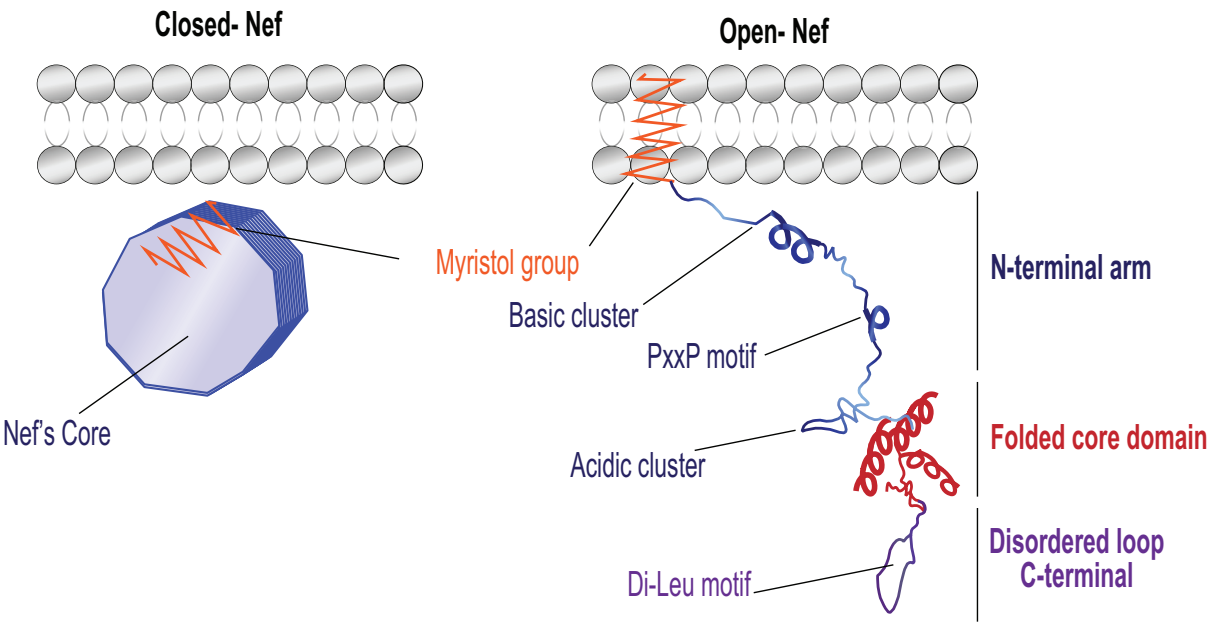


**Figure 1.6 Structural features of Nef**
A model of the full-length Nef representing the different forms of the protein. (A) Cartoon representation of (left-panel) the cytoplasmic closed form of Nef after protein translation, or (right-panel) the membrane anchored open form of Nef. The presented structure is adapted from Arold and Baur., Cell, 2001 [471].

#### 1.1.11.2 Functions

HIV-1 Nef is a multi-functional protein crucial for progression to AIDS (Figure 1.7). Nef hijacks several signal transductions and protein trafficking pathways. It is the flexible regions of Nef that provide an extensive accessible surface to connect with many host proteins, resulting in their trafficking away from the cell surface toward the *trans*-Golgi network (TGN) or lysosomes [471, 476]. Here, I will briefly extend the knowledge on Nef interference with three important proteins in the cell: downregulation of CD4, MHC-I, as well as NKG2D ligands.

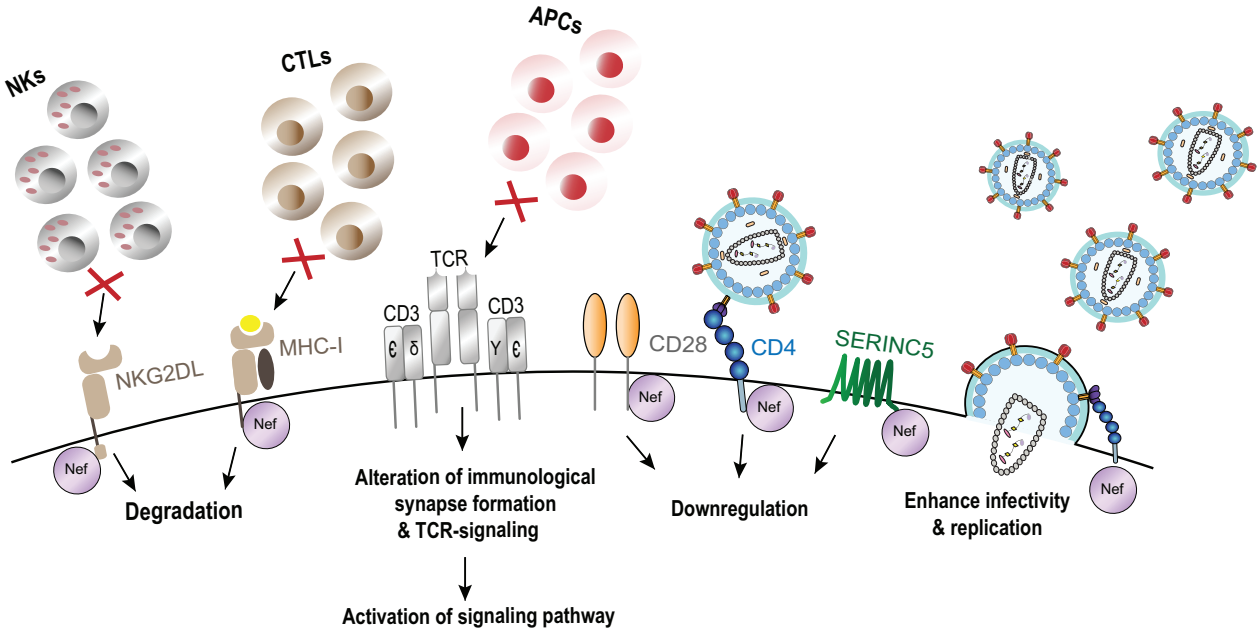


**Figure 1.7 Selected Nef functions in infected T cells**
Overview of some Nef's manipulations in infected CD4+ T cells: As outlined above, Nef promotes a variety of functions within virally infected cells that include implying of MHC-I antigen presentation to avoid CTLs lysis, affecting the formation of immunological synapse and TCR signalling via reducing the expression of CD28 and also CD3 from the cell surface, inducing downstream signalling events through interacting SFKs, minimizing the expression of SERIENC5 to promote viral release and spread, and down-modulating CD4 to prevent superinfection and protect infected cells from ADCC.

#### 1.1.11.2.1 Nef-induced CD4 downregulation

The most extensively characterized function of Nef is its ability to downregulate CD4 from the surface of infected cells. Briefly, the Nef motif $W_{57}$ - $L_{58}$ interacts with the cytoplasmic tail of CD4 and leads to the binding of Nef dileucine $L_{164}$ -$L_{165}$ motif to the clathrin adaptor protein complex (AP-2), thus connecting CD4 to the clathrin-coated vesicles for endocytosis and lysosomal degradation [477-484]. Moreover, Nef directly interacts with the β subunit of COP I, which is essential for the endosomal trafficking (ER-Golgi transport) along with the endosomal sorting complex required for transport machinery (ESCRT) for CD4 lysosomal degradation [485-488]. This rapid reduction in

surface CD4 levels by Nef prevents viral superinfection and limits the negative effects of CD4 molecules on the incorporating Env into the viral particle and their release [489, 490].

#### 1.1.11.2.2 Nef-induced MHC-I downregulation

Another conserved activity of Nef is its capacity to mediate MHC-I down-modulation [491]. This reduction on MHC-I expression enables infected cells to evade defences of the immune system during an active viral replication [492, 493]. To accomplish this, at least two primary pathways are known: (1) Nef directly binds to the cytoplasmic tail of MHC-I, leading to the recruitment of adaptor protein-1 (AP-1) and β-COP and its subsequent degradation [487, 494, 495], (2) Nef binds to the membrane trafficking proteins phosphofurin acidic cluster sorting protein 2 (PACS-2) to promote the endocytosis of surface MHC-I and its trafficking to TGN. At the TGN, Nef binds and subsequently activates specific Src-family kinases (SFK), which triggers the activation of PI3K-dependent internalization of MHC-I molecules [496-499]. Nef is also able to bind to the immature hypo-phosphorylated MHC-I in TGN and thereby block the transport of the newly synthesized MHC-I to the cell surface [500, 501]. Notably, it has been demonstrated that the MHC-I molecules are internalized into endosomal compartments coated with the small membrane associated GTPase, ARF-6 or ARF-1 (ADP ribosylation factor 1 or 6) [502, 503]. Three conserved sites of Nef are involved in mediating MHC-I downregulation; (i) the $EEEE_{65}$ acidic cluster on the N-terminal domain of Nef that initiates its binding to the sorting protein PACS-2 [497, 504, 505], (ii) the $PXXP_{75}$ motif that binds and activates the SFK [498], and (iii) the $M_{20}$ that is located within the N-proximal α-helical region of Nef and promotes the association of MHC-I with AP-1 protein [494].

Interestingly, this ability of Nef-mediated MHC-I downregulation is repressed in the presence of small chemical inhibitors (2c) that disrupts the Nef-SKF interaction [506]. Nef selectively limits the expression of HLA-A and B, but not HLA-C and -E molecules. A tyrosine residue unique to the cytoplasmic tail of HLA-A and -B proteins confers this specificity of Nef. Thus, the selective downregulation of HLA-A and B molecules reduces the susceptibility of infected cells to CTL-mediated lysis while the surface presence of HLA-C and -E helps to protect cells from lysis by natural killer cells [492, 507].

#### 1.1.11.2.3 Nef-induced NKG2D-L downregulation

It has been demonstrated that Nef down-modulates expression of ligands from the activating natural killer group 2, member D (NKG2D) receptor [508]. NKG2D is a type II membrane anchored of the C-type lectin family receptor that is expressed on all NK cells, $CD8^+$ T cells, $\gamma\delta$ T cells, and a small subset of $CD4^+$ T cells [509, 510]. NKG2D is a potent activating receptor and its expression could be stimulated by certain cytokines, such as IL-2, IL-15, or TNF-α, but is decreased by TGF-β [511-514]. NKG2D is a receptor for multiple ligands that have distinct binding affinities [515]. NKG2D ligands (NKG2D-L) consists of two classes of MHC-I like molecules: MHC-I related chain (MIC-A and B) and human cytomegalovirus UL16 binding protein (ULBP1-6) that are poorly expressed at the surface of normal cells and highly presented on stressed, transformed, and infected cells [510, 516]. NK cell activation through NKG2D receptor results in killing of infected or cancer cells. To do this, NKG2D forms a homodimer associating with a dimer of the adaptor protein DAP10, taking advantage of an opposite charge located within their transmembrane domains [517, 518]. Compared to other adaptor proteins, DAP10 does

not contain an immunoreceptor tyrosine-based activation motif (ITAM), but rather has a cytoplasmic YINM (YxxM) motif, which promotes the recruitment of P85 subunit of phosphoinositide kinase-3 (PI3K) and growth factor receptor-bound protein 2-Vav1 (Grb2) complex. The recruitment of these proteins result of the activation and phosphorylation of several downstream proteins that allows the induction of a calcium flux, survival, proliferation, or activation of the effector function of the NK cells[519-521]. Interestingly, NKG2D receptor also acts in conjunction with other NK cell receptors, such as 2B4, NKp46, and CD16 receptors to influence NK-cytotoxicity and cytokine secretion. It has been suggested that the ligation of certain activating receptors, including NKG2D and 2B4, synergizes with CD16 to enhance NK cell calcium flux [522, 523]. NKG2D receptor was recently identified as a co-receptor for NK cell-mediated ADCC response, acting in collaboration with CD16 receptor to improve NK cell cytotoxic activity [524]. It is also believed that the release of soluble NKG2D ligands during HIV-1 infection could impair NKG2D expression and $NKG2D^{+}$ cells cytotoxicity [525-527].

Remarkably, the effect of Nef protein on NKG2D-L expression might assist HIV-1 to evade the NK cell-mediated lysis of the target cell; thereby, establishing a state of chronic infection. Nef reduces the surface levels of MICA, ULBP1 and ULPB2 molecules through an undefined mechanism [508]. Nef mediates down-modulation of NKG2D-L in a mechanism that differs from the one used for CD4 or HLA molecules. Apparently, the tyrosine-based and di-leucine sorting motifs present in the MHC-I (HLA-A and -B) and CD4, respectively that are responsible for Nef-mediated downregulation activities [507, 528], have been shown to be absent from the GPI-anchored ULBPs and the cytoplasmic

tail of MIC-A [510]. It is thought that Nef-induced downregulation of NKG2D ligands could also occur during their trafficking within the cell, or at different steps of their biosynthesis [508]. The expression of NKG2D and its ligands has been linked to disease progression, yet the consequences of Nef-mediated NKG2D ligands downregulation on virus pathogenicity is still unclear.

## 1.2 ADCC response in HIV-1 infection

Antibody dependent cellular cytotoxicity (ADCC) represents an important arm of the immune system against viral infections. It connects both the innate and adaptive immune arms whereby an effector immune cell actively lyses an infected cell that presents antigens bound to one or more specific antibodies (Figure 1.8). Various cells of the innate immune arm have the ability to provide ADCC effector functions, including NK cells, neutrophils, and monocytes/macrophages [529, 530]. Several studies have shown an association between ADCC Ab responses and the rate HIV-1 disease progression [531-533] , viral load [531-536], and decrease of mother to child transmission of HIV and SIV infections [379, 537-543], yet these findings were not consistent among all studies [544-546]. Interestingly, the analysis of correlates of protection in the RV144 vaccine trial suggested that an increased ADCC activity could be linked to reduced HIV-1 acquisition [343], and antibodies with a potent ADCC phenotype were isolated from some RV144 vaccinees [349]. The importance of Fc-effector functions during HIV infection appear to be not only against infected cells, but also toward free viral particles [547]. Although some studies have failed to prove the ability of passively administrated non-NAbs with ADCC activity to protect against SIV or simian-HIV (SHIV) challenges in rhesus macaque [548-

552], a recent study suggested that the presence of non-NAbs with Fc-competent functions might be capable of altering the course of HIV-1 infection in humanized mice [553]. Nevertheless, vaccination studies in rhesus macaques have identified ADCC activity as one of the immunological parameters to reduce the risk of SHIV infection [375, 554].

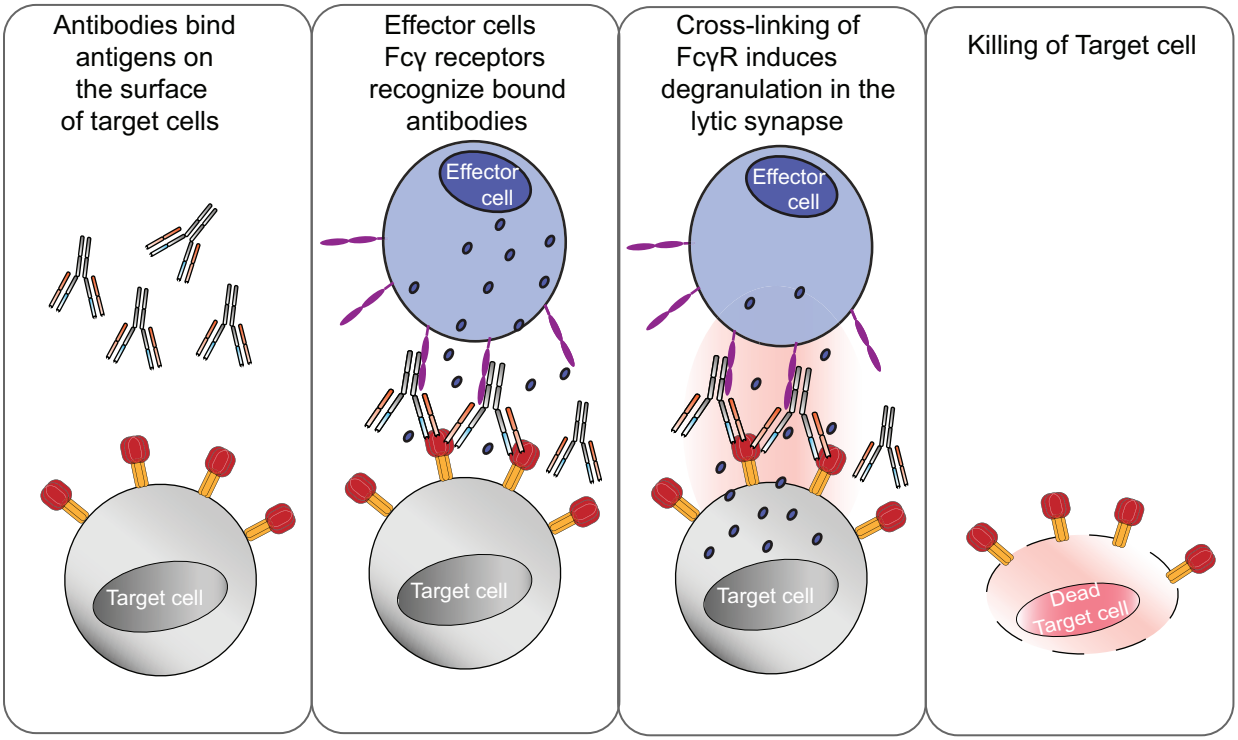


**Figure 1.8 Antibody dependent cellular cytotoxicity (ADCC)**
A diagram of ADCC process showing the ability of antibodies bound to viral protein on the surface of target cell to recruit Fc receptor bearing cells (or effector cells). Fc receptor cross-linking initiates the lysis of target cell.

### 1.2.1 Role of Env glycoproteins conformation on ADCC

Additional to its ability to mediate viral entry, the HIV-1 Env trimer also represents the only virus-specific antigen that locates at the surface of infected cells. This exposure of Env facilitates the generation of neutralizing and non-neutralizing Abs. The mature trimeric gp160 is composed of the surface gp120 and the transmembrane gp41

subunits [263, 265, 266]. The early steps of viral fusion consist of the interaction between gp120 subunit of the trimer and the cellular CD4 receptor [61, 555], triggering conformational changes in gp120 that would promote its further interaction with one of the co-receptor (CCR5 or CXCR4) [65, 69, 555-559]. In other words, this CD4-induced conformation changes transits the HIV-1 Env glycoprotein from its unliganded-close (state 1) to a partially open intermediate (state 2) rearrangement and then into an open state 3 conformation [560, 561]. This results in the exposure of epitopes within the highly conserved inner domain of gp120, which is otherwise buried deep inside the trimer [265, 562, 563]. The CD4-triggered changes lead to the reorganization of V1/V2 and V3 loops that impedes a spontaneous transition of the trimer toward the CD4-bound conformation, known as state 3 [560, 561]. The exposed epitopes of this conformation are preferentially recognized by ADCC mediating Abs that generally present within the sera of infected individuals [564]. To avoid this exposure, HIV-1 Env tightly controls its naïve closed conformation using complex approaches that involve several residues of the gp120 glycoprotein. These include residues within the topological layers, layer 1, 2, and 3, in the gp120 inner domain that play an important role in orchestrating the conformational rearrangements upon interaction with CD4 [265, 293, 295]. Mutation at the interface between these layers that involves highly conserved residue such as tryptophan 69 (W69) could reduce the formation of this conformation and recognition by CD4i-ADCC-mediating Abs [265, 562, 565]. HIV-1 Env also uses the transition of well-conserved V2 residues to keep the trimer at state 1 via restraining Env from forming the downstream state 2/3 conformations [560]. Another element that contributes in keeping the Env trimer in state 1 is the highly conserved pocket that contains the Phe-43 residue responsible for CD4

engagement and is located within the interface between the outer and inner domain of gp120 [268]. The size and nature of the residue at position 375 has a major impact on Env conformation. While the presence of small residues within the 375 position, such as serine (S375) results in an empty Ph-43 cavity that favors the state 1 conformation [265, 566], the substitution of S375 with larger hydrophobic residues, including histidine or tryptophan, leads to the filling of the Ph-43 cavity and allows the spontaneous sampling of a downstream state close to CD4-bound conformation [566]. Interestingly, the HIV-1 CRF01-AE Envs have a naturally filled Ph-43 cavity, as a result of a histidine residue at position 375 (H375), causing them to be better recognized by CD4i Abs, thus enhancing the sensitivity to ADCC responses [567].

Small CD4-mimetic compounds bind to the Ph-43 cavity, preventing CD4-gp120 interaction, causing thermodynamic changes similar to those induced by CD4 [376, 568] and shift Env toward a more open state 2/3 of Env-conformations [561]. Accordingly, CD4mc were shown to sensitize HIV-1 infected cells to ADCC mediated by Abs present in sera, cervicovaginal fluids, and breast milk of HIV-1 positive subjects [373, 374].

### 1.2.2 Role of HIV-1 accessory proteins on ADCC

It is becoming increasingly clear that the virus wants to avoid exposing the vulnerable CD4-bound conformation of Env at the cell surface. The virus puts in place several mechanisms to avoid this, including preventing Env-CD4 interaction [562]. HIV-1 accessory proteins Nef and Vpu reduce the levels of CD4 at the cell surface [226, 569]. Additionally, Vpu protein assists the release of viral particle by counteracting the

restriction factor BST-2, thus, preventing the accumulation of Env at the surface of infected cells [227, 228]. It has been shown that the production of viral particles in cells infected with viruses lacking Vpu results in a significant accumulation of new virions at the surface of infected cells [562, 564, 570, 571]. Hence, it is now known that these cellular activities of Nef and Vpu could indirectly control the interaction between Env and CD4 within the surface of infected cells; thus, affecting ADCC response. Indeed, cells infected with viruses defective for Nef and Vpu allow Env-CD4 interaction, causing the exposure of CD4i epitopes and increasing the susceptibility of these cells to ADCC-mediated killing [562, 564].Cells are infected with viruses lacking Vpu have increased Env at the surface of cells, as a result of trapped viral particles by BST-2. Interestingly, Env accumulated at the cell surface can be exacerbated upon addition of IFN-α, IFN-β, and IL-27 molecules, resulting in enhanced ADCC responses in the presence of CD4m [572]. Finally, the levels of Env at the surface of infected cells is also modulated through the effect of Env internalization, which protects from the elimination of HIV and SIV infected cells by ADCC [318].

### 1.2.3 Fc-mediated effector function of antibodies

Beside their neutralization capacity, Abs are able to initiate a myriad of immune responses combining the humoral and cellular immune axes. Antibodies are symmetrically composed of two light and heavy chains that are linked together via disulfide bonds (Figure 1.9). These chains contain regions of homologous repeat units of around 10 amino acids named immunoglobulin (Ig) domains. The heavy chain consists

of a variable region (V) comprising of an Ig domain and a conserved domain (C) containing three or four Ig domains.

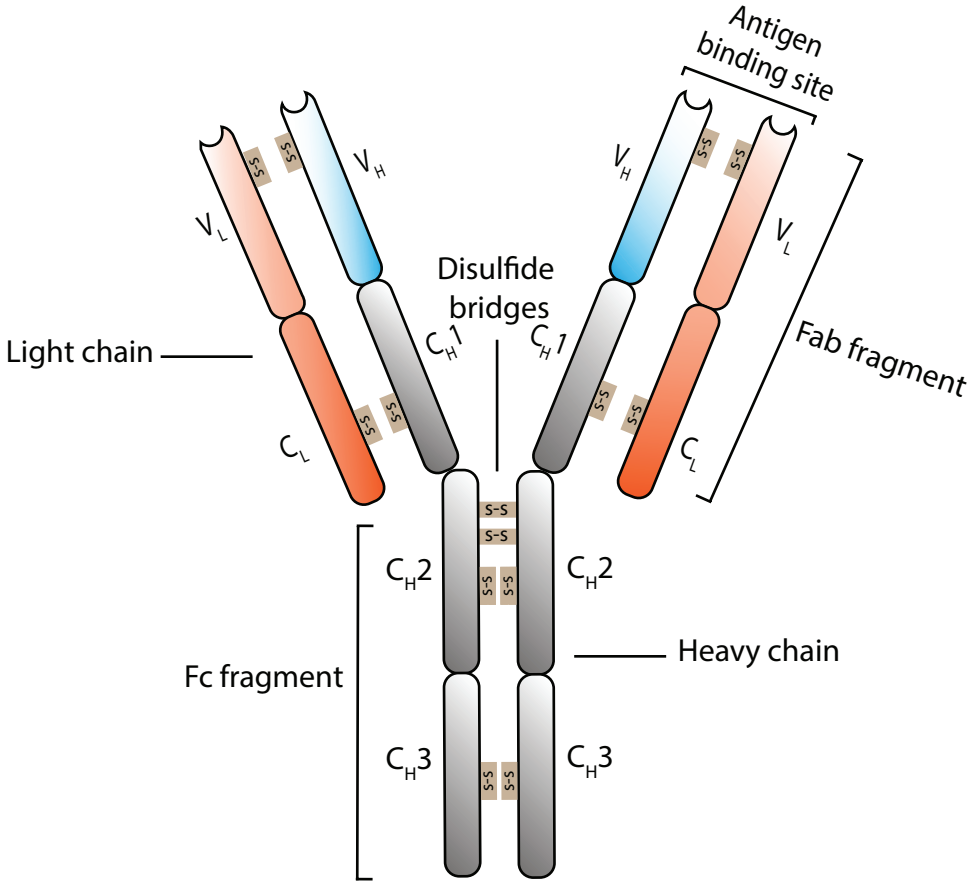


**Figure 1.9 Components of IgG antibody**
Diagram illustrating the structural composition of an IgG class of Ab.

The effector function of the cell-mediated Abs responses requires a recognition of the C region of the antibodies by a special cell surface receptor, termed Fc-receptor (FcR). These form a large group with various features that facilitate immune responses and are presented at the surface of a wide range of immune cells. For instance, phagocytosis of Ab-coated pathogens is largely carried out by macrophages and neutrophils that express FcγRI, FcγRIIA, and FcγIIc receptors and could interact with the Fc fragment of Abs [573]. For its part, an ADCC response would mainly be induced during

the recognition of IgG-Ab by the FcRIIIA receptor present at the surface of NK cells, with less contribution of macrophages and neutrophils [573]. The interaction of the Fc region of the Ab with the cellular receptor leads to the activation of the CD3ζ and / or FcεRIγ subunits that contains a single conserved motif known as ITAM (an immunoreceptor tyrosine-based activation motif) that favours the activation and degranulation of the effector cell [516, 574]. The effector activity of antibodies are determined by their Fc glycosylation profile [575]. In particular, the glycosylation of asparagine 297 that is known to affect the conformation of the Ab and to modulate its affinity for several Fc region [576-578].

### 1.2.4 CD4i Antibodies

One outcome of Env-CD4 interaction is the exposure of several CD4i epitopes. Numerous CD4i epitopes are targeted by various families of Abs, such as anti-CoRBS, anti-V1V2, anti-V3, anti-cluster A, and anti-gp41Abs. Two of these families (anti-cluster A and anti-CoRBS) could influence ADCC responses and will be addressed in this section.

#### 1.2.4.1 Anti-Cluster A Abs

CD4-induced conformational changes in Env causes the exposure of highly conserved epitopes that include those in the cluster A region located within the inner gp120 domain. This region contains the N- and C- terminals of gp120, seven-β-sandwich stranded, and the gp120 layers (1, 2, and 3). The cluster A epitopes were identified as potent target for ADCC responses and include Abs like A32 and C11 [579]. While A32 antibody maps to the interface of layer 1 and 2 of gp120 inner domain, C11 antibody binds

to the gp120 N-termini [265, 580-584]. Structural analysis of gp120 cores and CD4 in complex with the A32-like N5-i5 and 2.2c Abs revealed the importance of α0 and α1 helices in the C1 region to be crucial for their binding [563]. More importantly, the introduction of a disulfide linker that stabilizes the gp120 inner domain has facilitated the co-crystallization of A32 Ab [581] that support the role of W69 as a key residue for ADCC responses as mediated by anti-cluster A Abs [265, 565]. Anti-cluster A Abs are also known as non-neutralizing Abs (nnAbs) since they lack the potency to neutralize virions, but they have been described in many studies as potent ADCC mediators when bound to Env presenting the CD4-bound conformation [374, 562, 564, 567, 581, 585]. Nevertheless, cells infected with primary isolates expose Env in a closed state 1 conformation, limiting the recognition by anti-cluster A Abs and render them resistant to ADCC response [373, 374, 564, 572, 586, 587].

#### 1.2.4.2 Anti-CoRBS Abs

The co-receptor binding site is comprised of the V3 loop and the bridging sheet, which is formed by the β-sheets of V1V2 and β20-β21 loops that move closer upon interaction with CD4 [268]. The CoRBS epitopes are located within the bridging sheet that are presented after Env-CD4 binding and are recognized by CD4i Abs. These CoRBS epitopes are poorly expressed on HIV-1 infected cells presenting the Env trimer on state 1 [565, 586-589]. Although cells infected with viruses lacking Nef and Vpu can be well recognized by CoRBS Abs, these Abs are unable to promote an ADCC response [565, 585, 587, 588]. The inability of CoRBS Abs to mediate potent ADCC could be linked to

the angle of approach that they engage to interact with Env that might result in poor exposure of their Fc region to the FcR in effector cells [374, 563].

### 1.3 Natural Killer Cells (NKs)

NK cells are large granular lymphocytes that belong to the innate immune system and compose 10-15% of peripheral blood lymphocytes [590]. Besides presenting in the blood, these cells also populate in organs [591]. The function of these cells has been known to be central to innate immunity and has been implicated in controlling viral infections, modulating tumor immune surveillance [592], and contributing/protecting against autoimmune disorders [593]. They also can contribute in initiating adaptive immune responses through engaging in “cross talk” with DC leading to reciprocal activation of both cells. Ultimately, the activated DC initiates an adaptive T cell response [594]. Several lines of evidences supported the role of NK cells in directly clearing or contributing to control viral infections.

NK cells were originally described as cytotoxic agents capable of killing cancer or infected cells without the need for prior immunization. They induce the death of target cells through the release of granules containing cytolytic molecules (perforins and granzymes) in a process known as degranulation. These molecules are released within an immunological synapse complex at the surface of target cells. While the secretion of perforins allows the formation of pores within the plasma membrane of the target cell, granzymes initiates the apoptosis cascade via activation of the caspase pathway [595]. This degranulation is induced upon interaction with cells expressing activation ligands or

coated target cells. Additionally, NK cells are capable to promote apoptosis of target cells via expression of ligands associated with cytotoxic receptors such as Fas ligand, TRAIL cytokine, and membrane TNF-α (mTNF-α) [596-598]. NK cells are also considered important immunoregulatory cells that produce a multitude of cytokines and chemokines, such as TNF-α, GM-CSF (granulocyte macrophage colony-stimulating factor), IL-15, IL-13, IL-10, TGF-β, MIP-1α, MIP-1β, RANTES, and IFN-γ [599]. Notably, the production of these various cytokines and chemokines facilitates NK cells to promote a type-1 response, differentiation, and activation of macrophages and DCs [600, 601]. NK cells might be stimulated through several cytokines that are produced by DCs and macrophages, such as IFN-α / β, IL-2, IL-15, or IL-21 that could induce cell activation. Like other innate immune cells, NK cells also express several Toll-like receptors (TLR 2-9) that facilitate their activation via the recognition of certain viral proteins and nucleic acids [602-604]. Finally, the effector functions of NK cells are mainly regulated through the expression of a multitude of activator and inhibitory receptors that bind to various proteins on target cells (see below).

### 1.3.1 NK Cells Receptors

Inhibitory and activating receptors of NK cells recognize a various range of self and non-self ligands including MHC-I ligands, stress induced ligands, and some viral ligands [516]. Generally, inhibitory receptors modulate strong interactions with cells expressing MHC-I molecules whereas activating receptors mediate recognition of viral antigens or stress ligands expressed on the surface of virally infected or transformed cells [509, 605, 606]. The recognition patterns of NK cells facilitate the immune system to

spare healthy autologous cells expressing normal MHC-I, and allow NK effector cells to eliminate infected or transformed cells that exhibit downregulation of MHC-I or upregulation of stress ligands [607-609].

The ability of these cells to transmit signal upon ligation is determined through the features of their transmembrane regions and cytoplasmic tails [574, 610]. Inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) within their cytoplasmic tail that become phosphorylated upon ligation of the receptor causing the recruitment of phosphatases, including SHP-1, SHIP-2, and SHIP, which can subsequently decrease the phosphorylation of signalling molecules preventing the activation signals. Activating receptors lack the presence of these signaling motifs within their cytoplasmic tail. Instead, these receptors carry positively charged residues at their transmembrane region that assist their association with signalling adaptor molecules containing the ITAM motif, such as DAP10, DAP12, or FcγR [516, 611, 612]. Although the signalling approach of the inhibitory receptors is quite consistent, the adaptor protein and signalling pathways derived by the activating receptors depends on each receptor.

NK cells receptors have exhibited great diversity in humans that includes the conserved lectin-like receptors (NKG2A, NKG2C, and NKG2D) [516], natural cytotoxicity receptors (NCR) (NKp44 and NKp46) [613], and FcγRIIIa (CD16a) [614]. The later mediates the recognition of the constant IgG region bound to the surface of target cells; thereby, allowing the recognition of transformed or infected cells with the help of adaptive immunity. NK cells also express the killer immunoglobulin-like receptor (KIR) that

undertook a similar evolutionary path with simian primates [615]. Each one of these families of receptor modulates an important, yet mediate distinct activities an understanding of these receptors could in the future harness the therapeutic potential of NK cells.

### 1.3.2 Activating NKG2D receptor

The activating NKG2D receptor is an evolutionary conserved type II transmembrane protein. It is expressed at the cell surface through a disulfide-linked homodimeric glycoprotein. It has the ability to recognize a group of MHC-I like receptors that include MICA, MICB, ULBP1-6. These ligands have a structure similar to MHC-I, but they miss the ability to present peptides [616]. They are not normally expressed on the cell surface, but are upregulated during stress periods following cellular transformation or viral infection. The ligation of NKG2D via any of its ligands results in NK cell activation, as a result of the association of NKG2D receptors with ITAM-connected to DAP-10 adaptor proteins [517].

The importance of the activation of NK cells through NKG2D is supported by the fact that numerous viruses and tumors have developed various evasion approaches to avoid NKG2D-mediated NK cell responses. For example, solid tumors tend to increase the secretion of soluble NKG2D ligands, which could ultimately decline the surface level of NKG2D and impair NK cells functions. As mentioned above, HIV-1 Nef protein has the ability to downmodulate the expression of NKG2D ligands; thus, avoiding recognition of infected cells by NK cells [508].

### 1.3.3 The impact of HIV-1 infection on NK cells

Although NK cells appear to be active at the early stages of infection, the chronic phase of HIV-1 infection is associated with several phenotypic and changes in the NK cell population. These changes are largely attributed to the dramatic decrease in the cytolytic NK-subpopulation ($CD56^{low}$ $CD16^{+}$) that is preferentially found during the acute phase [617-620], and causes the expansion of a subpopulation of $CD56^{-}$ $CD16^{+}$ cells that has low lytic activity. Compared to the other populations of NK cells, these pathogenic $CD56^{-}$ $CD16^{+}$ cells have showed to be a dysfunctional subset of NK cells present in infected individuals [621-624]. Indeed, the progression of HIV-1 infection to the chronic phase has been associated with decreased NK cell direct or indirect cytolytic activity [621, 622, 625, 626]. This decreased cytosolic activity appears to be associated with a reduced expression of several NK cytotoxic receptors, such as NKp44, NKp46, and NKp30 in viremic patients [623, 624, 627]. Several studies have also shown that the level of most NK-inhibitory receptors is maintained or even increased in HIV-1 infected individuals [623, 624, 628, 629]. The cytokine and chemokine productions of NK cells are also reduced; thus, impairing their ability to interact with DCs [630, 631]. It has been also shown that the viral Env-gp120 is capable of suppressing the effector functions and proliferation of NK cells through its interaction with integrin α4β7 [632, 633].

Additionally, it has been shown that the reduction of NKG2D activity on NK cells was associated with the chronic phase of HIV infection, due to a decrease in its transcription and expression [525, 526]. Decreasing the level of expression of such activating receptors could then promote impairment of NK cell immunosurveillance

against opportunistic infections and cancers that are associated with the late stages of the disease. Interestingly, the control of viremia following ART treatment is associated with re-expression of NK-activating and inhibitory receptors; thus, indicating the importance of the regulation these receptors on the active viral replication [525, 623]. Several studies have indicated that these phenotypic and functional effects on NK cells are not observed in patients with low / undetectable viral load or non-progressing to AIDS, suggesting that these changes are induced by ongoing viral replication [624, 634-636]. The exact mechanism (s) involved in these phenotypic and functional changes in NK cells during HIV-1 infection are not completely understood. Therefore, a better understanding of the mechanisms regulating NK cells functions loss during chronic HIV infection could contribute to the development of new therapeutic strategies against HIV-1.

## 1.4 Thesis Rationale and Hypothesis

Over the past decades research has highlighted the crucial role of HIV-1 Nef protein in AIDS pathogenesis. Recent reports have indicated the significant implication of Nef protein on Env conformation and ADCC responses [562, 564]. Interestingly, Nef activities have been reported to be defective in ECs [637] and the unique controlling phenotype of these individuals suggest that an active ongoing immune response might play a role in their unique capacity to control viral replication and disease progression. Therefore, we decided to investigate the effect of Nef clones isolated from ECs and CPs on Env-conformational rearrangements and susceptibility of infected cells to ADCC with the goal to better understand the immune mechanisms involved in the EC's phenotype. **We hypothesized that Nef proteins from ECs unable to fully downregulate CD4 and NKG2D ligands might sensitize HIV-1-infected cells to ADCC (Chapter II and III).**

Env is a highly dynamic molecule that has the ability to transit from the "closed" unliganded state 1 conformation to an "open" CD4-bound state 3 conformation. Its engagement with CD4 induces an asymmetric intermediate "state 2" arrangement that represents a functional conformation on the entry pathway between state 1 and state 3 [560]. These conformational dynamics of HIV-1 Env provide an important balance between the requirement to interact with target cell receptors and the necessity to protect against anti-gp120 Abs. HIV-1 primary isolates carry Envs sampling the state 1 conformation (resistant to the easily elicited CD4i Abs) [587, 638]. The CD4-Env interaction pushes Env into state 2 and 3 conformations that renders it susceptible to recognition by some, but not all CD4i Abs, particularly those with high ADCC potential.

Therefore, the most vulnerable Env conformational state to ADCC remains to be defined. Thus, further understanding of Env-conformational landscape is needed to target the key Env-arrangements necessary for ADCC attack. **We hypothesized that CD4i ADCC-mediating Abs recognize a new Env conformation that is stabilized by CD4 binding.**

# CHAPTER II

## NEF PROTEINS FROM HIV-1 ELITE CONTROLLERS ARE INEFFICIENT AT PREVENTING ADCC

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**Running title**: Nef ADCC

**Key Words:** HIV-1, Nef, elite controllers, Env, gp120, CD4, ADCC, A32

Published in *The Journal of Virology*, February 2016

**Abstract**

Impairment of Nef function, including reduced CD4 downregulation, was described in a subset of HIV-1-infected individuals that control viral replication without antiretroviral treatment (elite controllers, EC). Elimination of HIV-1 infected cells by antibody-dependent cellular cytotoxicity (ADCC) requires the presence of envelope glycoproteins (Env) in the CD4-bound conformation, raising the possibility that accumulating CD4 at the surface of virus-infected cells in EC could interact with Env and thereby sensitize these cells to ADCC. We observed a significant increase in the exposure of Env epitopes targeted by ADCC-mediating antibodies at the surface of cells expressing Nef isolates from EC; this correlated with enhanced susceptibility to ADCC. Altogether, our results suggest that enhanced susceptibility of HIV-1-infected cells to ADCC may contribute to the EC phenotype.

**Importance**

Nef clones derived from elite controllers (EC) have been shown to be attenuated for CD4-downregulation; how this contributes to the non-progressor phenotype of these infected individuals remains uncertain. Increasing evidence supports a role for HIV-specific antibody dependent cellular cytotoxicity (ADCC) in controlling viral infection and replication. Here we show that residual CD4 left at the surface of cells expressing Nef proteins isolated from ECs are sufficient to allow Env-CD4 interaction, leading to increased exposure of Env CD4-induced epitopes and increased susceptibility of infected cells to ADCC. Our results suggest that ADCC might be an active immune mechanism in EC that helps to maintain durable suppression of viral replication and low plasma

viremia level in this rare subset of infected individuals. Therefore, targeting Nef's ability to downregulate CD4 could render HIV-1-infected cells susceptible to ADCC and thus have therapeutic utility.

**Introduction**

HIV-1 Nef is a small (27-35 kDa) accessory protein critical for viral replication and progression to AIDS (1). Infection with *nef*-deleted or -defective strains of HIV or SIV was shown to lead to a slow or non-progressive disease phenotype (2-4). Nef possesses several activities that are important for viral replication and pathogenesis including down-regulation of CD4 (5, 6) and HLA class I (7) molecules and enhancement of viral infectivity and replication (8, 9). Impairment of these Nef activities was demonstrated in HIV-1 elite controllers (EC), rare infected individuals who spontaneously suppress plasma viremia to < 50 RNA copies/ml without antiretroviral therapy (10). In particular, Nef clones derived from EC displayed a significantly lower ability to down-regulate CD4 when compared to clones isolated from individuals during chronic progressive infection (CP) (10, 11). However, how impaired Nef function contributes to the EC phenotype remains unclear.

Increasing evidence supports a role for HIV-specific antibody dependent cellular cytotoxicity (ADCC) in controlling viral infection and replication (12-18). Analysis of the correlates of protection in the RV144 vaccine trial suggested that increased ADCC activity was linked with decreased HIV-1 acquisition (19); and antibodies (Abs) with potent ADCC activity were isolated from some RV144 vaccinees (20). We reported that the CD4-bound conformation of HIV-1 envelope glycoproteins (Env) was preferentially targeted by ADCC-

mediating Abs or sera from HIV-1-infected individuals (21-24). Of note, ADCC-mediating (non-neutralizing) Abs represent a significant proportion of anti-Env Abs elicited during natural HIV infection (24, 25), and particularly high levels of ADCC-mediating Abs have been observed in EC subjects (26, 27). However, in order to limit the exposure of CD4-bound Env at the surface of infected cells, HIV-1 has developed sophisticated mechanisms to efficiently internalize Env (28), to counteract host restriction factor BST-2 by Vpu (23, 29, 30), and to downregulate CD4 by Nef and Vpu (23, 24). It was also reported that the Env-CD4 interaction plays a role in CD4 downregulation (31). Here we evaluated whether the inability of EC Nef clones to fully downregulate CD4 results in the adoption of a CD4-bound Env conformation on HIV-1-infected cells and enhanced susceptibility of these cells to ADCC.

## Materials and Methods

### Cell lines and isolation of primary cells

293T human embryonic kidney and HOS cell lines were obtained from ATCC and NIH AIDS and Research and Reference Reagent Program, respectively. PBMCs from healthy donors were obtained under research regulations approved by the CRCHUM; written informed consent was obtained from each individual. Cells were grown as previously described (23, 32). CD4 T lymphocytes were purified from rested PBMCs by negative selection and activated as previously described (24).

**Study Participants and nef cloning.**

Plasma from 47 untreated EC (pVL< 50 RNA copies /ml plasma) and 48 untreated CP (median pVL 80500 (interquartile range, IQR) 25121-221250) was used to amplify Nef sequences (10, 11, 33-36), see below. All EC and CP were HIV-1 subtype B-infected from the Boston area, and comparable with respect to ethnicity and diagnosis date of HIV (EC: 1985-2006 vs CP: 1981-2003). The study was approved by the institutional review board of Massachusetts General Hospital, Boston USA; all participants provided written informed consent.

HIV RNA was extracted from plasma of EC and CP subjects and amplified using nested RT-PCR, as described (36, 37). Nef amplicons were cloned into pIRES2-EGFP expression vector (Clontech). At least three Nef clones were sequenced per patient, and a single clone having an intact Nef reading frame that closely resembled the sequence of the original bulk plasma RNA was chosen, as described (10). Nef clones were transferred into a pNL4.3 lacking Nef (N-) plasmid and confirmed by DNA sequencing as described (38). Recombinant viruses harbouring *nef* from HIV SF2 strain (wt Nef $_{SF2}$) and lacking *nef* (N-) were used as positive and negative controls, respectively.

**Viral production and infections**

Vesicular stomatitis virus G (VSVG)-pseudotyped pNL4.3-encoding Nef SF2, deleted *nef*, and *nef* clones from EC (15 clones) or CP (15 clones) viruses were produced in 293T cells and titrated as previously described (23). A random number generator (GraphPad QuickCalcs) was used to randomly select EC and CP *nef* proviruses for this

study. Viruses were then used to infect approximately 20 % to 30 % of primary CD4 T cells from healthy donors by spin infection at 800 g for 1 h in 96-well plates at 25 °C.

**Antibodies and sera**

The gp120 outer-domain recognizing antibody 2G12 was obtained from the NIH AIDS and Research and Reference Reagent Program. The broadly-neutralizing CD4-binding site VRC01 antibody was obtained from Dr. Peter Kwong (VRC, NIAID). The anti-gp120 cluster A (A32, L9-i1, L9-i2, N26-i1) and anti-gp41 antibodies (7B2, M785-U1) were previously reported (23, 39-41). The monoclonal antibody anti-CD4 OKT4 (BioLegend) binds to the D3 domain of CD4 and was used to measure cell surface levels of CD4, as described (23). The secondary goat anti-mouse and anti-human antibodies coupled to Alexa Fluor 647 (Invitrogen) were used in flow cytometry experiments.

HIV+ sera was obtained from the Montreal Primary HIV Infection Cohort (42, 43) and the Canadian Cohort of HIV Infected Slow Progressors (33, 44, 45). Research adhered to the ethical guidelines of the CRCHUM and informed consent was obtained from each volunteer. 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 and used in subsequent experiments as shown (23, 24). A random number generator (GraphPad QuickCalcs) was used to randomly select a number of sera from each cohort for testing.

**Cell-based ELISA**

Detection of trimeric Env at the surface of HOS cells was performed by cell-based ELISA, as previously described (23, 46, 47). Briefly, HOS cells were seeded in 96-white well plates (2 x $10^4$ cells per well) and transfected the next day with a cytoplasmic-tail deleted HIV-1 $Env_{YU2}$ variant together with a human CD4 expressor and *nef*-encoding plasmid from EC or CP using standard a polyethylenimine (PEI, Polyscience Inc, PA, USA) transfection method. Two days post transfection, cells were blocked (10mg/ml non-fat dry milk, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 25mM Tris, pH 7.5 and 140mM NaCl) and then incubated with 1:1000 dilutions of HIV+ sera or 1 µg/mL of relevant mAbs. Env/CD4-specific IgGs were detected using an HRP-conjugated anti-human/mouseIgG-specific secondary Ab (Pierce) with a TriStar LB 941 luminometer (Berthold Technologies).

**Flow cytometry: cell-surface staining and ADCC responses**

Cell-surface staining was performed as previously described (23, 24). Binding of HIV-1 infected cells by sera (1:1000 dilution) or relevant mAbs (1 µg/mL) was performed 48 hr after infection. After surface staining, infected cells were permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) to detect infected cells (p24+ cells) with the fluorescent anti-p24 mAb (PE-anti-p24, clone KC57; Beckman Coulter/Immunotech, Hialeah, FL) (1:100 final concentration), as previously described (23, 48). The percentage of infected cells was determined by gating on the living cell population based on the viability AquaVivid dye (Invitrogen). 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 or A32-mediated ADCC responses was performed using a FACS-based ADCC assay (21, 23, 24, 48). Briefly, infected primary CD4+ T cells were stained with viability (AquaVivid; Invitrogen) and cellular (cell proliferation dye eFluor670; eBiosciences) markers and used as Target cells (T). Autologous PBMC effectors cells (used as Effector cells, E), 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; infected cells were identified by intracellular p24 staining, as described above. Samples were analyzed on an LSRII cytometer (BD Biosciences). 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 or A32) / (% of $p24^+$ cells in Targets).

**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.

## Results

### Attenuated CD4 downregulation by Nef alleles from elite controllers enhances exposure of ADCC-mediating epitopes on HIV-1 Env

To evaluate the effect of CD4 downregulation mediated by Nef clones isolated from previously characterized EC (47 clones) or chronic progressors (48 clones) (10, 11, 49-51), on the exposure of Env ADCC-mediating epitopes, we used a previously-described cell-based ELISA which allows measurement of Env conformation at the cell surface (22). Briefly, HOS cells were transfected with plasmids expressing Env, CD4 and Nef clones. Two days later, transfected cells were washed and incubated with anti-CD4 and anti-Env Abs or sera from HIV-1-infected individuals. As previously reported (10, 11), Nef clones isolated from EC were less efficient for CD4 downregulation compared to Nef clones from CP (Figure 1A), but no difference was observed in the overall levels of Env, as measured by the outer-domain recognizing 2G12 Ab (Figure 1B) or anti-gp41 Abs (Figure 2). CD4 remaining at the surface of cells expressing EC Nef clones competed for ligands that recognize the CD4-binding site, such as VRC01 Ab (Figure 1C), suggesting that CD4 recognized its binding site on these cells in a manner similar to that of Env in the context of viral particles, as previously-reported (23). Importantly, in presence of EC Nef clones, Env exposed CD4-induced (CD4i) ADCC-mediating epitopes, such as A32 (Figure 1D) and other anti-Cluster A Abs (Figure 1 E-G), significantly better. Moreover, in the presence of EC Nef clones, we observed that Env was recognized better by sera from HIV-1-infected individuals (Figure 1H), suggesting that inefficient CD4 downregulation by EC Nef clones might affect Env conformation and sensitize infected cells to ADCC.

**Cells infected with viruses coding for Nef from elite controllers expose Env ADCC-mediating epitopes.**

To address whether ADCC-mediating epitopes were better exposed at the surface of infected cells, 15 randomly selected Nef clones from EC and 15 from CP were cloned into replication-competent pNL4.3 proviruses. Infectious viral particles were generated by transfection into 293T cells and used to infect primary CD4 T cells isolated from healthy donors. As positive and negative controls for CD4 downregulation, viruses coding for wild-type $Nef_{SF2}$ or deleted for Nef (Nef-) were also generated. At two days post-infection, cells were surface stained with anti-CD4, anti-Env or HIV+ sera followed by permeabilization and intracellular p24 staining. As expected, cells infected with wild-type virus displayed greater CD4 downregulation than those infected with Nef- virus (Figure 3A). No difference was observed in the amount of Env at the cell surface between the two viruses, based on recognition by 2G12 Ab (Figure 3B). Remarkably, relatively modest difference in CD4 levels at the surface of wild-type versus Nef- virus infected cells was sufficient to induce Env conformational changes and expose CD4i epitopes such as those recognized by A32 (Figure 3C), N26i1 (Figure 4 A) or HIV+ sera (Figure 3D). As expected, sera from healthy uninfected individuals failed to recognize HIV-1-infected cells (Figure 5). Of note, recognition of infected cells by N26i1 correlated with the amount of CD4 remaining at the cell surface (Figure 4C).

Similar differences were seen for cells infected with viruses encoding EC or CP Nef proteins. Indeed, cells infected with EC Nef viruses presented significantly more CD4 at their surface when compared to CP (Figure 3E); while the overall levels of Env

remained similar (Figure 3F). However, in the context of EC Nef, Env appeared to sample a conformation closer to the CD4-bound state, since it was better recognized by A32 (Figure 3G), N26i1 (Figure 4B) and HIV+ sera (Figure 3H).

**Enhanced susceptibility of cells infected with EC Nef viruses to ADCC-mediated killing.**

Using a previously described FACS-based ADCC assay (21, 23), we then asked whether the higher levels of A32 and HIV+ sera staining at the surface of HIV-1-infected cells observed for EC Nef viruses enhanced their susceptibility to ADCC mediated by PBMCs from healthy individuals. Primary CD4 T cells infected with wild-type and Nef- viruses were used as positive and negative controls. As previously reported (21, 23, 24), wild-type-infected cells were not sensitive to ADCC mediated by autologous PBMCs (Figure 6A and B); thus, highlighting the ability of HIV-1 to escape from this adaptative immune response. Confirming Nef's role in protection from ADCC (21, 23, 24), cells infected with Nef- virus were susceptible to ADCC killing mediated by A32 (Figure 6A) and HIV+ sera from five HIV-1-infected individuals (Figure 6B). Mirroring Env recognition by A32 and HIV+ sera presented in Figure 3, ADCC activity was higher in the presence of cells infected with viruses encoding Nef proteins from EC compared to those from CP. Indeed, using the same PBMCs, we observed that A32 (Figure 6C) and HIV+ sera (Figure 6D) mediated significantly greater killing of infected cells expressing Nefs from EC than CP ($p<0.0001$). Interestingly, recognition of infected cells by HIV+ sera positively correlated with higher levels of CD4 at the cell surface (Figure 7A) and with enhanced ADCC responses (Figure 7B and C). This is true for cells expressing Nefs from EC and

CP as well. But, as mentioned above, ADCC responses were higher for EC due to attenuated CD4 downregulation. Thus, highlighting the necessity of HIV-1 to downregulate CD4 from the surface of infected cells to escape from ADCC.

**Discussion**

Previous studies have shown that HIV-1 has evolved several mechanisms to prevent exposing the CD4-bound conformation of Env at the cell surface (23, 24). Accordingly, by forcing Env to sample this conformation using a CD4 mimetic, we found that it was sufficient to sensitize infected cells to ADCC-mediated killing (48). The HIV-1 accessory protein Nef protects HIV-1-infected cells from ADCC by decreasing cell-surface levels of CD4 (23, 24), which otherwise engages with Env, induces the CD4-bound conformation and exposes CD4i epitopes. These epitopes are recognized by well-established CD4i ADCC-mediating Abs such as A32 (23), or by sera from HIV-1-infected individuals (24, 25, 52). Importantly, in this study Env conformational changes observed at the surface of HIV-1-infected cells depended on Nef proteins since EC and CP Nefs were cloned into isogenic proviruses coding for the same Vpu and Env proteins. Interestingly, a role for ADCC in immune control of HIV-1 in EC was previously suggested based on the presence of high levels of ADCC-mediating Abs in these individuals (27). Moreover, it was recently reported that several Nef functions, including CD4 downregulation, are attenuated in this subset of HIV-1-infected individuals (10, 11), but whether this affected ADCC responses was unknown. Here we evaluated whether the inability of EC Nef clones to fully downregulate CD4 affected the conformation of Env at the cell surface, thereby enhancing susceptibility of HIV-1-infected cells to ADCC.

We observed that Env levels at the surface of cells expressing Nef clones from EC were similar to those of cells expressing CP Nefs. However, in the presence of EC Nef clones, Env sampled the CD4-bound conformation more readily and exposed ADCC-mediating epitopes such as those recognized by the anti-Cluster A class of Abs or HIV+ sera from several HIV-1-infected individuals. Enhanced recognition by these ligands was correlated with the amount of CD4 molecules present at the cell surface and resulted in increased ADCC-mediated killing. These results suggest a model (Figure 8) where a limited amount of CD4 still present at the surface of HIV-1-infected cells in EC is sufficient to induce the CD4-bound conformation of Env, thereby exposing epitopes that are readily recognized by ADCC-mediating Abs, which are also prevalent in sera from these individuals. Upon recognition of Env, these Abs recruit effector cells such as NK cells to eliminate the infected cells through ADCC. These observations may also help to explain the functional and immunological pressure on HIV-1 Nef to maintain its ability to downregulate CD4.

Revealing mechanisms associated with spontaneous control of HIV might provide important insight with regard to HIV pathogenesis and critical information to develop strategies aimed at eliciting a functional cure in non-controller subjects. For example, highly effective HLA class I-restricted T cell responses targeting conserved viral peptide epitopes is a major factor modulating durable control of HIV infection in EC (53). In addition, non-canonical HLA-associated escape mutations in EC have been shown to affect the proper function of several viral proteins, including Nef (10). Indeed, multiple Nef activities, including HLA class I and CD4 downregulation, enhancement of viral infectivity

and replication, were reported to be significantly attenuated in EC Nef clones compared to those from CP (10, 49-51). An extensive analysis of Nef sequences from ECs and CPs used in the current study was previously conducted and found no common residue signatures involved in decreased CD4 downregulation (10). However, inverse associations between the number of EC-specific B*57-associated polymorphisms and Nef-mediated replication, HLA-I down-regulation and CD74 up-regulation were observed (10). CD4 downregulation also displayed a modest, albeit not significant, negative relationship between the burden of B*57-associated escape mutations and function (10). Here we report that impaired CD4 downregulation by EC Nef clones results in a functional consequence; namely, that infected cells become more susceptible to ADCC.

In conclusion, we observed an incomplete downregulation of CD4 molecules by Nef clones isolated from EC. Residual levels of CD4 at the surface of infected cells were sufficient to allow Env-CD4 interaction, leading to increased exposure of Env CD4i epitopes and increased susceptibility of infected cells to ADCC. Our results suggest that ADCC might be an active immune mechanism in EC that helps to maintain durable suppression of viral replication and low plasma viremia level in this rare subset of infected individuals. Therefore, targeting Nef's ability to downregulate CD4 could render HIV-1-infected cells susceptible to ADCC and thus might have therapeutic utility in strategies aiming to elicit a functional cure in HIV-1-infected individuals.

**Funding Information**

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. This study was possible thanks to the International HIV Controllers study, funded by Bill and Melinda Gates Foundation, the Schwartz Foundation and the Harvard University Center for AIDS Research. This work was supported by a Canada Foundation for Innovation Program Leader grant, by Canadian Institutes for Health Research (CIHR) operating grants #119334 and #134117, by a FRQS Establishment of Young Scientist grant #26702 to A.F, by the FRQS AIDS and Infectious Diseases Network and by NIH AI100645 and AI100663 Center for HIV/AIDS Vaccine Immunology and Immunogen Design (CHAVI-ID), and by RO1 HL092565. A.F. is the recipient of a Canada Research Chair on Retroviral Entry. M.A.B. holds a Canada Research Chair of Viral Pathogenesis and Immunity. N.A. is the recipient of a King Abdullah scholarship for higher education from the Saudi Government. J.R. is the recipient of a CIHR Fellowship Award #135349. D.E.K is supported by a Research Scholar Career Award of the Quebec Health Research Fund (FRQS).

**Acknowledgments**

The authors thank the CRCHUM Flow Cytometry Platform for technical assistance as well as Mario Legault for cohort coordination. We thank Maxime Veillette, Mathieu Coutu, and Jean-Philippe Chapleau for helpful discussions; Toshiyuki Miura for isolation of EC virus specimens and all subjects for their participation. The authors have no conflicts of interest to report.

## Figures and Figure Legends

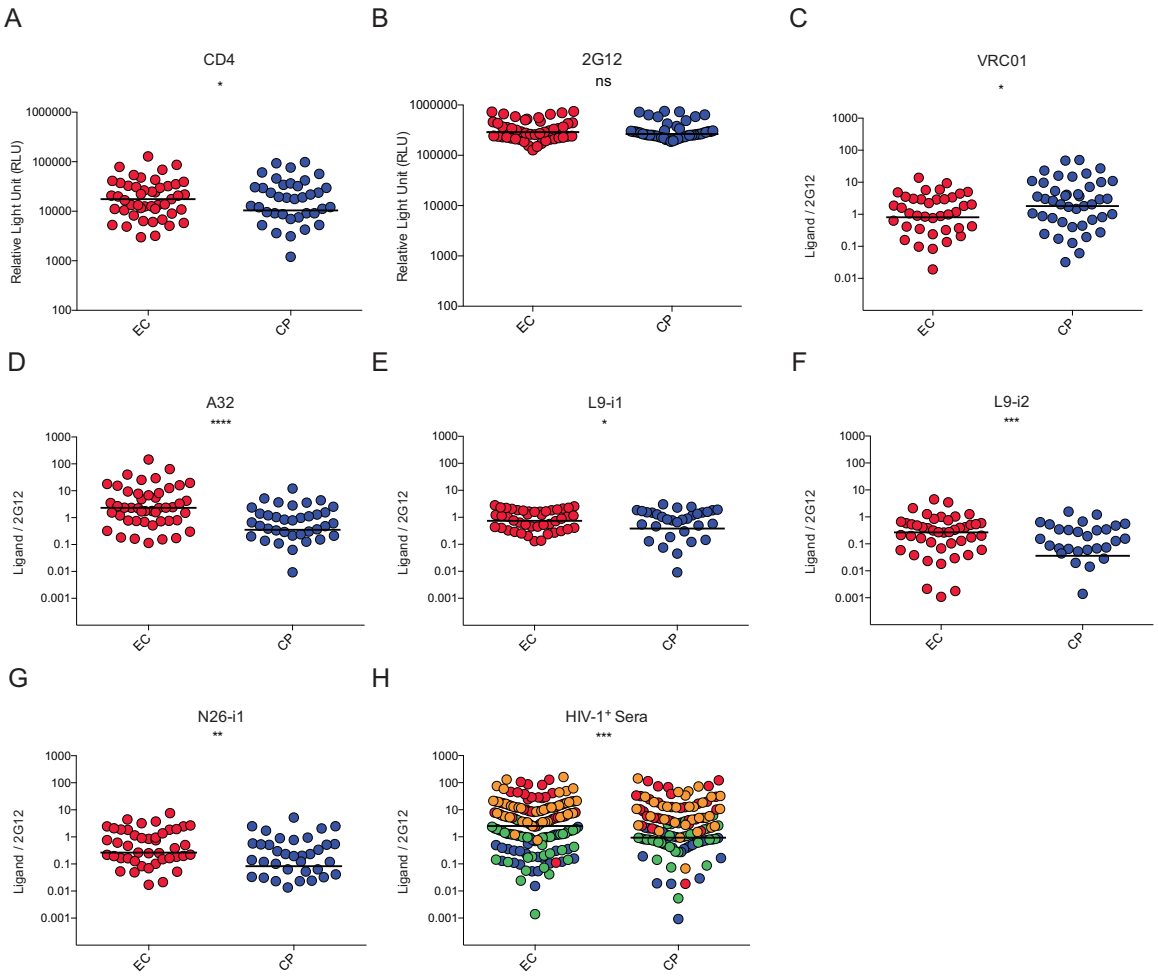


**Figure 2.1 (Figure 1 in Article). Attenuated CD4 downregulation by Nef alleles from elite controllers enhance exposure of ADCC-mediating epitopes.**

Hos cells co-expressing HIV-$1_{YU2}$ Env and CD4 in the presence of Nef isolates from EC (47 clones) or CP (48 clones) were evaluated 48h post-transfection by cell-based ELISA (22). Nef clones from EC present attenuated CD4-downregulation compared to CP (**A**) but do not affect levels of Env present at the cell surface, as measured by 2G12 (**B**). Inefficient CD4 downregulation by Nef from EC resulted in competition for the CD4-binding site VRC01 antibody (**C**) but enhanced recognition by anti-cluster A ADCC-mediating antibodies A32 (**D**), L9i1 (**E**), L9i2 (**F**), N26i1 (**G**) and sera from four HIV-1-infected individuals (**H**). Results for each serum are depicted in a different color. Data shown are representative of those of at least 2 independent experiments acquired in triplicate. In A and B, relative light units (RLU) are shown; in (C-H) signals were

normalized to Env levels as evaluated by 2G12 binding. Shown is median of EC (in red) vs CP (in blue) clones. Statistical significance was tested using unpaired t test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; ns, not significant).

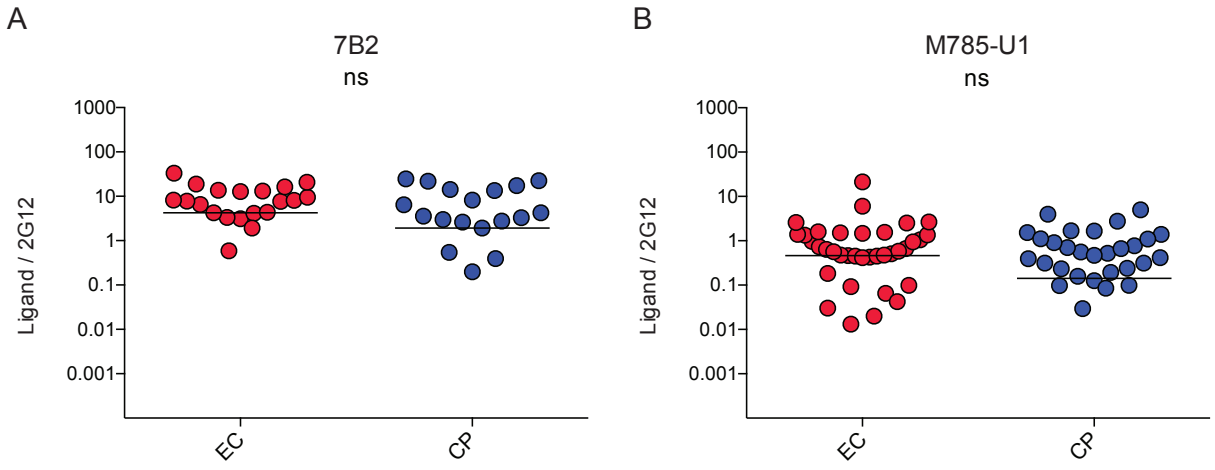


**Figure 2.2 (Figure 2 in Article). Gp41 detection is not affected in the presence of Nef proteins from ECs.**

Env at the surface of Hos cells co-expressing HIV-$1_{YU2}$ Env and CD4 in the presence of Nef isolates from EC or CP is detected to similar levels by 7B2 (**A**) and M785-U1 (**B**) anti-gp41 antibodies. Data shown are representative of those of at least 2 independent experiments acquired in triplicate. Signals were normalized to Env levels as evaluated by 2G12 binding. Shown is median of ECs (in red) vs CPs (in blue) clones. ns, not significant.

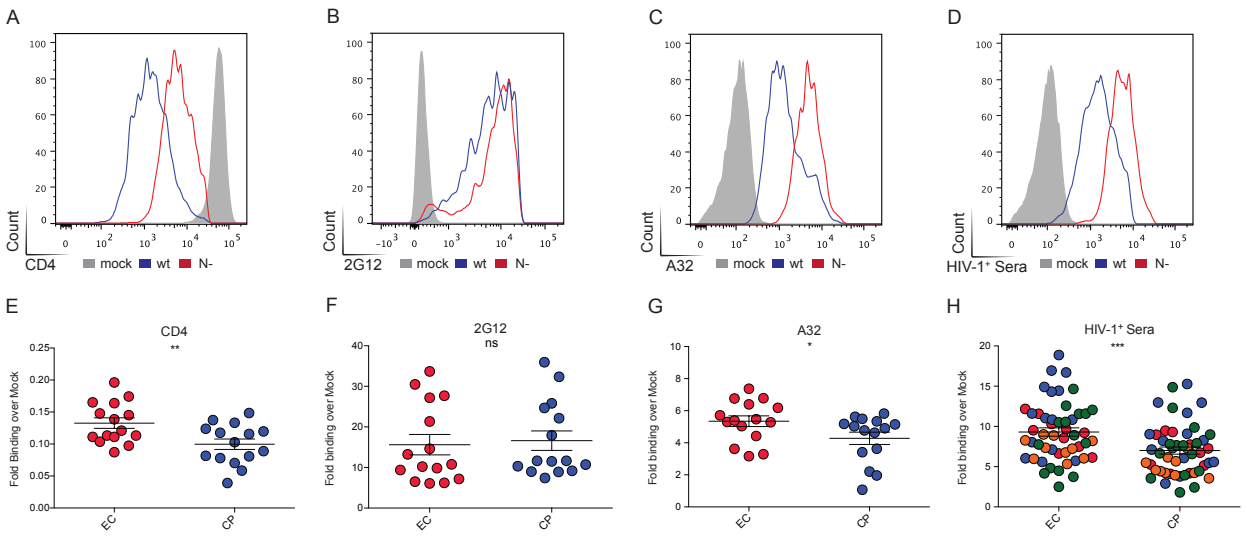


**Figure 2.3 (Figure 3 in Article). Env conformation at the surface of cells infected with viruses coding for Nef proteins from ECs vs CPs.**

Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 15 randomly-selected clones from ECs or CPs. Infected primary CD4 T cells were stained at 48 h post-infection with an anti-CD4 (OKT4, **A** and **E**) or anti Env 2G12 (**B** and **F**), A32 (**C** and **G**) antibodies or with HIV+ sera from 4 different donors (shown in different colors) (**D** and **H**). In the upper panels, results obtained with wt vs Nef- infected cells are shown. In the lower panels, results from cells infected with viruses coding for Nef from ECs (in red) or CPs (blue) are shown. Quantification of data represented as fold binding over mock representative of at least 3 independent experiments is shown. Error bars indicate mean ±SEM. Statistical significance was evaluated using unpaired t test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns, not significant).

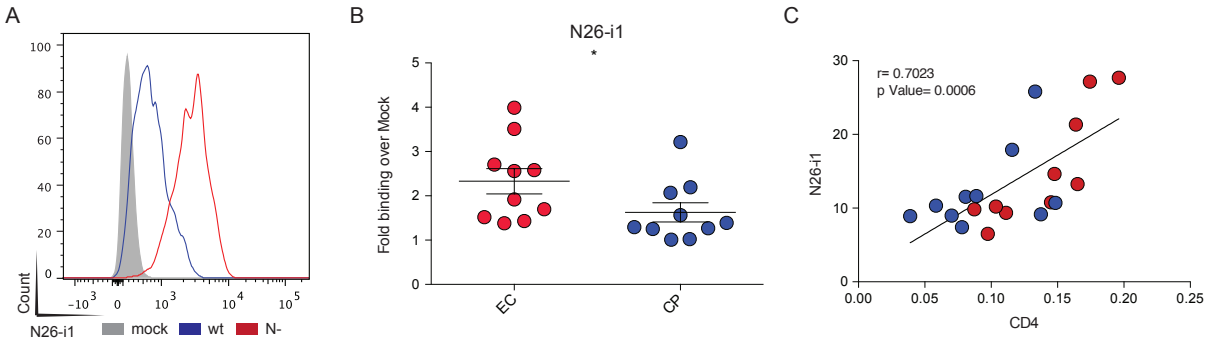


**Figure 2.4 (Figure 4 in Article). Env conformation at the surface of cells infected with viruses coding for Nef proteins from ECs vs CPs.**

Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 10 randomly-selected clones from ECs or CPs. Infected primary CD4 T cells were stained at 48 h post-infection with an anti-cluster A ADCC-mediating antibody N26-i1 (**A** and **B**). In A, results obtained with wt vs Nef- infected cells are shown. In B, results from cells infected with viruses coding for Nef from ECs (in red) or CPs (blue) are shown. Quantification of data represented as fold binding over mock representative of at least 3 independent experiments is shown. (**C**) A positive correlation between cell-surface levels of CD4 and N26-i1 recognition was observed with a Spearman rank correlation. Error bars indicate mean ±SEM. Statistical significance was evaluated using unpaired t test. (*p<0.05).

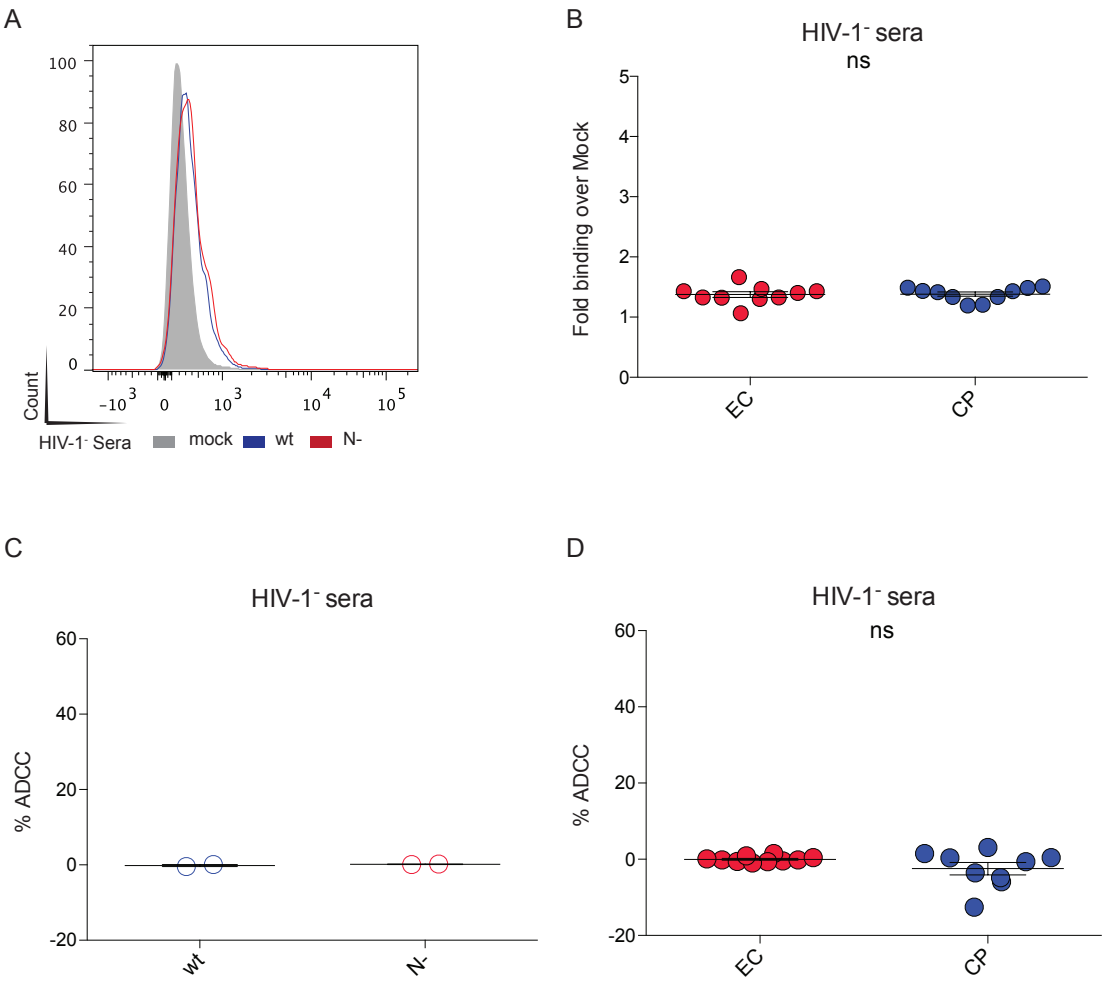


**Figure 2.5 (Figure 5 in Article). HIV-negative sera does not recognize or mediate ADCC of cells infected with viruses coding either Nef from ECs or CPs.**

Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 10 randomly-selected clones from ECs or CPs. Infected primary CD4 T cells were stained at 48 h post-infection with sera from healthy HIV-negative donors (**A** and **B**). Cells were also used to evaluate the ability of HIV-negative sera to mediate ADCC (**C** and **D**). In the left panels, results obtained with wt vs Nef- infected cells are shown. In the right panels, results from cells infected with viruses coding for Nef from ECs (in red) or CPs (blue) are shown. In B data is presented as the quantification of fold binding over mock in at least 3 independent experiments. ns, not significant.

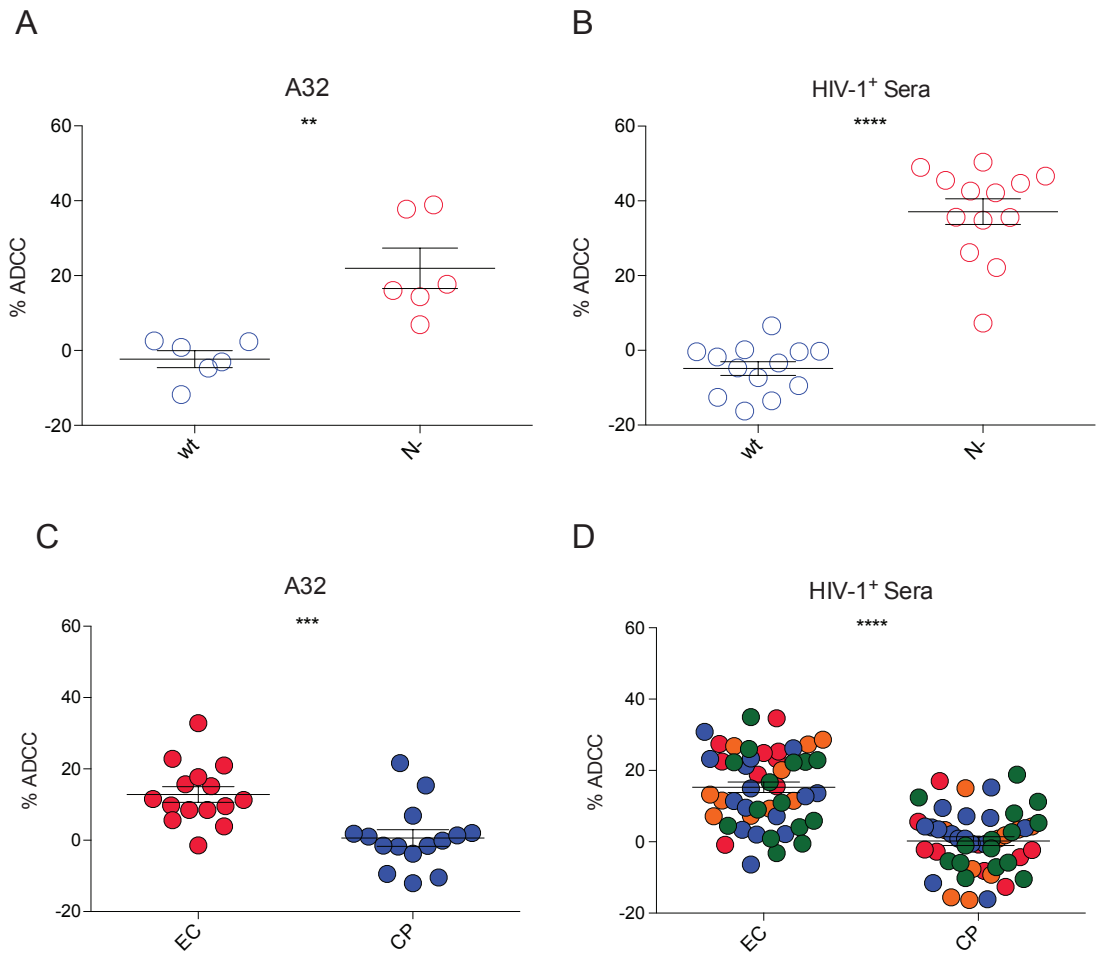


**Figure 2.6 (Figure 6 in Article). Primary CD4 + T cells infected with viruses coding Nef proteins from elite controllers are more susceptible to ADCC-mediated killing.** Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 15 randomly-selected clones from ECs or CPs and used 48h after-infection as target cells in a FACS-based ADCC assay (23) to determine their susceptibility to ADCC, by autologous PBMCs. ADCC mediated by A32 (**A** and **C**) or HIV+ sera from two to four different donors (shown in different colors) (**B** and **D**) is shown. In the upper panels, results obtained with wt vs Nef- infected cells are shown. In the lower panels, results from cells infected with viruses coding for Nef from ECs (in red) or CPs (blue) are shown. Data shown are representative of at least 4 independent experiments, with mean ± SEM. Statistical significance was tested using unpaired t test (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

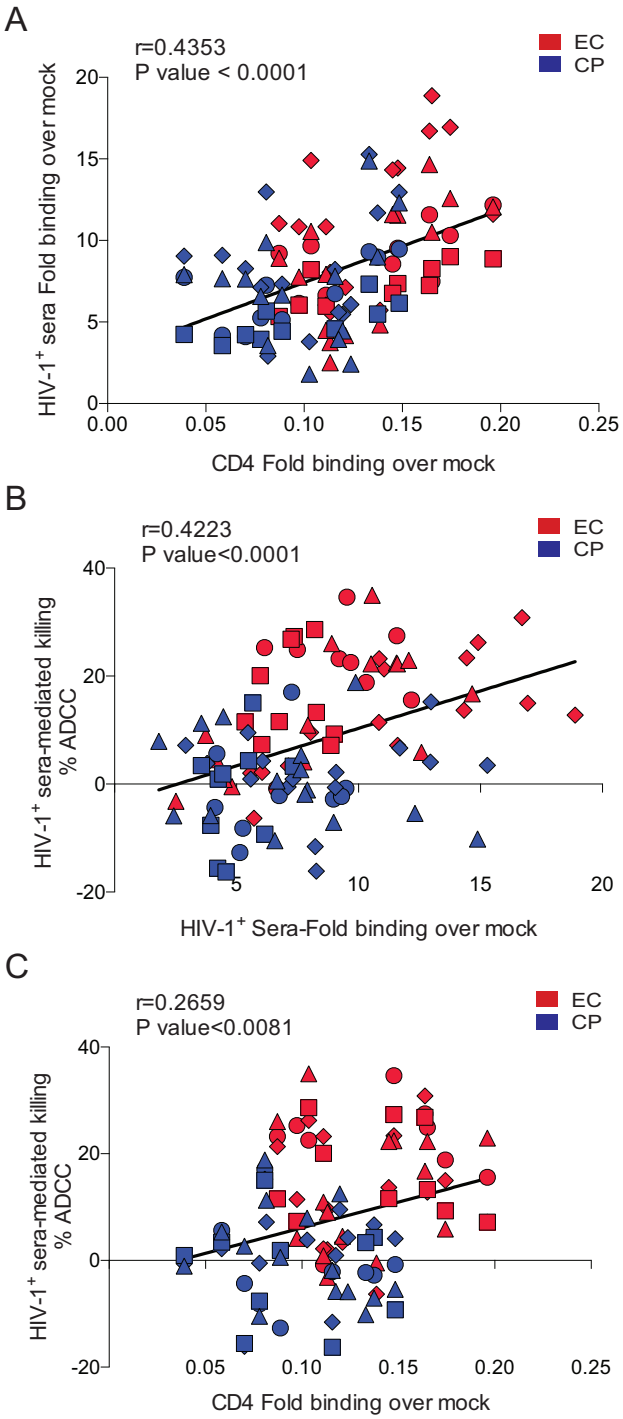


**Figure 2.7 (Figure 7 in Article). The presence of CD4 at the surface of HIV-1-infected cells enhances the susceptibility of infected cells to ADCC mediated by HIV+ sera.** CD4 and HIV+ sera staining of primary CD4 T cells infected with viruses coding for Nef from 15 randomly selected clones from ECs (in red) or CPs (in blue) exhibited a positive correlation (**A**). Sera from two to four HIV-1-infected individuals (represented with different

shapes) were evaluated for their ability to recognize infected cells and correlated with ADCC killing (**B**). Cell surface levels of CD4 correlated with ADCC responses mediated by HIV+ sera (**C**). Statistical analysis was tested utilizing a Spearman rank correlation.

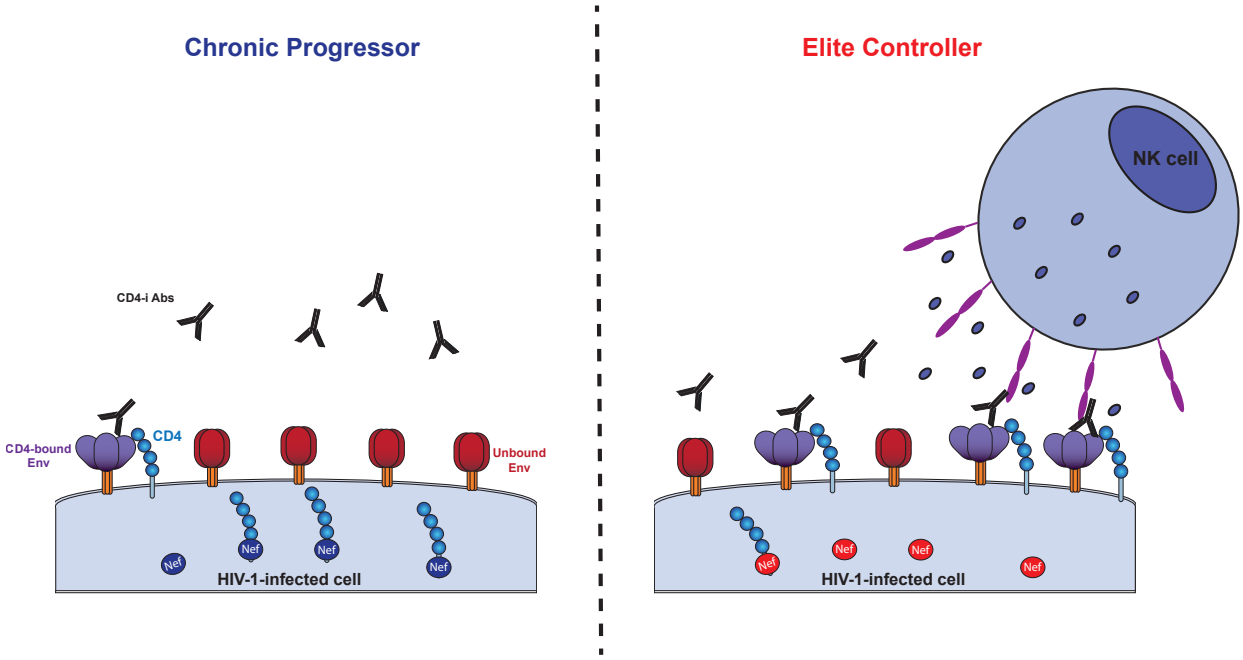


**Figure 2.8 (Figure 8 in Article). Nef-mediated CD4 down-regulation affects the susceptibility of infected cells to ADCC.**

Nef proteins from CPs (left panel, shown in dark blue) downregulate CD4 molecules (light blue) more efficiently than Nef proteins from ECs (right panel, shown in light red). Efficient Nef-mediated CD4-downregulation in CPs allows Env to stay in its unbound conformation (shown in red); thus, hiding ADCC-mediating epitopes. In ECs, limited amounts of CD4 remain at the cell-surface due to attenuated Nef-mediated CD4-downregulation forcing Env to sample the CD4-bound conformation (shown in purple), thereby exposing epitopes recognized by CD4-induced ADCC-mediating Abs (shown in black) which are highly-prevalent in ECs (27). Upon Env recognition, the Fc portion of these antibodies recruits effector cells such as NK cells trough their Fcγ receptors (shown in pink). Activation of effector cells results in degranulation and secretion of perforin and granzymes which ultimately leads to cell death.

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## Bridge from Chapter II to III

Previous research from the groups of Andrés Finzi and Stephen J Kent had demonstrated that the involvement of NKG2D activating receptor in NK-cell mediated-anti-HIV-1 ADCC. The study revealed that NKG2D activating receptor expressed at the surface of NK cells can act in conjunction with CD16 receptor to enhance ADCC response against HIV-1 infected cells [524]. Since the expression of NKG2D ligands was shown to be downregulated by HIV-1 Nef [508], we decided to evaluate whether Nef clones isolated from ECs or CPs differentially modulated NKG2D ligands expression.

# CHAPTER III

## IMPAIRED DOWNREGULATION OF NKG2D LIGANDS BY NEF PROTEIN FROM ELITE CONTROLLERS SENSITIZE HIV-1-INFECTED CELLS TO ADCC

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**Running title:** Nef NKG2D ligands

**Key Words:** HIV-1, Nef, elite controllers, ADCC, NKG2D, gp120, CD4

Published in *The Journal of Virology*, July 2017

**Abstract**

HIV-1 Nef clones isolated from a rare subset of HIV-1-infected elite controllers (EC), with the ability to suppress viral load to undetectable levels in the absence of antiretroviral therapy, are unable to fully downregulate CD4 from the plasma membrane of CD4+ T cells. Residual CD4 left at the plasma membrane allows Env-CD4 interaction, which leads to increased exposure of Env CD4-induced epitopes and increases susceptibility of infected cells to antibody-dependent cellular cytotoxicity (ADCC). ADCC is mediated largely by natural killer (NK) cells, which control their activation status through the cumulative signals received through activating and inhibitory receptors. Recently, the activating NKG2D receptor was demonstrated to positively influence ADCC responses. Since HIV-1 Nef has been reported to reduce the expression of NKG2D ligands, we evaluated the relative abilities of Nef from EC and progressors to downmodulate NKG2D ligands. Furthermore, we assessed the impact of EC and progressor Nef on the ADCC susceptibility of HIV-1-infected cells. We observed a significantly increased expression of NKG2D ligands on cells infected with viruses coding for Nef from EC. Importantly, NKG2D ligand expression levels correlated with enhanced susceptibility of HIV-1-infected cells to ADCC. The biological significance of this correlation was corroborated by the demonstration that antibody-mediated blockade of NKG2D significantly reduced ADCC of cells infected with viruses carrying Nef from EC. These results suggest the involvement of NKG2D/NKG2D ligand interactions in the enhanced susceptibility of EC HIV-1-infected cells to ADCC responses.

## Importance

Attenuated Nef functions have been reported in HIV-1 isolated from elite controllers (EC). The inability of elite controller Nef to fully remove CD4 from the surface of infected cells enhanced their susceptibility to elimination by ADCC. We now show that downregulation of NKG2D ligands by HIV-1 Nef from EC is inefficient and leaves infected cells susceptible to ADCC. These data suggest a critical role for NKG2D ligands in anti-HIV-1 ADCC responses.

## Introduction

Human Immunodeficiency virus type 1 (HIV-1) has developed multiple mechanisms to evade the immune system and establish a chronic infection. Several of these strategies are mediated by viral accessory proteins, including Vif, Vpr, Vpu, and Nef, which together ensure viral persistence and survival. The negative regulatory factor (Nef) is a 27-35 kDa accessory protein that is highly expressed in the early phase of HIV-1 infection (1, 2). It has been shown that infection with HIV-1 or SIV strains unable to code for functional Nef proteins leads to a slow or non-progressive disease (3, 4). The positive effects of Nef on viral-pathogenicity and persistence are based largely on its ability to decrease the surface expression levels of important cellular molecules present on the surface of infected cells, including CD4, CD28, NKG2D ligands and HLA-I molecules (5-8). The ability of Nef to decrease surface expression of certain cell surface proteins, such as CD4 and HLA-I, is impaired in Nef clones isolated from elite controllers (EC) (9, 10), a rare group of infected individuals with the ability to suppress plasma viremia to <50 RNA copies/ml in the absence of antiretroviral therapy. The impaired ability of Nef proteins

isolated from EC to decrease CD4 expression is associated with increased susceptibility of HIV-1-infected cells to antibody-dependent cellular cytotoxicity (ADCC) (11).

ADCC efficacy relies on several parameters imposed by the characteristics of the target and effector cells involved, as well as the features of the antibodies. Natural killer (NK) cells, monocytes and neutrophils serve as effector cells for anti-HIV-1 ADCC (12). The ADCC function of NK cells is a topic of much research interest due to the extensiveness of NK cell regulation. Indeed, NK cells regulation occurs on two levels (reviewed in (13)). Initially, NK cells undergo a process termed “NK cell education”, where NK cells that carry inhibitory receptors capable of interacting with self major histocompatibility complex class I (MHC-I or HLA-I) ligands are conferred with functional potential. Alternatively, NK cells that do not carry self HLA-I-binding inhibitory NK cell receptors remain hypofunctional. The second level of NK cell regulation occurs when NK cells encounter a putative target cell. At this point the outcome of the effector/target cell interaction is determined by cumulative signals received through the plethora of inhibitory and activating receptors on the NK cell surface. It is through these co-signaling molecules that the characteristics of the target cell influence the capacity of the effector cell to mediate ADCC. If the target cell expresses sufficient HLA-I that can bind to inhibitory NK cell receptors, such as killer immunoglobulin receptors of the NKG2A receptor, to create a cumulative inhibitory signal, the target will be spared from cytolysis. Alternatively, if the target cell expresses a sufficiently high enough density of antigen targeted by ADCC antibodies that are recognized by the activating receptor FcγRIIIa/CD16, the target will likely induce a cumulative activating signal and be lysed. HIV-1 utilizes this level of NK

cell regulation to evade ADCC. Indeed, HIV-1 Nef downregulates HLA-I molecules important for cytotoxic T-lymphocyte recognition of infected cells, including HLA-A and HLA-B, but spares HLA-C and HLA-E, which interact with inhibitory NK cell receptors and can inhibit anti-viral ADCC (14, 15). Besides characteristics of effector and target cells, the features of the antibodies involved are important determinants of ADCC. Indeed, glycosylation features of the antibody constant region (Fc) influence the interaction of the antibody with Fc receptors and the degree of ADCC observed (16). Additionally, access of the antibody paratope to the antigenic epitope is essential for efficient ADCC. In the case of HIV-1, it was recently shown that ADCC-mediating antibodies present in HIV sera preferentially recognize Env in its CD4-bound conformation (17). However, to limit the exposure of CD4-bound Env on the surface of infected cells, HIV-1 evolved sophisticated mechanisms to efficiently internalize Env (18), to counteract the host restriction factor BST-2 with the viral Vpu protein (19-21), and to downregulate CD4 by Nef and Vpu (17, 21).

In addition to FcγRIIIa/CD16, there are several other activating and co-activating receptors capable to activate NK cell effector functions. Ligands for these receptors are generally induced as a result of activation, cellular stress, transformation, or viral infection. Accordingly, previous studies demonstrated that ligands for the activating NK cell receptors NKG2D are induced during HIV-1 infection (22-25). Importantly, cell-surface expression of these ligands on HIV-1-infected cells was found to trigger autologous NK cells to lyse infected cells (22-26). While NK cell-mediated killing of HIV-1-infected cells occurs mainly through NKG2D, this response appear to be optimal when the activating

receptor DNAM-1 and the co-activating receptor NTB-A are also simultaneously triggered (27-29). Interestingly, a previous study reported that NKG2D contributes to HIV-1 disease status.

In addition to its capacity to influence NK cell functionality through decreasing surface expression of select HLA-I and decreasing the availability of cell surface CD4 required to CD4-induced (CD4i) ADCC-mediating epitopes (11), Nef has been demonstrated to interfere with NK cell activation by limiting the expression of NKG2D ligands on the surface of infected cells (8). This observation is interesting in the context of additional research suggesting that NKG2D contributes to HIV-1 disease status. Indeed, a previous study linked low viral set point and low viral loads in long-term non-progression patients (LTNPs) with the expression level of the NKG2D (30). The role of Nef in decreasing NKG2D ligand expression on infected cells is also interesting in the context of recent research demonstrating that NKG2D acts as a co-receptor for NK-mediated ADCC against HIV-1 infected cells (31). Since Nef is known to diminish the expression of NKG2D ligands including MICA, ULBP1, and ULBP2 at the cell surface (8), here we investigated whether enhanced susceptibility of HIV-1-infected cells expressing EC Nef to ADCC was linked to modulation of NKG2D ligands.

## Materials and Methods

### Cell lines and isolation of primary cells

293T human embryonic kidney cell line was obtained from ATCC. PBMCs from uninfected healthy donors (n=5) were obtained from leukapheresis under research regulations approved by the CRCHUM; written informed consent was obtained from each individual. Cells were grown as previously described (11, 21) CD4 T lymphocytes were purified from rested PBMCs by negative selection and activated as previously described (32).

### Study Participants and nef cloning.

Nef clones were obtained from plasma of 47 untreated EC (pVL< 50 RNA copies /ml plasma) and 48 untreated CP (chronic progressor) (median pVL 80500 (interquartile range, IQR) 25121-221250) that were previously described (9, 10, 33-36). HIV RNA was extracted and amplified using nested RT-PCR, as described (36, 37). At least three Nef clones were sequenced per patient, and a single clone having an intact Nef reading frame that closely resembled the sequence of the original bulk plasma RNA was chosen (9). Nef clones were transferred into a pNL4.3 lacking Nef (N-) plasmid and confirmed by DNA sequencing, as described (38). Recombinant viruses harbouring *nef* from HIV-$1_{SF2}$ (wt $Nef_{SF2}$) and lacking *nef* (N-) were used as positive and negative controls, respectively. All EC and CP were HIV-1 subtype B-infected from the Boston area, and comparable with respect to ethnicity and diagnosis date of HIV (EC: 1985-2006 vs CP: 1981-2003). The study was approved by the institutional review board of Massachusetts General Hospital, Boston USA; all participants provided written informed consent.

**Viral production and infections**

Vesicular stomatitis virus G (VSVG)-pseudotyped pNL4.3-encoding $Nef_{SF2}$, deleted nef and nef clones from EC (18 clones) or CP (19 clones) viruses were produced in 293T cells and titrated as previously described (21). A random number generator (GraphPad QuickCalcs) was used to randomly select EC and CP nef proviruses. Viruses were then used to infect approximately 20 % to 30 % of primary CD4 T cells from healthy donors by spin infection at 800 g for 1 h in 96-well plates at 25 °C. The same viral stock was used to perform all the experiments reported in this manuscript.

**Antibodies and sera**

The A32 anti-gp120 cluster A antibody was previously reported (39). The monoclonal antibody anti-CD4 OKT4 (BioLegend) binds to the D3 domain of CD4 and was used to measure cell surface levels of CD4, as described (21). The soluble NKG2D-IgG Fc fusion proteins (R&D systems) bind to NKG2D ligands (ULBPs, MICA and B), while matched IgG Fc fusion molecules (R&D systems) were used as controls as reported (22). The secondary goat anti-mouse and anti-human antibodies coupled to Alexa Fluor 647 (Invitrogen) were used in flow cytometry experiments.

HIV+ sera was obtained from the Montreal Primary HIV Infection Cohort (40, 41) and the Canadian Cohort of HIV Infected Slow Progressors (33, 42, 43). Research adhered to the ethical guidelines of the CRCHUM and informed consent was obtained from each volunteer. Sera was collected during Ficoll isolation of PBMCs and stored at -80 °C. Sera aliquots were heat-inactivated for 30 min at 56 °C and stored at 4 °C until

ready and used in subsequent experiments, as previously reported (17, 21). A random number generator (GraphPad QuickCalcs) was used to randomly select a number of sera from each cohort.

**Flow cytometry: cell-surface staining and ADCC responses**

Cell-surface staining was performed as previously described (17, 21). Briefly, binding of HIV-1 infected cells by plasma antibodies (1:1000 dilution) or relevant mAbs (1 µg/mL) were performed 48 hr after infection. The soluble NKG2D-IgG Fc fusion proteins was used at 50 µg/ml and binding was detected with an Alexa-fluor-647-conjugated goat anti-human IgG antibody. After surface staining, infected cells were permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) to detect infected cells (p24+ cells) with the fluorescent anti-p24 mAb (PE-anti-p24, clone KC57; Beckman Coulter/Immunotech, Hialeah, FL) (1:100 final concentration), as previously described (11, 32). The percentage of infected cells was determined by gating on the living cell population based on the viability AquaVivid dye (Invitrogen). Samples were acquired on an LSRII cytometer (BD Biosciences, Mississauga, ON, Canada) and data analyses were performed using FlowJo vX.0.7 (Tree Star, Ashland, OR, USA).

Measurement of serum or A32-mediated ADCC responses was performed using a previously described FACS-based ADCC assay (11, 17, 21, 32, 44-50). Briefly, infected primary $CD4^+$ T cells were stained with viability (AquaVivid; Invitrogen) and cellular (cell proliferation dye eFluor670; eBiosciences) markers and used as target cells. Autologous

PBMC effectors cells, stained with another cellular marker (cell proliferation dye eFluor450; eBiosciences), 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 plasma or 5µg/ml of the A32 mAb was added in the presence of 5µg/ml of purified anti-human CD314 (NKG2D) (R&D system, 139-NK-50) or matched IgG isotype control 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. Samples were acquired on an LSRII cytometer (BD Biosciences). Infected cells were identified by intracellular p24 staining, as described above. 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 or A32) / (% of $p24^+$ cells in Targets), as reported (44). Of note, in some samples some "negative cytotoxicity" was observed. This is due to the killing of gp120-coated uninfected bystander CD4+ T cells which are present in the culture of HIV-1-infected cells and affects ADCC calculation and was previously reported in detail elsewhere (49).

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

## Results

### CD4 and NKG2D ligands are inefficiently downregulated by Nef proteins from elite controllers

The interaction of Env and CD4 induces conformational changes in Env that result in the exposure of CD4i epitopes, including those recognized by anti-cluster A antibodies, known to mediate potent ADCC responses (17, 21, 32, 46, 51). As previously reported, when primary $CD4^+$ T cells were infected with HIV-$1_{NL4.3}$ infectious viral particles coding for Nef clones from ECs, we observed an impaired CD4 down-regulation when compared to cells infected with viruses coding for Nef clones isolated from CPs (Figure 1 A-B) (9, 11). Of note, no mutations in motif L166-L168 involved in CD4-downregulation (7) were found in Nef clones from ECs (not shown). Suboptimal CD4 downregulation translated into a significant exposure of anti-cluster A epitopes, such as the one recognized by the ADCC-mediating A32 antibody (Figure 1 C-D) and those recognized by antibodies within HIV+ sera (Figure 1E and F), despite similar levels of Env being expressed at the cell surface (Figure 1G and H). Enhanced recognition of infected cells by A32 and HIV+ sera antibodies was previously reported to result in enhanced susceptibility of infected cells to ADCC (11). To mediate ADCC, however, effector cells, such as NK cells, must be activated. It is well known that NK cells effector functions are modulated by a tight balance between signals delivered through inhibitory (KIR, CD94/NKG2A), activating (CD16, NKG2D, DNAM-1) or co-activating (NTB-A, 2B4) receptors that either enhance or suppress NK cell activity, respectively (52). Interestingly, Nef decreases the expression of NKG2D ligands (MICA, ULBP1, and ULBP2) on infected cells (8) thus preventing their interaction with the NK cell activating NKG2D receptor. Since previous work indicated

that Nef proteins from EC are impaired in several functions (9), we evaluated if the enhanced susceptibility of HIV-1-infected cells, infected with viruses coding for Nef proteins from EC, to ADCC could be linked to an incomplete downregulation of NKG2D ligands. To evaluate this, primary $CD4^+$ T cells were isolated from HIV-uninfected individuals and infected with pNL4.3 viruses encoding Nef from EC or CP. Primary $CD4^+$ T cells were also infected with wild-type $Nef_{SF2}$ (wt) or Nef-deleted (N-) viruses as positive and negative controls for NKG2D ligand down-regulation. Cultures containing infected cells were then stained with a recombinant human NKG2D-Fc chimera which recognizes several NKG2D ligands (23-25, 31) or a matched isotype control, and infected cells within these cultures were identified by intracellular p24 staining. As previously reported (22), HIV-1 infection enhanced NKG2D-Fc detection compared to mock-infected cells (Figure 2 A and B). This enhancement, however, was significantly higher when cells were infected with the *nef*-defective virus compared to its wt counterpart. Interestingly, we observed that cells infected with viruses coding for Nef from EC expressed higher levels of NKG2D ligands as demonstrated by a significant increase in NKG2D-Fc binding compared to cells infected with viruses coding for Nef isolated from CP (Figure 2C). Despite the differential expression of NKG2D ligands at the surface of cells expressing Nef from CP vs EC, we failed to identify a particular motif linked to Nef-mediated NKG2D ligand downregulation (not shown).

**Attenuated downregulation of NKG2D ligands by Nef proteins from elite controllers enhances susceptibility of infected cells to ADCC**

Using a previously described FACS-based ADCC assay (21, 44), we next addressed whether the increased expression of NKG2D ligands on cells infected with viruses coding for Nef alleles from EC enhanced susceptibility to ADCC. Primary $CD4^+$ T cells infected with wild-type and Nef-defective viruses were used as positive and negative controls for NKG2D ligand downregulation, respectively. As previously reported (11, 17, 21, 46), wild-type-infected cells were not sensitive to ADCC mediated by A32 (Figure 3A) or antibodies within HIV+ sera (Figure 3B) using autologous PBMCs as effector cells. Supportive of the role for Nef in viral evasion of ADCC (11, 17, 21, 46), cells infected with Nef-deleted virus were more susceptible to ADCC mediated by either A32 (Figure 3A) or HIV+ sera from seven different HIV-1-infected individuals (Figure 3B). Intriguingly, this enhanced susceptibility to ADCC was observed despite similar levels of Env expression, as determined by detection of binding of the conformational independent 2G12 antibody, on cells infected with viruses encoding wild type Nef or having Nef deleted (Figure 1 G-H). Corroborating a role for NKG2D ligands in ADCC responses against HIV-1-infected cells (31), addition of a blocking anti-NKG2D antibody significantly decreased, but did not abrogate, the susceptibility of Nef-deleted infected cells to ADCC mediated by A32 and antibodies within HIV-1+ sera (Figure 3 A and B).

Given the capacity of Nef to decrease surface expression of NKG2D ligands and our data suggesting Nef from EC are inefficient at decreasing NKG2D ligand expression, we next evaluated the role played by NKG2D ligands in ADCC responses against primary

$CD4^+$ T cells infected with viruses coding for Nef proteins from EC or CP. As previously reported (11), we observed enhanced susceptibility of cells infected with viruses coding for Nef from EC versus CP to ADCC mediated by A32 or antibodies within HIV-infected sera (Figure 3 C and D). Suggestive of a role for NKG2D in this enhanced susceptibility to ADCC, addition of an anti-NKG2D blocking antibody, but not of an isotype control, significantly decreased ADCC of cells infected with viruses encoding Nef from EC, of cells infected with viruses encoding Nef from EC, but not CP (Figure 3C and D). In further agreement of a role for NKG2D in anti-HIV-1 ADCC, ADCC mediated by A32 or antibodies within HIV-1-infected sera correlated with not only cell surface levels of CD4 (Figure 4A), but also with NKG2D-Fc binding to infected cells (Figure 4B). Cumulatively, this suggests that in addition to the exposure of ADCC-mediating epitopes induced by the presence of CD4 at the cell surface, the accumulation of NKG2D activating ligands might promote NK cell cytotoxicity.

**Discussion**

HIV-1 has evolved several mechanisms to prevent exposing the CD4-bound conformation of Env at the cell surface. These mechanisms are potentially important, as ADCC-mediating antibodies present in HIV+ sera preferentially target the CD4-bound conformation of Env (11, 17, 46). Accordingly, the HIV-1 Nef accessory protein protects HIV-1-infected cells from ADCC by decreasing cell-surface levels of CD4 (11, 17, 21, 46), which otherwise engages with Env and induces the CD4-bound conformation to expose CD4i epitopes. These epitopes are recognized by well-established CD4i ADCC-mediating Abs, such as A32 (21), or antibodies within sera from HIV-1-infected individuals (11, 17,

21, 46). Here we confirmed that infection of cells with viruses encoding Nef from EC inefficiently decreased cell surface CD4 expression and resulted in the exposure of viral epitopes recognized by ADCC-mediating antibodies, such as the A32 antibody and antibodies within HIV+ sera. We now show that in addition to an impaired capacity to decrease cell surface CD4 expression, Nef from EC also inefficiently decreases the expression of NKG2D ligands on HIV-1-infected cells. This observation is highly important in the context of recently published data demonstrating NKG2D to act as a co-receptor for anti-HIV-1 ADCC (31).

While we and others (8, 53, 54) observed Nef-mediated interference with NKG2D ligand expression another report failed to see any effect of Nef on NKG2D ligands (23). The reasons for this are unclear but could be due to the different viral constructs used in the different studies. Indeed we used fully-replicative viruses while in the Ward study, Env-defective and therefore non-replicative viruses were used.

It has been reported that Nef-mediated NKG2D ligand downregulation occurs through the utilization of domains that differ from those required for CD4 downregulation (8) Accordingly, we found that the capacity of Nef to decrease cell surface CD4 expression did not correlate with their capacity to decrease cell surface NKG2D ligand expression (Figure 4C). This could represent an overall impaired function of Nef clones from EC, as illustrated by their decreased ability to downregulate CD4, HLA-I, CCR5, and their reduced capacity to enhance HIV-1 infectivity and replication (9, 10).

While the presented data are highly relevant for understanding viral mechanisms of susceptibility and evasion of ADCC, they also provide clues to how NK cell-mediated anti-HIV-1 ADCC is regulated on the level of effector/target cell interactions. The mechanism through which NKG2D is contributing to anti-HIV-1 ADCC remains open to debate. Assuming, however, that NKG2D contributes to anti-HIV-1 ADCC through the propagation of activating signals, this observation provides further confirmation of the importance of cumulative activating/inhibitory signals in the functionality of NK cells (52). Indeed, this observation might direct us towards a better understanding of why cells infected with viruses coding for wild type Nef are poorly susceptible to ADCC. While it is evident that cells infected with wild type virus inadequately bind antibodies directed to CD4-induced epitopes, these cells do bind low levels of antibodies within the sera of HIV-1-infected donors. This low level of antibody binding to cells infected with viruses encoding wild type Nef might be sufficient to trigger signals through FcγRIIIa/CD16, but little to no ADCC is observed against these target cells. This raises the possibility that cells infected with viruses encoding wild type Nef are protected from NK cell-mediated ADCC by their incapacity to ligate sufficient activating receptors and capacity to ligate sufficient inhibitory NK cell receptors to generate a cumulative inhibitory NK cell signal. Indeed, previous research has demonstrated that HIV-1-infected targets express sufficient HLA-C and HLA-E to inhibit anti-HIV-1 ADCC (14, 15) despite the ability of primary, but not lab-adapted, Vpu to downregulate HLA-C (55). Given that cumulative signals are important determinants of ADCC susceptibility, future research should assess if target cells infected with viruses coding for wild type Nef become susceptible to ADCC after blockade of inhibitory NK cell receptors. Such studies could generate potential

therapeutic avenues for eliminating HIV-1-infected cells important for curative efforts to eliminate reactivated HIV-1 (56). Finally, whether ligands for others activating or co-activating receptors expressed on HIV-1-infected cells also participate to this cumulative activating signal and modulate ADCC susceptibility remain to be determined.

Altogether, our results suggest a model (Figure 5) where the activities of Nef from CP, including efficient downregulation of CD4 and NKG2D ligands, protect infected cells from ADCC by diminishing the propensity of Env to sample the CD4-bound conformation, and also by decreasing the amount of NK cell activating ligands. On the other hand, infected cells expressing Nef from EC become more susceptible to ADCC responses by exposing Env in its CD4-bound conformation, as well as their inability to remove NK cell activating ligands from the cell surface. The accumulation of NKG2D ligands at the surface of infected cells synergizes with CD16 to elevate ADCC killing. Among different mechanism of control, including the presence of protective HLA alleles (33), we believe that enhanced susceptibility of cells infected with HIV-1 from EC to anti-HIV-1 ADCC might contribute to the durable suppression of viral replication and low plasma viremia in this rare subset of infected individuals.

**Acknowledgments**

The authors thank Florencia Pereyra, Bruce D Walker and all investigators who have contributed to The International HIV Controllers Study (www.hivcontrollers.org), funded by the Mark and Lisa Schwarz Foundation, the Bill and Melinda Gates Foundation and the AIDS Healthcare Foundation; the CRCHUM Flow Cytometry Platform for technical assistance as well as Mario Legault for cohort coordination; and all subjects for their participation and collaboration. This work was supported by a Canada Foundation for Innovation Program Leader grant, CIHR foundation grant #352417, by an amfAR Innovation Grant #109343-59-RGRL with support from FAIR to A.F. and by the FRQS AIDS and Infectious Diseases Network. A.F. and M.A.B. are supported by the Canada Research Chairs program. J.R. is the recipient of a CIHR Fellowship Award #135349. N.A. is the recipient of a King Abdullah scholarship for higher education from the Saudi Government. M.P. is a recipient of a CIHR Fellowship Award. D.E.K. is supported by a Research Scholar Career Award of the Quebec Health Research Fund (FRQS). This study was also supported by NIH AI100645 and AI100663 Center for HIV/AIDS Vaccine Immunology and Immunogen Design (CHAVI-ID). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no conflicts of interest to report.

## Figures and Figure Legends

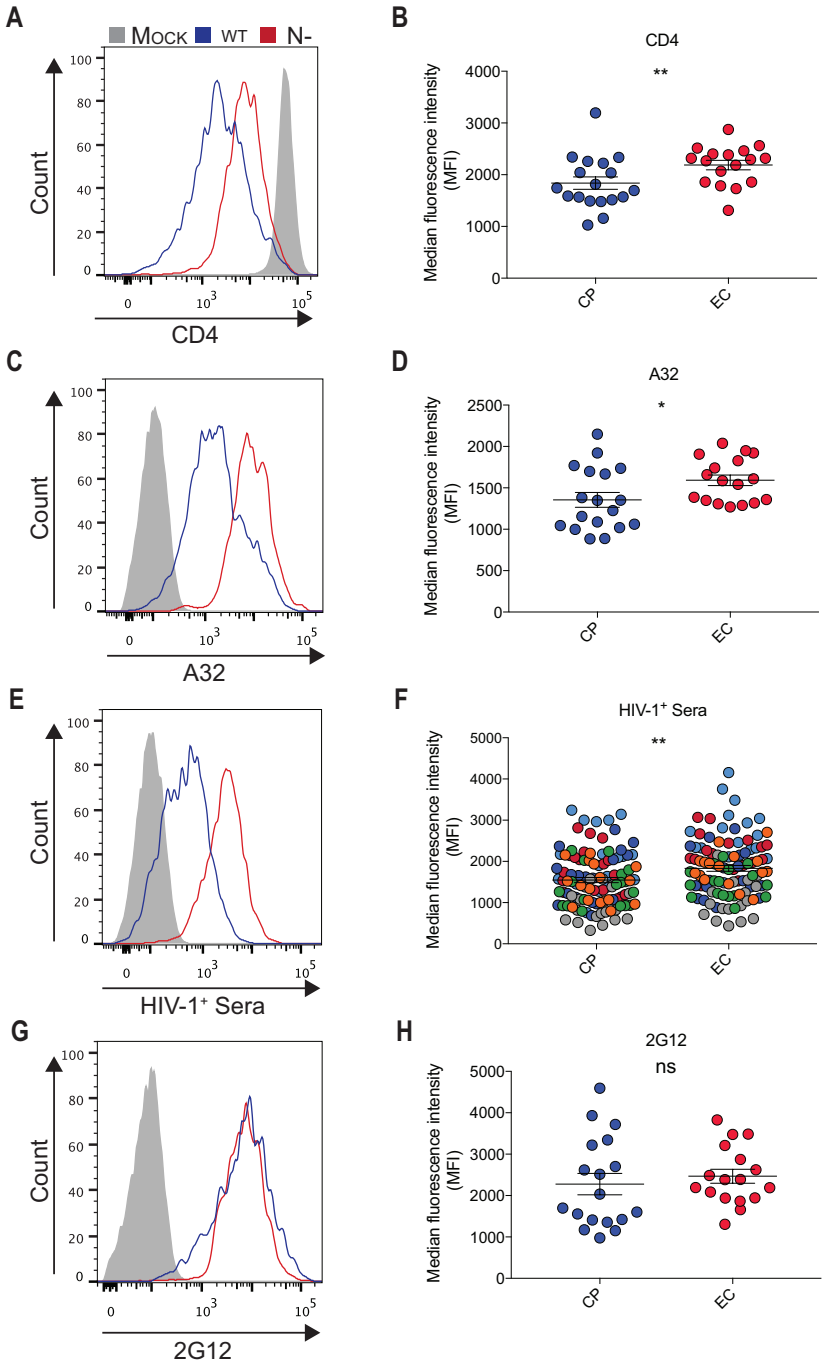


**Figure 3.1 (Figure 1 in Article). Attenuated CD4 downregulation by Nef alleles from elite controllers enhance exposure of ADCC-mediating epitopes.**

Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 18 and 19

randomly-selected clones from ECs or CPs, respectively. Infected primary CD4 T cells were stained at 48 h post-infection with an anti-CD4 (OKT4, **A** and **B**) or A32 (**C** and **D**) antibodies, with HIV+ sera from 7 different donors (shown in different colors) (**E** and **F**) or with the conformational independent 2G12 antibody (**G** and **H**). In the left panels, are histograms depicting representative staining obtained with wt vs Nef- infected. In the right panels, the mean fluorescence intensities (MFI) obtained for multiple staining using cells infected with viruses coding for Nef from ECs (in red) or CPs (blue) are shown. Quantification of data represented as Median Fluorescence Intensity (MFI) binding is shown. Error bars indicate mean ±SEM. Statistical significance was evaluated using unpaired t test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns, not significant).

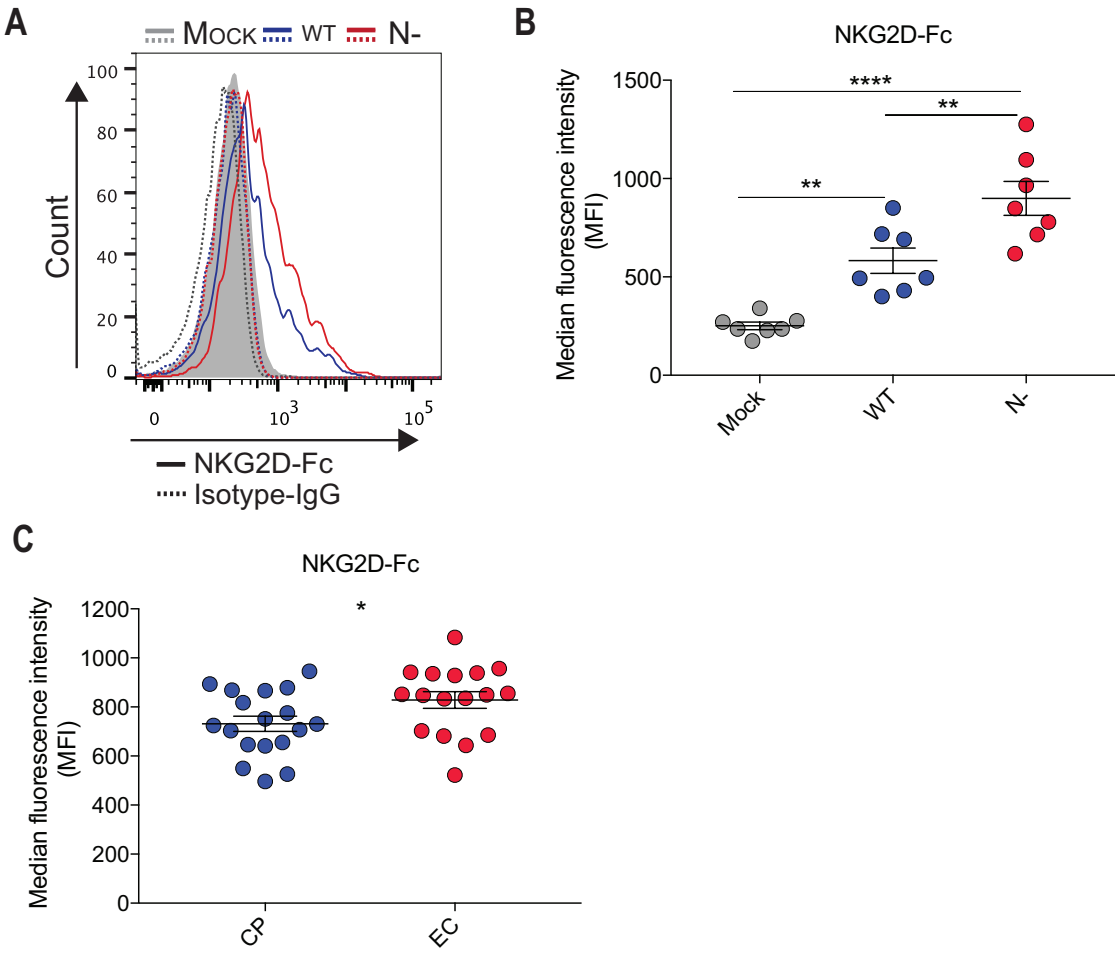


**Figure 3.2 (Figure 2 in Article). Attenuated downregulation of NKG2D ligands by Nef alleles from elite controllers.**

Primary CD4+ T cells from healthy donors were infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from 18 and 19 randomly-selected clones from ECs or CPs, respectively. Infected primary CD4 T cells were stained at 48 h post-infection with 50 µg/mL of a recombinant NKG2D-Fc chimera or matched IgG Fc molecules and then fluorescently labeled with an Alexa-Fluor-647 conjugated anti-human IgG secondary Ab. Shown in (**A** and **B**) are histogram and graph, respectively depicting representative staining of infected (p24+) cells with mock (in grey), wt (in blue), and N- (in red). (**C**) Recognition of infected (p24+) cells by viruses coding for Nef from ECs (in red) or CPs (blue) with NKG2D-Fc. Data shown are the results of four different experiments and error bars depict the SEM. Statistical significance was tested using paired one-way ANOVAs and unpaired t test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

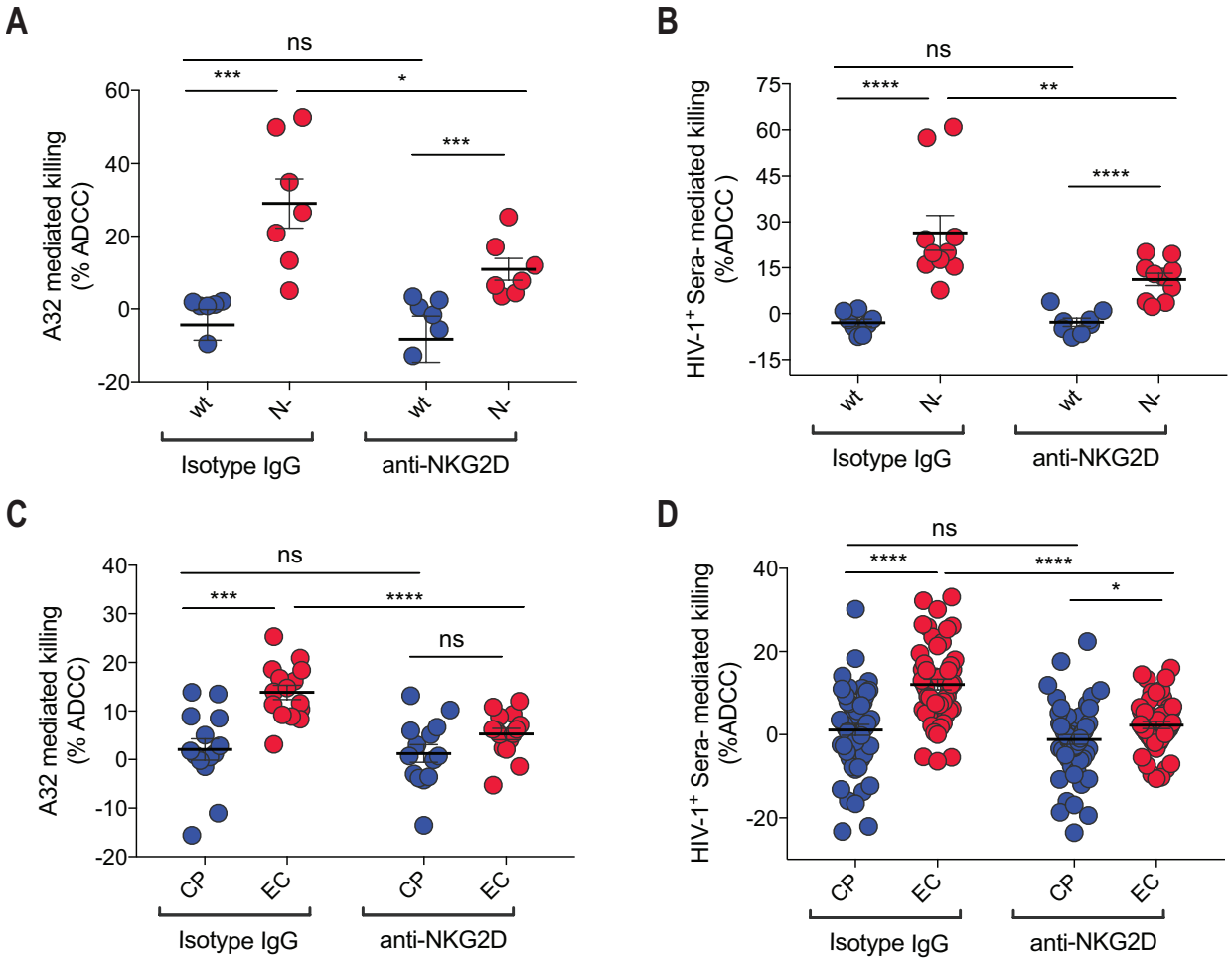


**Figure 3.3 (Figure 3 in Article). Attenuated downregulation of NKG2D ligands by Nef alleles from elite controllers enhances the susceptibility of infected cells to ADCC mediated by A32 and HIV+ sera.**

Primary CD4+ T cells from 5 healthy individuals infected with a panel of pNL4.3-based viruses: wild-type (wt, coding for $Nef_{SF2}$), lacking Nef (N-) or encoding Nef from at least 18 randomly-selected clones from ECs or CPs were used at 48h post-infection as target cells using a previously-reported FACS-based ADCC assay (11, 21) to determine their susceptibility to ADCC, by autologous PBMCs. ADCC mediated by A32 (**A** and **C**) or HIV+ sera from two to seven different donors (**B** and **D**) is shown. Data shown are the results of at least six different experiments, with mean ± SEM. Statistical significance was tested using paired or unpaired t test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns, not significant).

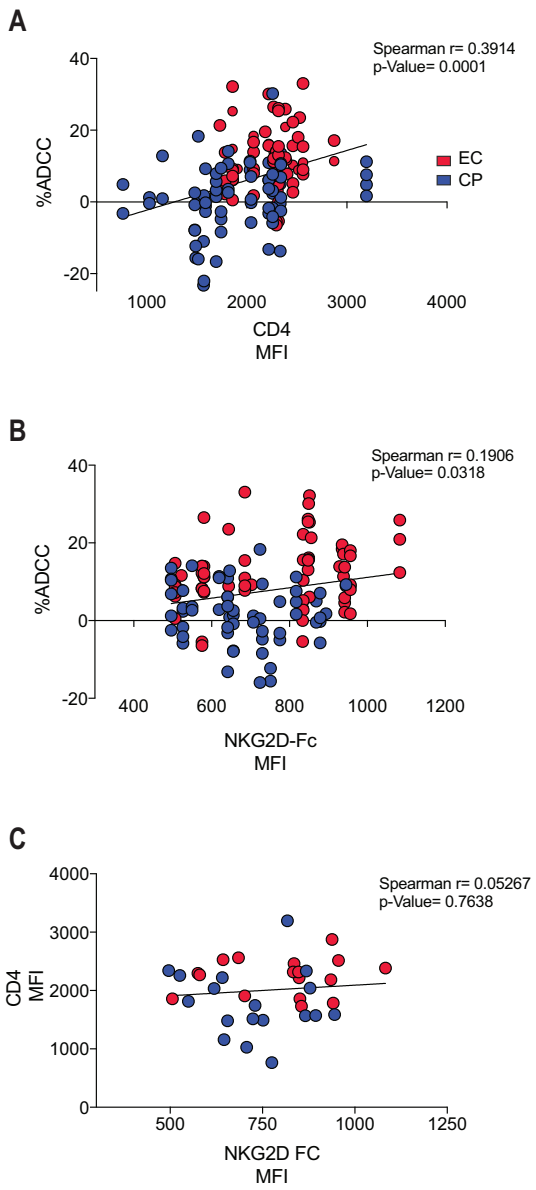


**Figure 3.4 (Figure 4 in Article). Enhanced levels of CD4 and NKG2D ligands at the surface of HIV-1-infected cells correlate with enhanced ADCC.**

The presence of CD4 and NKG2D ligands at the surface of HIV-1 infected cells enhances the sensitivity of infected cells to ADCC mediated by HIV$^+$ sera and A32 monoclonal antibody. CD4 expression (**A**) and the presence of NKG2D ligands (**B**) on the surface of cells infected with viruses encoding Nef from at least 18 randomly-selected clones from ECs (in red) or CPs (in blue) correlated positively with ADCC killing mediated by HIV$^+$ sera and A32 antibody. No statistically significant correlation was observed between cell surface levels of CD4 and NKG2D ligands expression (**C**). Statistical analysis was tested utilizing a Spearman rank correlation.

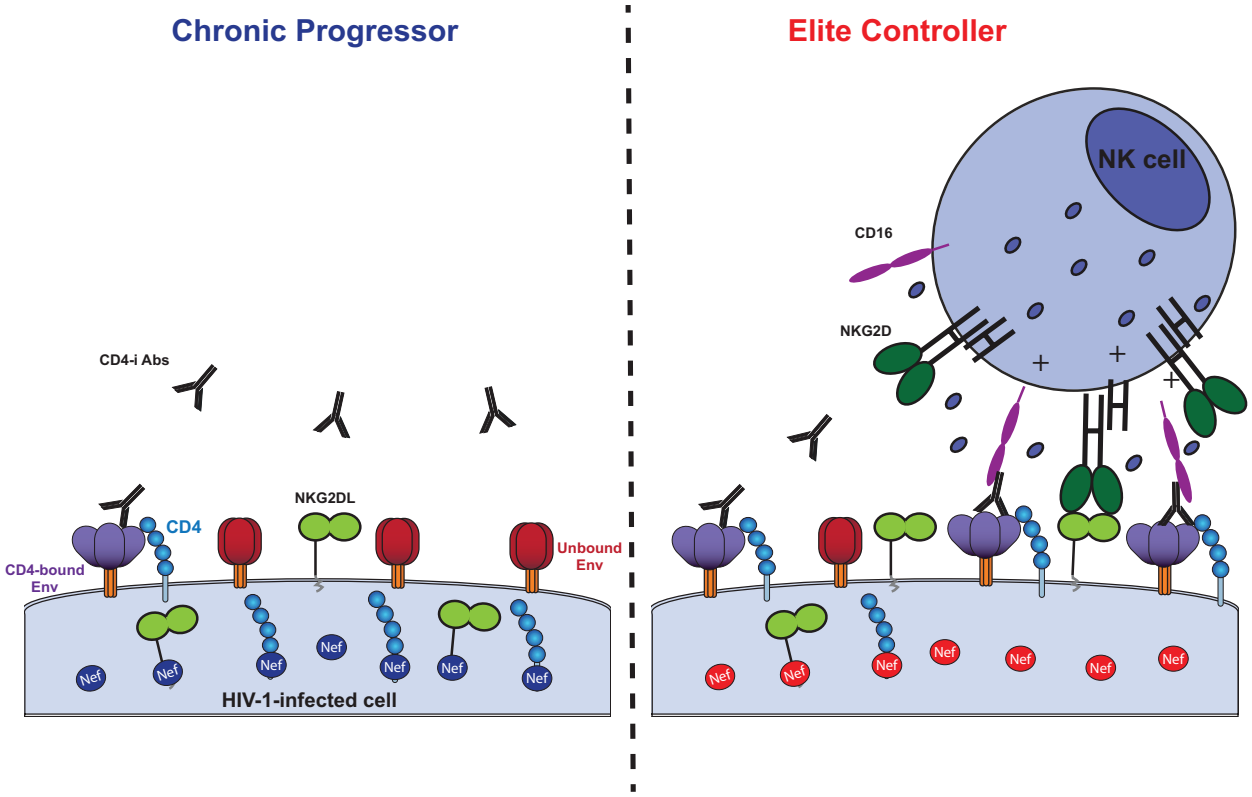


**Figure 3.5 (Figure 5 in Article). Nef-mediated CD4 and NKG2D ligands down-regulation modulate susceptibility of HIV-1 infected cells to ADCC.**

Nef clones isolated from CPs provide protection to infected cells from ADCC by downregulating CD4 and NKG2D ligands from the surface of infected cells (**A**). Impaired abilities of Nef clones from ECs to reduce surface levels of CD4 and NKG2D ligands expose Env CD4i epitopes targeted by ADCC-mediating Abs and contribute to strength the activation of NK cells (**B**).

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## Bridge from Chapter III to IV

The data presented in Chapter II and III reaffirmed the importance of Env-CD4 interaction on the exposure of epitopes targeted by ADCC. Studies on the single-molecule fluorescence resonance energy transfer (smFRET) revealed new insights into the Env-conformational landscape and determined that HIV-1 Env samples three distinct conformational states. Binding to CD4 triggers a cascade of conformational changes in HIV-1 Env that causes its transition from the high energy, metastable, unliganded “state 1” to the low energy, downstream open “states 2 and 3” conformations [560, 561].

The experiments presented in the next chapter were designed to identify the Env-conformational state that is stabilized by CD4 engagement and is susceptible to ADCC. We uncovered a new state of Env conformation using smFRET imaging, cell surface staining and ADCC analysis.

# CHAPTER IV

## HIV-1 ENVELOPE GLYCOPROTEINS SAMPLE A NEW CONFORMATION VULNERABLE TO ANTIBODY ATTACK

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**Running title:** HIV-1 Env State 2A

**Key words:** HIV-1, Envelope glycoproteins, State 2A, smFRET, ADCC, CD4i Abs

Submitted to *Cell Host & Microbe*, September 2018 (*under review*)

**SUMMARY**

The HIV-1 envelope glycoprotein trimer (Env) $(gp120\text{-}gp41)_3$ is the only viral protein on the surface of virions and on the surface of infected cells. As such, Env is the main target for neutralizing antibodies and antibody-dependent cellular cytotoxicity (ADCC). HIV-1 Env is a flexible molecule known to sample three different conformational states. Prior to engaging the primary receptor, CD4, Env preferentially adopts a compact, closed conformation that is largely antibody resistant. In contrast, CD4 binding increases the vulnerability to ADCC. However, which Env conformational state is most vulnerable to ADCC has remained unknown. Here, we evaluated the conformational landscape of Env in complex with different classes of non-neutralizing antibodies that bind CD4-induced epitopes. We uncovered a new Env conformation recognized by antibodies that target the gp120 inner domain and efficiently mediate antibody-dependent cellular cytotoxicity.

**Introduction**

Env resides on the surface of the HIV-1 virion, engages CD4 and the coreceptor, and promotes entry into the target cell. Single-molecule Förster resonance energy transfer (smFRET) imaging experiments directly demonstrated that Env is a highly dynamic molecule which transitions from a “closed” conformation (State 1) to an “open” conformation that is recognised by CD4 (State 3). CD4 engagement induces an asymmetric intermediate (State 2) adopted on the pathway to State 3 (Herschhorn et al., 2016; Ma et al., 2018; Munro et al., 2014). Env represents the only viral antigen exposed on the surface of virions and infected cells, and thus is the primary target for antibodies (Abs). Env-targeting Abs can neutralize the virus, or induce the death of infected cells

through antibody-dependent cellular cytotoxicity (ADCC). Envs from primary HIV-1 isolates are relatively resistant to easily elicited CD4-induced (CD4i) Abs, which are predominant in HIV-1-infected individuals (Decker et al., 2005; Richard J., 2018; von Bredow et al., 2016). This resistance is likely due to the stability of State 1 in primary Envs, which rarely transition to conformations recognized by CD4 (Munro et al., 2014). CD4 engagement drives Env into States 2 and 3 and renders Env susceptible to CD4i Ab attack (Herschhorn et al., 2016; Madani et al., 2017; Prevost et al., 2018a; Prevost et al., 2017; Veillette et al., 2015; Veillette et al., 2014b). To avoid the exposure of the epitopes targeted by CD4i Abs, Env tightly controls the transition from State 1 to States 2 and 3. For example, the V1/V2 and V3 loops of gp120 play a critical role in preventing spontaneous transitions out of State 1 (Kwon et al., 2012; Liu et al., 2008; Mao et al., 2012). Similarly, filling the Phe43 cavity, a highly conserved ~150-$Å^3$ pocket where phenylalanine 43 of CD4 engages gp120, with large hydrophobic residues can predispose Env to spontaneously assume States 2 or 3 (Prevost et al., 2017; Xiang et al., 2002). This might explain why the vast majority of circulating HIV-1 strains have a serine or threonine at position 375 that leaves the Phe43 cavity “empty”.

Despite the mechanisms put in place by primary Envs to limit the spontaneous exposure of CD4i epitopes, the susceptibility of the cell to ADCC mediated by CD4i Abs dramatically increases when CD4 interacts with Env on the surface of infected cells (Veillette et al., 2015; Veillette et al., 2014a; Veillette et al., 2014b). To avoid this, HIV-1 utilizes its accessory proteins Nef and Vpu to decrease cell surface expression of CD4 (Alsahafi et al., 2016; Veillette et al., 2015; Veillette et al., 2014b). The requirement to

evade ADCC provides one explanation for why the majority of circulating HIV-1 strains express functional Nef and Vpu proteins, which limit the exposure of CD4i Env epitopes. Small CD4-mimetic compounds (CD4mc) are able to induce conformational changes in Env similar to those observed upon CD4 binding (Melillo et al., 2016; Schon et al., 2006). This results in the sensitization of HIV-1-infected cells to ADCC mediated by CD4i Abs present in biological fluids, such as sera, breast-milk and cervicovaginal fluids (Richard et al., 2016; Richard et al., 2017; Richard et al., 2015). CD4mc expose CD4i epitopes located in different Env elements such as the co-receptor binding site (CoRBS), the V1/V2 and V3 regions, as well as in gp41 (Gohain et al., 2016; Herschhorn et al., 2016; Madani et al., 2017; Melillo et al., 2016). However, only CD4i anti-cluster A Abs mediate potent ADCC (Ding et al., 2016; Richard et al., 2016). These antibodies recognize the first and second (C1-C2) regions of the inner domain of gp120 in its CD4-bound conformation (Finzi et al., 2010; Guan et al., 2013). There are two prototypes of this class of Abs: A32 and C11, recognizing distinct and non-overlapping epitopes within the Cluster A region (Acharya et al., 2014; Tolbert et al., 2017; Tolbert et al., 2016). This region is buried inside the Env trimer and is not readily accessible when Env is in the State 1 conformation (Acharya et al., 2014; Ding et al., 2016; Tolbert et al., 2016; Veillette et al., 2014b; von Bredow et al., 2016). However, Env binding to CD4 at the cell surface readily exposes this region, leading to ADCC mediated by anti-cluster A Abs (Acharya et al., 2014; Alsahafi et al., 2016; Prevost et al., 2018b; Veillette et al., 2015; Veillette et al., 2014b). Interestingly, while soluble CD4 or CD4mc can readily expose different CD4i epitopes, they fail to expose cluster A epitopes on their own (Richard et al., 2016). A sequential opening of Env appears to be required to expose these epitopes. Indeed, it was shown

that CD4mc initially open the Env trimer allowing for CoRBS Abs engagement, which further opens the trimer, exposing cluster A epitopes (Richard et al., 2016).

Here, using a combination of smFRET imaging, antibody-binding and ADCC assays, we have investigated the conformational dynamics required to expose cluster A epitopes. We report the surprising observation that antibodies against this region stabilize a previously uncharacterized conformation of Env (State 2A). This conformation can be triggered with a CD4mc, a CoRBS Ab, and two different anti-cluster A Abs. Patient HIV+ sera also contains these antibodies, and in the presence of a CD4mc, triggers transition of Env into State 2A. Finally, we show that the conditions that trigger State 2A also expose anti-cluster A epitopes on the surface of primary CD4+ T cells isolated from HIV-1-infected individuals.

## STAR Methods

### Ethics Statement

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 the CRCHUM and was reviewed and approved by the CRCHUM institutional review board (ethics committee, approval number CE 16.164 - CA). Research adhered to the standards indicated by the Declaration of Helsinki. All participants were adult and provided informed written consent prior to enrolment in accordance with Institutional Review Board approval.

**Cell lines and isolation of primary cells**

HEK293T human embryonic kidney cells (obtained from ATCC) were grown as previously described (Richard et al., 2015; Veillette et al., 2014b). Primary human PBMCs, and CD4+ T cells were isolated, activated and cultured as previously described (Richard et al., 2015; Veillette et al., 2014b). Briefly, PBMC were obtained by leukapheresis. CD4+ T lymphocytes were purified from resting PBMCs by a negative selection using immunomagnetic beads per the manufacturer's instructions (StemCell Technologies, Vancouver, BC). CD4+ T lymphocytes were activated with phytohemagglutinin-L (PHA-L) (10 µg/ mL) for 48 hours and then maintained in RPMI 1640 complete medium supplemented with rIL-2 (100 U/mL). 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).

**Plasmids and site-directed mutagenesis**

The sequence of full-length clade B HIV-$1_{JRFL}$ Env (Wu et al., 2006) was codon-optimized (GenScript) and cloned into the expression plasmid pcDNA3.1 or PCGGS. Site-directed mutagenesis was performed using the QuikChange II XL site-directed mutagenesis protocol (Stratagene), and a stop codon was introduced to replace the codon for Gly 711, truncating the cytoplasmic tail ($\Delta$CT) and enhancing cell-surface expression of selected HIV-$1_{JRFL}$ Env. Point mutations were introduced at positions R508S and R511S to allow changes on the gp120-gp41 cleavage site (Bosch V., 1990).

Mutations were either added individually or in combination into pcDNA3.1-JRFL Env or the PCGGS-JRFL Env. The presence of the desired mutations was determined by automated DNA sequencing. The numbering of the HIV-1 Env amino acid residues is based on that of the prototypic HXBc2 strain of HIV-1, where position 1 is the initial methionine (Korber, 1998).

**Viral production, infections, *ex vivo* amplification and detection of infected cells**

HIV-1 viruses were produced and titrated as previously described (Veillette et al., 2015). Viruses were then used to infect activated primary CD4 T cells from healthy HIV-1 negative 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) (Richard et al., 2015).

**Antibodies and sera**

Anti-HIV-1 cluster A monoclonal antibodies A32, and C11 were either conjugated with Alexa-Flour 647 probe (Invitrogen) or not and used for cell-surface staining of HIV-1 Env expressing cells. The following Abs were used alone or in combination with anti-cluster A mAbs for cell-surface staining: A32, C11, 17b, 19b, GE2JG8, N12-i2, (1μg/ml) and the Fab fragments, Fab'2 fragments or full 17b (5μg/ml) mAb.. The monoclonal anti-CD4 OKT4 (1μg/ml) (14-0048-82; eBiosciences) was used to measure cell surface levels of CD4, as previously shown (Richard et al., 2015). Sera from different HIV-infected an

uninfected donors were collected, heat-inactivated and conserved as previously described (Richard et al., 2015; Veillette et al., 2014b). The secondary goat anti-mouse and anti-human antibodies coupled to Alexa Fluor 647 (Invitrogen) were used as secondary Abs.

**smFRET imaging**

HIV-$1_{JR-FL}$ virions with a single peptide-tagged gp120 domain were generated, purified, and fluorescently labeled as described (Munro et al., 2014). Briefly, HEK293T cells were transfected with pNL4-3 ΔEnv ΔRT plasmid, and a 20:1 ratio of wild-type HIV-$1_{JR-FL}$ gp160 plasmid to gp160 with the V1-Q3 and V4-A1 peptide insertions. Virus was collected 24 hours post-transfection and pelleted in PBS over a 5% sucrose cushion at 20,000x g for 2 hours. The virus was resuspended in labeling buffer (50 mM HEPES PH7, 10 mM $CaCl_2$, 10 mM $MgCl_2$), and incubated with 0.5 $\mu$M each of LD550-cadaverine and LD650-coenzye A (Lumidyne Technologies), 0.65 $\mu$M transglutaminase (Sigma), and 5 $\mu$M acyl carrier protein synthase (AcpS). The virus was then incubated with 0.02 mg/ml DSPE-$PEG_{2000}$-biotin for 30 minutes at room temperature. Virus was purified away from unbound fluorophore and lipid by ultracentrifugation over a 5-20% Optiprep (Sigma) gradient in an SW41 rotor for 1 hour at 35,000 rpms. The gradients were fractionated and the fractions containing labeled HIV-$1^{JR-FL}$ virions were identified by p24 Western blot. The purified labeled virions were immobilized on passivated, streptavidin-coated quartz microscope slides. smFRET imaging was performed on a custom-built prism-based TIRF microscope as described (Das et al., 2018). All ligands were introduced to the labeled virus and incubated for 1 hour at room temperature prior to surface immobilization. The

smFRET traces were processed and analyzed using the SPARTAN software package in Matlab (Juette et al., 2016). The number of distinct states present in the smFRET trajectories was quantitatively determined by fitting the traces to 3- and 4-state models using the Baum-Welch algorithm. The maximized likelihood determined during fitting was corrected for the different numbers of model parameters using the Akaike Information Criterium (AIC), where AIC = 2*k*-2*L*, *k* is the number of model parameters (12 for the 3-state model; 20 for the 4-state model), and *L* is the maximum likelihood. Reduction in the AIC indicates improved model fitness.

**Flow cytometry analysis of cell-surface staining**

To assess the Env conformation of wild-type codon-optimized HIV-$1_{JR-FL}$ Env by flow cytometry analysis, 3 X $10^5$ 293T cells were transfected by the calcium phosphate method with the Env-expressing plasmids along with a pIRES-GFP vector, at a ratio of 2 µg of pcDNA3.1 or JRFL ∆CT Env variants to 0.5 µg of green fluorescence protein (GFP). Sixteen hours post-transfection, cells were washed with fresh medium and epitope exposure was evaluated 24 h later. The recombinant Alexa-Fluor-conjugated C34-Ig protein (Si et al., 2004) was used to detect HR1 exposure in the presence or absence of sCD4 (10µg/ml), as previously shown (Pacheco et al., 2017).

Cell-surface staining was performed as previously described (Richard et al., 2015; Veillette et al., 2015). Binding of HIV-1-Env expressing cells by sera (1:1000 dilution), anti-Env mAbs or anti-CD4 mAbs was performed with or without BNM-III-170 (50µM) or its equivalent volume of vehicle (DMSO) at 48h post-infection/transfection. Cells infected

with HIV-1 primary isolates were stained intracellularly for HIV-1 p24, using the Cytofix/Cytoperm Fixation/ Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) and the fluorescent anti-p24 mAb (PE-conjugated anti-p24, clone KC57; Beckman Coulter/Immunotech). The percentage of infected or transfected cells ($p24^+$ cells or $GFP^+$, respectively) was determined by gating the living cell population on the basis of the AquaVivid viability dye staining. Samples were analyzed on an LSRII cytometer (BD Biosciences), and data analysis was performed using FlowJo vX.0.7 (Tree Star, Ashland, OR, USA).

**ADCC FACS-based assay**

Measurement of ADCC using the FACS-based assay was performed at 48h post-infection as previously described (Richard et al., 2014; Richard et al., 2015; Richard J., 2018; Veillette et al., 2014b). Briefly, infected primary CD4+ T cells were stained with viability (AquaVivid; Thermo Fisher Scientific) and cellular (cell proliferation dye eFluor670; eBioscience) markers and used as target cells. Autologous PBMC effectors cells, stained with another cellular marker (cell proliferation dye eFluor450; eBioscience), were added at an effector: target ratio of 10:1 in 96-well V-bottom plates (Corning, Corning, NY). Briefly, infected primary CD4+ T cells were incubated with autologous PBMC (Effector: Target ratio of 10:1) in presence of A32 (0.3125, 0,625, 1,25, 2,5 or 5 µg/ml,) and 17b (5 µg/ml) or 17b Fab fragments alone or in combination, or with HIV+ sera (1:1000), in presence of 50 µM of BNM-III-170 or with equivalent volume of vehicle (DMSO). The plates were subsequently centrifuged for 1 min at 300 g, and incubated at 37°C, 5% $CO_2$ for 5 to 6 h before being fixed in a 2% PBS-formaldehyde solution.

Samples were analyzed on an LSRII cytometer (BD Biosciences). Data analysis was performed using FlowJo vX.0.7 (Tree Star). The percentage of ADCC was calculated with the following formula: (% of p24+ cells in Targets plus Effectors) − (% of p24+ cells in Targets plus Effectors plus sera) / (% of p24+ cells in Targets) by gating on infected lived target cells.

**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

### Anti-cluster A Antibodies Recognize a Conformation that Differs from States 2 and 3

Small molecule CD4mc induce conformational changes in Env similar to those observed upon CD4 binding (Lalonde et al., 2012; Schon et al., 2006). This triggers the exposure of several CD4i epitopes including the CoRBS and the V3 loop (Herschhorn et al., 2016; Lalonde et al., 2012; Madani et al., 2017; Schon et al., 2006), which is consistent with the stabilization of Env in States 2 and 3 (Herschhorn et al., 2016; Munro et al., 2014). Interestingly, despite exposing several CD4i epitopes, CD4mc fail to expose those recognized by the anti-cluster A family of Abs (Richard et al., 2016). To gain a better understanding of this differential exposure of CD4i epitopes, we expressed HIV-$1_{JRFL}$ Env or Env $\Delta$CT in 293T cells and evaluated their conformational state in the presence or absence of CD4mc using a panel of CD4i Abs recognizing the CoRBS (17b, N12i2), the V3 loop (19b, GE2JG8) and cluster A (A32, C11). In agreement with the CD4-induced nature of the epitopes that they recognize, the CoRBS and cluster A Abs bound poorly to Env in the absence of CD4mc (Figures 1A and S1A). V3 loop Abs exhibited a moderate level of Env or Env ΔCT recognition in the absence of CD4mc. However, upon CD4mc addition, anti-CoRBS and V3 Abs efficiently recognized the CD4mc-bound trimer (Figures 1A and S1A). Interestingly, neither of the anti-cluster A Abs tested bound Env, regardless of the presence of CD4mc. This suggests that anti-cluster A Abs recognize an Env conformation that differs from that recognized by anti-CoRBS and anti-V3 Abs. In agreement with the requirement for a sequential opening of the trimer in order to enable anti-cluster A Ab binding to Env (Richard et al., 2016), addition of the 17b anti-CoRBS Ab

together with CD4mc was required to obtain efficient anti-cluster A Ab binding (Figures 1B and S1B). The 17b Fab'2, but not the 17b Fab fragment, was able to recapitulate cluster A epitope exposure in the presence of CD4mc (Figure S2), suggesting that the bivalent recognition of Env by CoRBS Abs is required to facilitate access to anti-cluster A Abs. Altogether, these data suggest that anti-cluster A Abs recognize an Env conformation that differs from the one stabilized by CD4mc and that bivalent binding by anti-CoRBS Abs is required to stabilize this potential new Env conformation.

To determine the conformation recognized by anti-cluster A antibodies, we performed smFRET imaging on HIV-$1_{JRFL}$ Env and Env ΔCT with donor and acceptors fluorophores attached to the V1 and V4 loops of gp120 (Munro et al., 2014). Introduction of the Q3 and A1 peptides into the V1 and V4 loops, respectively, minimally affected Env processing or stability (Figure S3). Labeled virions were immobilized on quartz microscope slides, and imaged using total internal reflection fluorescence (TIRF) microscopy. smFRET trajectories acquired from individual Env or Env ΔCT molecules indicated the same three FRET states previously reported: State 1 (0.18 ± 0.09), State 2 (0.65 ± 0.09), and State 3 (0.38 ± 0.09; Figures 2A and S4A). Incubation with CD4mc led to a slight stabilization of State 3 in Env (Figure S4B), and to the emergence of a small but detectable occupancy in a fourth FRET state (0.84 ± 0.06), which was more pronounced in Env ΔCT (Figure 2B). This state was present at only trace levels in unbound Env and Env ΔCT. Hidden Markov modeling (HMM) of the smFRET trajectories confirmed the existence of four distinct FRET states (Figure S5), as was also seen during visual inspection of the traces (Figure S6). The additional presence of 17b Ab further

stabilized State 3 in Env, and the fourth FRET state in Env ΔCT. The FRET value of the fourth state shifted subtly higher in the presence of 17b (0.90 ± 0.08; Figure 2C and Figure S4C). This increase in the FRET value of the fourth state may indicate a further shift in Env conformation, which is not promoted by the small-molecule CD4mc alone and requires interaction with an Ab. Introduction of the A32 Ab in addition to CD4mc and 17b led to dramatic stabilization of the 0.9-FRET state, which we now denote as State 2A (Figures 2 and S4). Incubation of the labeled virus with C11 Ab, in the presence of CD4mc and 17b, showed a similar, although less dramatic, stabilization of State 2A, and showed greater occupancy of State 3. In all cases, the effect of the anti-cluster A Abs was consistent regardless of the presence of the cytoplasmic tail of Env, although the extent of State 2A stabilization was always greater for Env ΔCT, consistent with greater reactivity of Env ΔCT with anti-cluster A Abs as compared to full-length Env. HMM analysis indicated that transitions of Env ΔCT primarily occurred between States 2 and 3 as shown in a transition density plot (TDP), similarly to previous reports of Env (Ma et al., 2018; Munro et al., 2014). Transitions to State 2A increased upon addition of 17b, and further increased in the presence of the anti-cluster A Abs. While most transitions to State 2A appeared to occur from State 2 (Figures 2 and S6), we cannot exclude the possibility that a transient dwell in State 3 occurred that was not detectable at our current time resolution. Overall, these data support the presence of an additional conformational state of HIV-1 Env that is associated with anti-cluster A Ab binding in the presence of CD4mc and 17b.

**CD4 downregulation prevents the spontaneous sampling of State 2A**

Members of the first generation of CD4mc, NBD-556 and NBD-557, were identified in a screen for inhibitors of the gp120-CD4 interaction (Zhao et al., 2005). These small-molecule ~337-dalton compounds and recently designed derivatives (BNM-III-170) bind in the gp120 Phe 43 cavity (Melillo et al., 2016), block the gp120-CD4 interaction and induce thermodynamic changes in gp120 similar to those observed upon soluble CD4 (sCD4) binding (Schon et al., 2006). However, similarly to soluble CD4 they fail to expose cluster A epitopes (Figure 1). This is unlike membrane-bound CD4 which is able to expose these epitopes without the cooperation of CoRBS Abs (Acharya et al., 2014; Veillette et al., 2014b). This unique ability of membrane-bound CD4 to expose cluster A epitopes explains why cells infected with HIV-1 defective for *nef* are readily recognized by anti-cluster A Abs. Indeed, in cells infected with *nef*-deleted viruses, CD4 accumulates at the cell surface and engages Env, readily exposing cluster A epitopes (Alsahafi et al., 2016; Veillette et al., 2015; Veillette et al., 2014b). However, cells infected with a wild-type virus fail to do so, even in the presence of CD4mc (Richard et al., 2016). In order to get a better understanding of the conformation induced by the interaction with membrane-bound CD4 we cotransfected increasing concentrations of membrane-bound CD4 together with the HIV-$1_{JRFL}$ Env expressor and evaluated the ability of anti-cluster A Abs to bind to Env. As shown in Figure 3A, in absence of CD4 co-expression, Env recognition by anti-cluster A Abs A32 and C11 was inefficient. However, as CD4 expression increased, we observed a concomitant increase in recognition of Env by these Abs. This supports the ability of membrane-bound CD4 to expose cluster A epitopes without the need for CoRBS Abs. We then produced HIV-1 particles using a *nef*-deleted virus in the

absence or presence of membrane-bound CD4 and evaluated Env conformational dynamics by smFRET. We observed no effect of *nef* deletion on the conformational equilibrium of unliganded Env (compare Figures 3B and 2A). In agreement with the ability of membrane-bound CD4 to directly expose cluster A epitopes, incorporation of CD4 into viral particles readily promoted transitions to open Env conformations. In particular, the stabilization of State 2A is consistent with the enhanced exposure of the cluster A epitopes. Altogether, these data suggest that Nef-mediated CD4-downregulation might be a mechanism put in place by the virus to avoid exposure of the antibody-vulnerable State 2A conformation.

**Impact of proteolytic processing on Env conformational dynamics**

Previous reports have shown that proteolytic processing decreases conformational flexibility of Env resulting in reduced exposure of certain CD4i epitopes (Chakrabarti et al., 2011; Haim et al., 2013). However, to our knowledge the impact of Env cleavage on anti-cluster A Abs recognition was not previously reported. We therefore evaluated the impact of Env cleavage on A32 and C11 recognition. As shown in Figure 4A, alteration of the Env cleavage site significantly enhanced recognition by anti-cluster A A32 and C11 Abs. In agreement with a recent report that suggested that the C11 epitope is buried deeper than A32 within the trimer (Tolbert et al., 2017), addition of the CD4mc BNM-III-170 slightly enhanced A32 but not C11 recognition of the cleavage-deficient Env. Accordingly, while the anti-CoRBS 17b antibody and Fab'2 fragment was still required to expose the epitope recognized by C11, a 17b single fragment was sufficient to expose the epitope bound by A32 (Figure 4B). Apparently, differences exist between recognition

of the Env trimer by different anti-cluster A Abs, and the epitope recognized by A32 is exposed before the one seen by C11 in agreement with previous observations (Tolbert et al., 2017). To confirm the impact that Env processing has on Env conformational dynamics we performed smFRET analysis. As shown in Figure 4C, the unliganded Env precursor displayed greater intrinsic sampling of States 2 and 3 as compared to cleaved Env or Env ΔCT, confirming greater conformational flexibility prior to cleavage. Addition of BNM-III-170 in combination with 17b and A32 resulted in significant stabilization of State 2A (Figure 4D). The extent of State 2A stabilization was comparable to that seen for Env ΔCT (Figure 2), but markedly more significant than for Env (Figure S4D).

**Antibodies present in HIV+ sera can stabilize State 2A in presence of CD4mc**

It was previously shown that small molecule CD4mc enhance the susceptibility of HIV-1-infected cells to ADCC mediated by HIV+ sera (Richard et al., 2017; Richard et al., 2015). Importantly, this activity depends on the presence of anti-cluster A Abs (Ding et al., 2016; Tolbert et al., 2016), which stabilize State 2A (Figures 2 and 4). Therefore, in order to evaluate whether HIV+ sera are able to stabilize this new conformation, we first evaluated the ability of HIV+ sera from 9 chronically-infected HIV-1 individuals, and 1 HIV- serum, for their ability to allow A32 recognition of HIV-$1_{JRFL}$ Env. As shown in Figure 5, HIV+ sera was unable to facilitate binding of A32 in the absence of the CD4mc BNM-III-170. However, upon addition of BNM-III-170, all HIV+ sera increased A32 recognition. This suggests that HIV+ sera can stabilize State 2A in the presence of a CD4mc. Accordingly, smFRET analysis of HIV-$1_{JRFL}$ Env in the presence of the same 9 HIV+ sera showed that the small molecule CD4mc was required in order to stabilize State 2A (Figure

6). In contrast, HIV- serum in the presence CD4mc did not promote State 2A beyond what was seen in the presence of CD4mc alone.

Of particular note, we observed that the serum from one HIV-1-infected individual stabilized Env in State 1. Addition of BNM-III-170 did not result in a shift to other Env conformational states (Figure 6B). Since stabilization of State 1 was previously observed with broadly-neutralizing Abs (bNAbs) (Munro et al., 2014; Pancera et al., 2014), we screened the different serum used in this study for their ability to neutralize a panel of 8 viruses representing clades A, B and C. From the 9 sera tested, only Serum #1 presented broadly neutralizing activity and correlated with its ability to stabilize State 1 (Table S1). This data suggests that the presence of neutralizing antibodies in sera, which stabilize State 1, outweighs the effect of anti-cluster A Abs that stabilize State 2A.

**State 2A is susceptible to ADCC responses**

Previous reports indicated that anti-cluster A Abs mediate potent ADCC against cells expressing Env in the CD4-bound conformation (Alsahafi et al., 2016; Ding et al., 2016; Prevost et al., 2017; Richard et al., 2016; Veillette et al., 2015; Veillette et al., 2014b). To evaluate whether the different conditions tested here to stabilize State 2A resulted in enhanced susceptibility to ADCC, we incubated primary CD4+ T cells infected with the transmitted/founder (T/F) CH58 virus in the presence or absence of CD4mc BNM-III-170 with the full 17b antibody or its Fab fragment and evaluated the ability of A32 to recognize infected cells. Incubation with the 17b antibody, but not its Fab fragment, was sufficient to allow recognition of HIV-1-infected cells by A32 (Figure 7A) and this

translated into potent ADCC activity (Figure 7B). We also tested the ability of the sera presenting different degrees of State 2A stabilization activity (Figure 6) for their ability to mediate ADCC in the absence or presence of the CD4mc. All HIV+ sera recognized HIV-1-infected cells more efficiently upon CD4mc BNM-III-170 addition (Supplemental Figure 7). Enhanced recognition translated into a significant improvement of their capacity to eliminate HIV-1-infected cells (Figure 7D).

In order to evaluate whether it was possible to expose cluster-A epitopes at the surface of infected cells from clinically relevant samples, we purified primary CD4+ T cells from seven 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 (Figure 7E) and the ability of A32 to recognize infected (p24+ cells) was evaluated. We observed that infected cells isolated from five out of seven individuals were efficiently recognized by A32 upon CD4mc BNM-III-170 and 17b addition (Figure 7F).

## Discussion

HIV-1 infection elicits a robust humoral immune response against the viral envelope glycoprotein. But the vast majority of the elicited antibodies are non-neutralizing (nnAbs). These Abs target variable regions, or epitopes that are not exposed in the untriggered trimer. HIV-1 and its envelope glycoproteins are formidable machines of immune evasion. Env is extensively glycosylated, exposes sequence-variable regions and is very efficient at conformationally masking vulnerable epitopes (Kwong et al., 2002; Wyatt et al., 1998; Wyatt and Sodroski, 1998). Moreover, the conformational dynamics of Env also constitute a major barrier for viral neutralization. The functional Env trimer mainly exists in an untriggered "closed" conformation (State 1) that cannot be seen by nnAbs. However, Env has intrinsic access to the "open" CD4-bound conformation (State 3) through one necessary conformational intermediate (State 2) in which only one protomer is competent for CD4 binding (Ma et al., 2018; Munro et al., 2014). Primary Tier 2 viruses are more "closed", with elevated State 1 occupancy, explaining why they are resistant to neutralization by nnAbs (Munro et al., 2014). CD4 interaction modifies the conformational landscape towards State 3 by lowering the energy barrier to the "open" states (Herschhorn et al., 2016; Ma et al., 2017; Munro et al., 2014). By exploring the Env conformational landscape in association with the epitopes recognized by different classes of CD4i Abs, we found that Env is able to sample a fourth conformation (State 2A) which is vulnerable to antibody attack. Env occupancy of States 2 and 3 can be enhanced with CD4mc (Herschhorn et al., 2016). When Env samples these states it can be recognized by CD4i Abs targeting the CoRBS and the V3 loop, but the cluster A epitopes remain obscured. Stabilization of State 2A is required to expose the cluster A epitopes. In

addition to CD4mc, engagement with the CoRBS Abs in a bivalent mode of recognition is required to stabilize this new Env state, which is recognized by anti-cluster A Abs such as A32 and C11. These data are reminiscent of a recent report that suggested that upon CD4 interaction, binding to two co-receptor molecules is required in order to allow Env-mediated fusion (Ozorowski et al., 2017). There may be similar consequences with respect to Env conformation of binding CoRBS Abs and the co-receptor.

The low State 2A occupancy is consistent with the occluded position of cluster A epitopes, which are buried deep within the trimer axis (Acharya et al., 2014; Gohain et al., 2015; Tolbert et al., 2017). Interestingly, anti-cluster A Abs are those that present the most potent ADCC activity among easily-elicited CD4i Abs (Ding et al., 2016; Richard et al., 2016). It is therefore tempting to speculate that barriers limiting transitions to State 2A represent a mechanism put in place by HIV-1 to avoid exposure of vulnerable epitopes. Interestingly, while CD4mc require the addition of CoRBS Abs in order to stabilize State 2A, we found that binding to membrane-bound CD4 is sufficient to trigger this conformation. These data are in agreement with previous reports that showed the critical role played by Nef-mediated CD4-downregulation in avoiding ADCC responses mediated by HIV+ sera and anti-cluster A Abs (Alsahafi et al., 2016; Prevost et al., 2018b; Veillette et al., 2015; Veillette et al., 2014b). Our data also confirm the formidable flexibility of the uncleaved HIV-1 Env which is able to spontaneously sample all four States. Env cleavage dramatically shifts this conformational landscape favoring the untriggered, most neutralization-resistant State 1 conformation. Therefore, our data suggest that exposure of only efficiently cleaved Envs at the cell surface appears to be

important to avoid premature elimination of infected cells via ADCC. Altogether, our data identify a new conformational state of HIV-1 Env, which is vulnerable to attack by easy-to-elicit CD4i Abs. Strategies aimed at stabilizing this conformation might facilitate the design of new approaches to fight HIV-1 infection.

**Acknowledgments**

The authors thank Maolin Lu and Xiaochu Ma for helpful discussions and critical reading of the manuscript, Dominique Gauchat from the CRCHUM Flow Cytometry Platform for technical assistance, the FRQS AIDS and Infectious Diseases network and Mario Legault for cohort coordination and clinical samples. This work was supported by a CIHR foundation grant #352417 to A.F. Support for this work was also provided by NIH R01 to A.F. and M.P. (AI129769), to A.F. and X.W. (AI122953), to J.S. (AI124982) and NIAID R01 to M.P. (AI116274). This study was also supported by NIH AI100663 Center for HIV/AIDS Vaccine Immunology and Immunogen Design (CHAVI-ID) (D.E.K; PI: Dennis Burton) and by R01-GM56550 to A.S. Work by J.M. was supported by the National Institutes of Health (1K22AI116262) and the Gilead Sciences Research Scholars Program in HIV. A.F. is the recipient of a Canada Research Chair on Retroviral Entry # RCHS0235. N.A. is the recipient of a King Abdullah scholarship for higher education from the Saudi Government. J.R. is the recipient of a Mathilde Krim Fellowships in Basic Biomedical Research from amfAR. S.D. is the recipient of a FRSQ postdoctoral fellowship award. D.E.K is supported by a FRQS Senior Research Scholar Award. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Author Contributions**

AF and JM conceived the study, AF, NA, NB and JM designed experimental approaches and AF and JM wrote the paper. AF, NA, NB, JR, SD, SPA, HL, HM, NB, SK, XW, WM and JM performed, analyzed and interpreted the experiments and WDT, VV, ASIII, MP and DEK supplied novel reagents. All authors have read, edited and approved the final manuscript.

## Figure and Figure legends

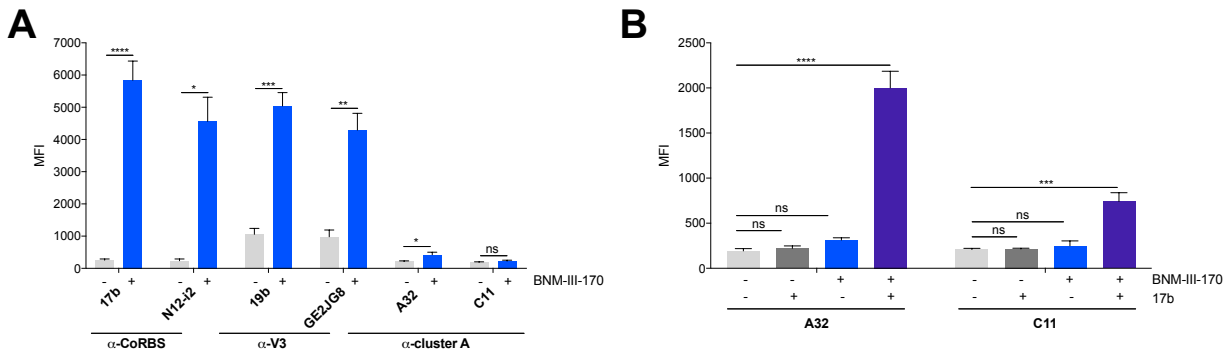


**Figure 4.1 (Figure 1 in Article). CD4mc fail to enhance recognition of HIV-1 Env by anti-Cluster A Abs in the absence of CoRBS Abs.**

293T cells were transfected with a plasmid expressing the HIV-$1_{JRFL}$ ΔCT Env. **(A)** Env recognition by CD4i Abs against the CoRBS, the V3 and cluster A was evaluated in the presence or absence of the CD4mc BNM-III-170 using an Alexa-Fluor 647 (AF647)-conjugated secondary anti-human IgG Abs. **(B)** The exposure of cluster A epitopes was assessed with the Alexa-Fluor 647 (AF647)-conjugated A32 or C11 Abs in the presence of the CoRBS 17b antibody with or without BNM-III-170, as described in Material and Methods. Data are presented as means and SEM of the mean fluorescence intensity (MFI). Data are the averages from at least three independent experiments. Statistical significance was tested using **(A)** multiple-t tests, correcting for multiple comparisons using the Holm-Sidak method and **(B)** one-way ANOVAs (* P<0,05,** *P* <0.01, ****P* <0.001, ns, not significant).

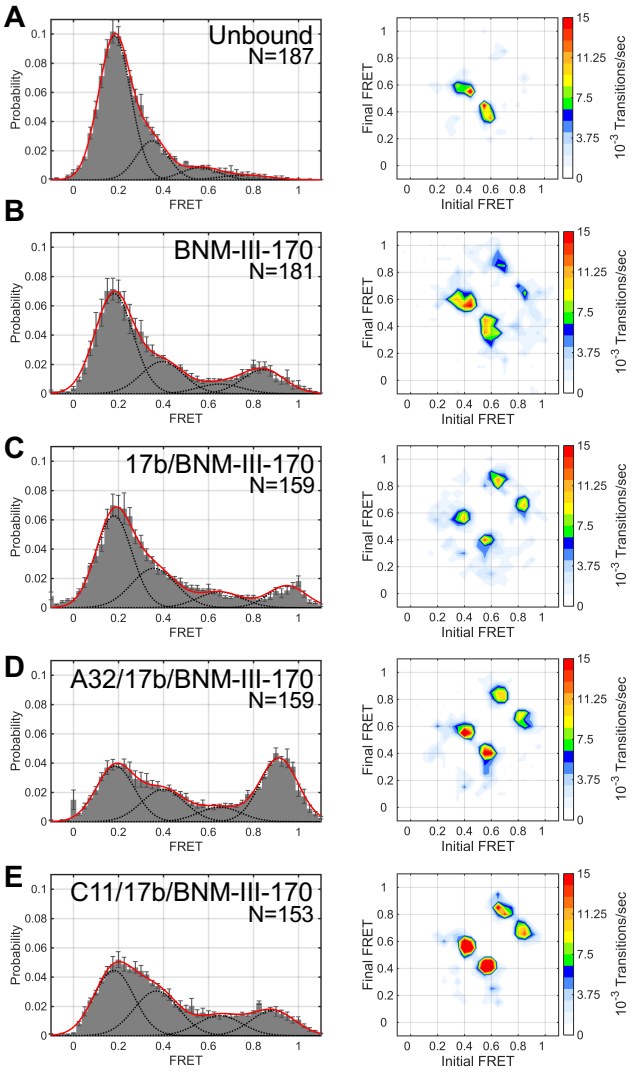


**Figure 4.2 (Figure 2 in Article). CD4mc in combination with anti-cluster A and CoRBS Abs stabilize a new Env conformation.**

Histograms of FRET values (left) and TDPs resulting from HMM analysis (right) observed for HIV-$1_{JR-FL}$ Env ΔCT **(A)** in the absence of bound ligands, **(B)** in the presence of the CD4mc BNM-III-170 (100 $\mu$M), **(C)** in the presence of both BNM-III-170 (100 $\mu$M) and the CD4i Ab 17b (5 $\mu$g/ml), **(D)** in the presence of BNM-III-170 (100 $\mu$M), 17b (5 $\mu$g/ml), and the anti-cluster A Ab A32 (5 $\mu$g/ml) or **(E)** C11. In all cases the histograms were formed by compiling the indicated number (N) of smFRET traces. Overlaid on the FRET histograms is the sum of four Gaussian distributions with means and standard deviations determined by HMM analysis (0.18 ± 0.09, 0.38 ± 0.09, 0.65 ± 0.09, 0.90 ± 0.08). Error bars reflect the standard deviation in the number of data points per histogram bin determined from three independent groups of smFRET traces.

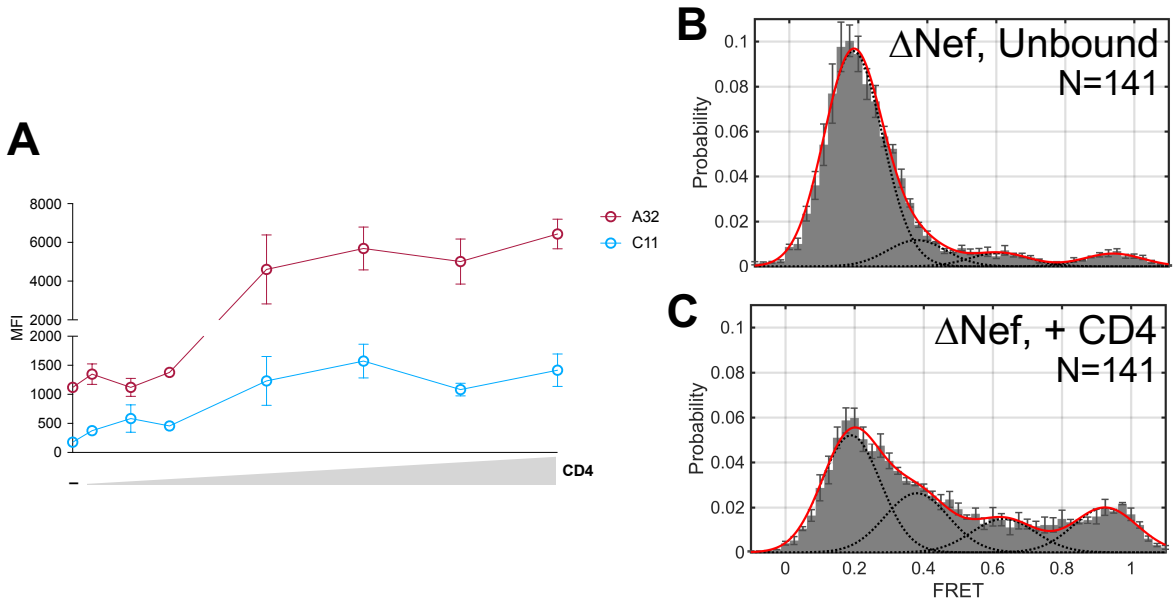


**Figure 4.3 (Figure 3 in Article). Membrane-bound CD4 exposes cluster-A epitopes and stabilizes Env State 2A.**

293T cells were transfected with a plasmid expressing the cytoplasmic-tail-deleted HIV-$1_{JRFL}$ Env, together with increasing concentrations of a human CD4 expressor. **(A)** Cell-surface Env recognition by anti-cluster A Abs A32 and C11. Data are presented as means and SEM of the mean fluorescence intensity (MFI) derived from at least three independent experiments. **(B)** FRET histograms observed for HIV-$1_{JR-FL}$ Env in the absence of Nef, and in the absence of any bound ligand, and **(C)** in the presence of membrane-bound CD4. Histograms are displayed as in Figure 2.

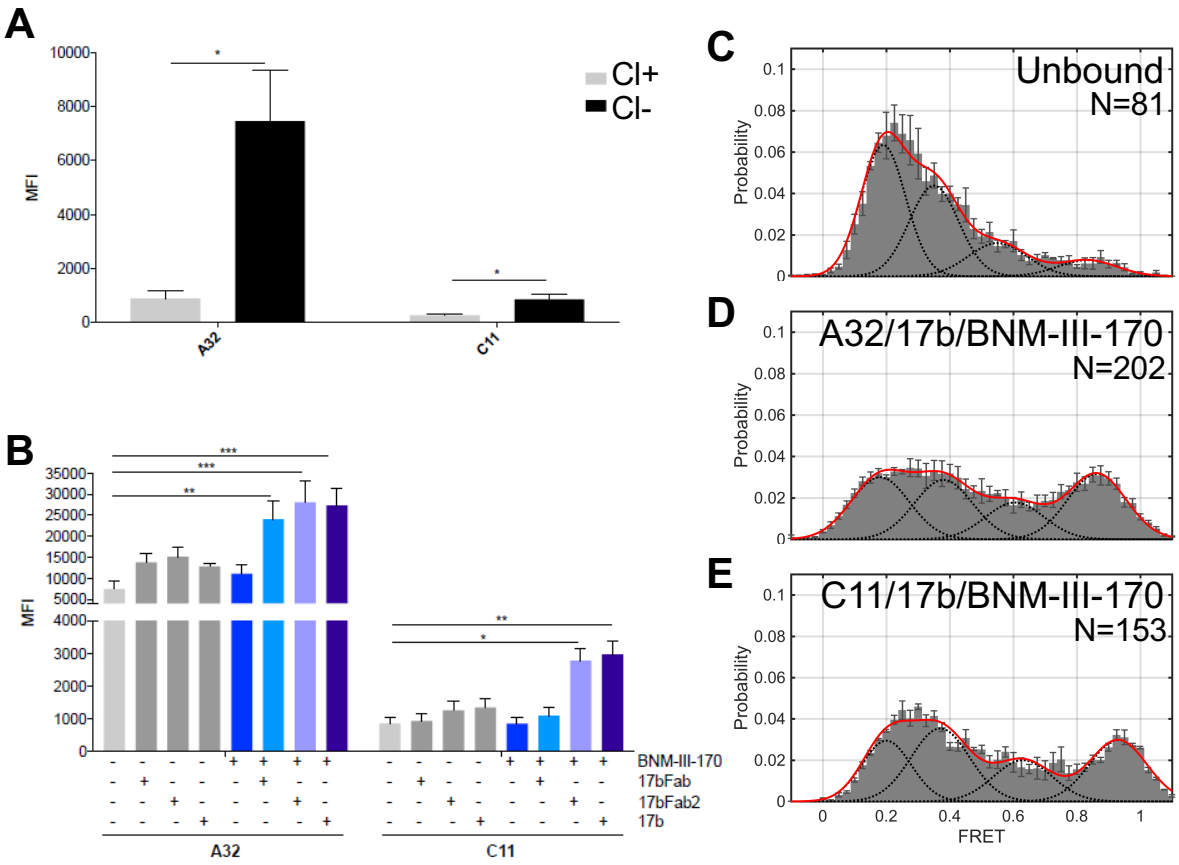


**Figure 4.4 (Figure 4 in Article). Env cleavage decreases the spontaneous sampling of State 2A.**

**(A)** The impact of Env cleavage on cluster A epitope exposure was evaluated by cell surface staining of 293T cells transfected with HIV-$1_{JRFL}$ ΔCT Env (Cl+) or its cleavage defective (Cl-) counterpart using A32 and C11 anti-cluster A Abs. **(B)** The impact of CD4mc BNM-III-170 and CoRBS 17b full Ab, Fab or Fab'2 fragment on cluster A epitope exposure on the uncleaved Env was evaluated with Alexa-Fluor647-conjugated A32 and C11. Data are presented as means and SEM of the mean fluorescence intensity (MFI). Data are the averages from at least three independent experiments. Statistical significance was evaluated using multiple-comparison-one-way ANOVAs or Kruskal-Wallis test and multiple-comparison using Holm-Sidak method, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$; ns, not significant. **(C)** FRET histograms observed for the uncleaved HIV-$1_{JR-FL}$ Env precursor in the absence of any bound ligand; **(D)** in the presence of BNM-III-170 (100 $\mu$M), 17b (5 $\mu$g/ml), and the anti-cluster A Ab A32 (5 $\mu$g/ml); and, **(E)** in the presence of BNM-III-170 (100 $\mu$M), 17b (5 $\mu$g/ml), and C11 (5 $\mu$g/ml). Histograms are displayed as in Figure 2.

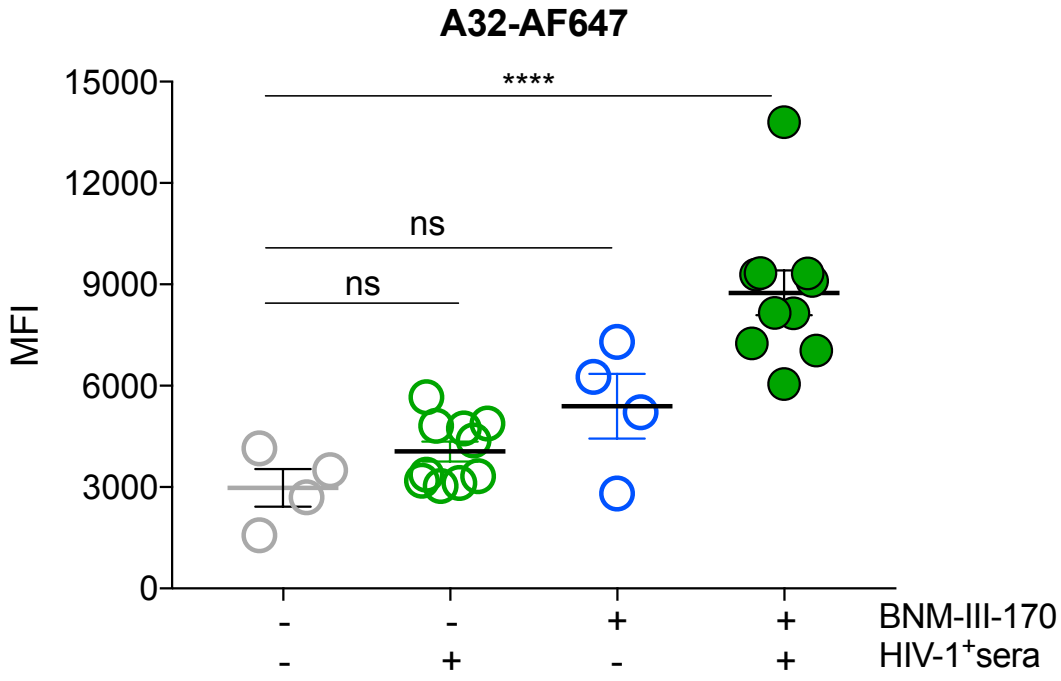


**Figure 4.5 (Figure 5 in Article). HIV+ sera facilitates the exposure of anti-cluster A epitopes in the presence of a CD4mc.**

HIV-$1_{JRFL}$ ΔCT Env recognition by Alexa-Fluo r647 conjugated A32 (A32-AF647) in the presence of a 1/1000 dilution of sera from nine chronically HIV-1-infected individuals in the presence of the CD4mc BNM-III-170 (50μM) or equivalent volume of vehicle (DMSO). Data are presented as means and SEM of the mean fluorescence intensity (MFI). Statistical significance was evaluated using Kruskal-Wallis test, ****$P$ <0.0001; ns, not significant.

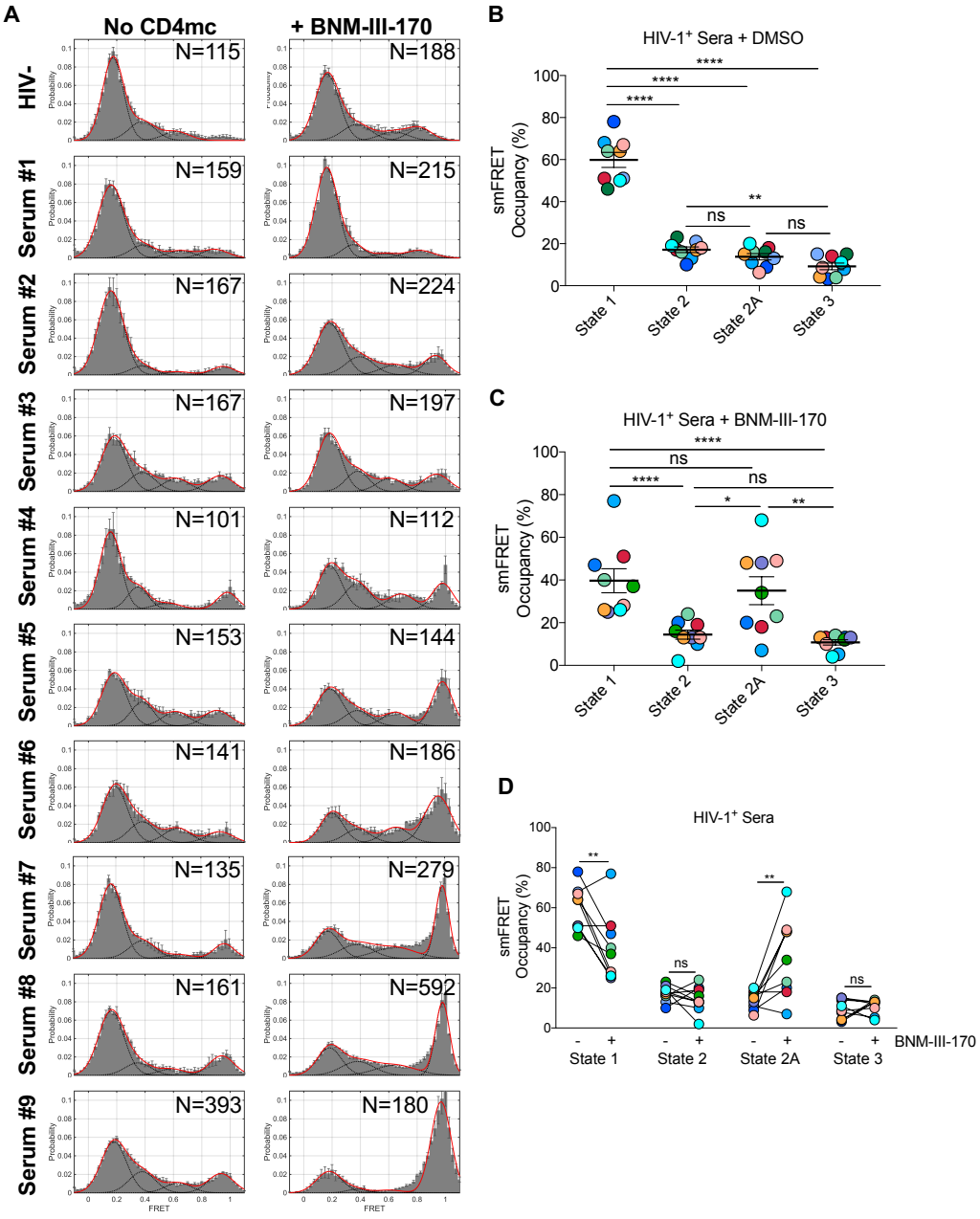


**Figure 4.6 (Figure 6 in Article). HIV+ sera stabilizes State 2A in the presence of CD4mc.**

FRET histograms obtained for HIV-$1_{JR-FL}$ Env ΔCT in the absence or presence of CD4mc BNM-III-170 (100 $\mu$M), as indicated, and in the presence of sera from 9 chronically HIV-1-infected individuals (**A**). The ability of each individual serum to neutralize a panel of 8 primary viruses is indicated in Table S1. Histograms are displayed as in Figure 2. Graphs of the percentage of smFRET occupancy shown in (A) for HIV+ sera in presence of DMSO (**B**) or CD4mc BNM-III-170 (**C**) are shown. The impact of CD4mc BNM-III-170

addition on smFRET occupancy is shown in (**D**). Data are presented as mean and SEM of sera from 9 HIV-1-infected individuals presented in different colors. Statistical significance was evaluated using an unpaired t-test or Mann-Whiney test (** $P$ <0.01, **** $P$ < 0.0001, ns, not significant).

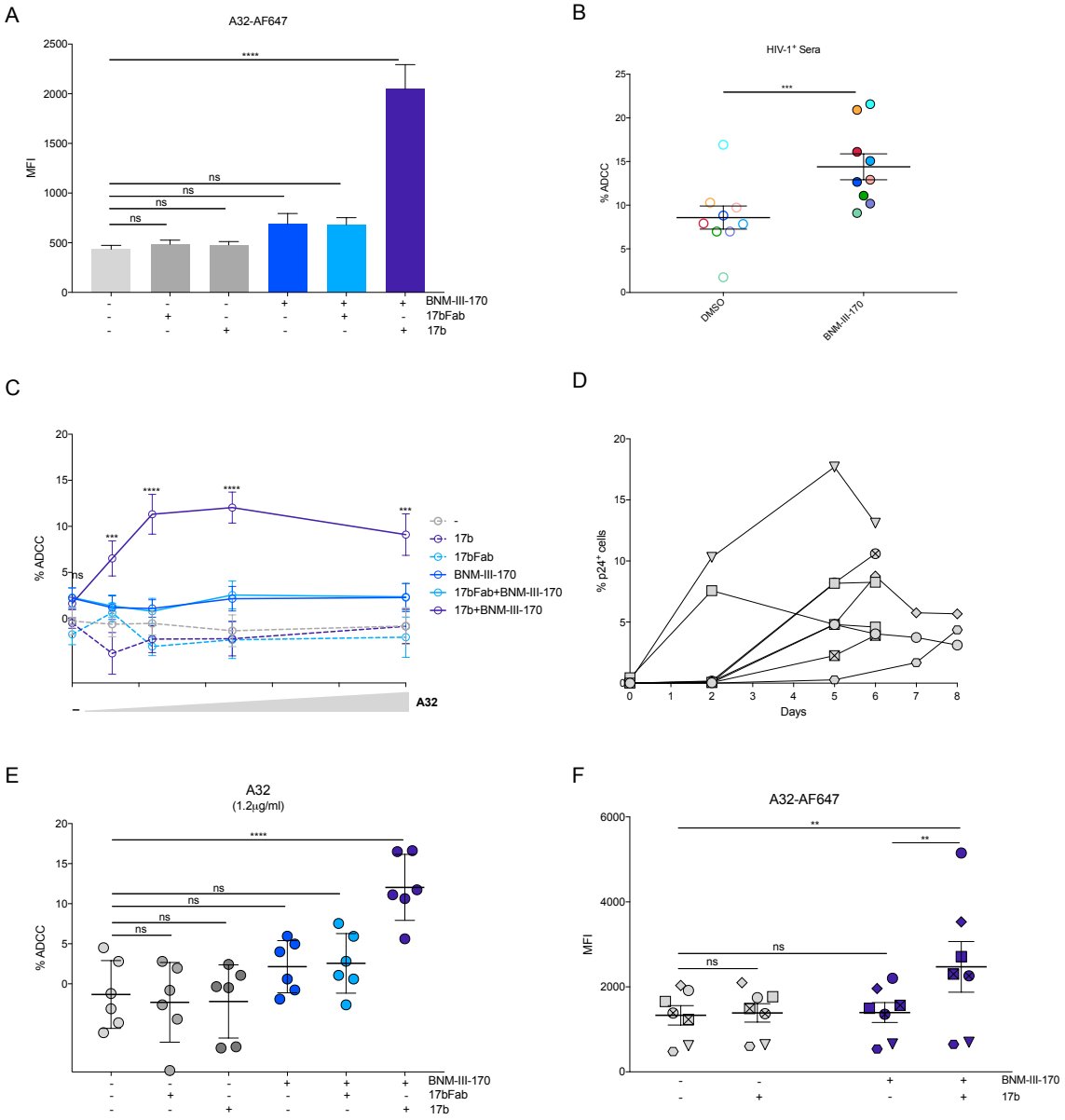


**Figure 4.7 (Figure 7 in Article). CD4mc-mediated exposure of cluster A epitopes sensitizes HIV-1 infected primary CD4+ T cells to ADCC.** Primary CD4+ T cells infected with the transmitter/founder CH58 virus were used to evaluate **(A)** CD4mc BNM-III-170-mediated exposure of cluster A epitopes, alone or in combination with 17b or its

Fab fragment, using Alexa-Fluor 647 conjugated A32 Ab (A32-AF647). Only combination of BNM-III-170 with the full 17b Ab enabled A32-AF647 recognition and correlated with **(B)** the susceptibility of infected cells to ADCC. ADCC was performed using serial dilutions of A32 (0.3125, 0.625, 1.25, 2.5 $\mu$g/ml) alone or in combination with 17b or its Fab fragment (5 $\mu$g/ml), in the presence of the CD4mc BNM-III-170 or equivalent volume of DMSO. Shown is the average of 6 independent experiments. Shown in **(C)** is a snapshot of the results obtained at 1.25 $\mu$g/ml of A32. **(D)** CD4mc BNM-III-170 (50 µM) also enhanced the susceptibility of HIV-1-infected cells to ADCC mediated by sera from nine chronically HIV-1-infected individuals. **(E)** Cluster A epitopes can get exposed *ex vivo*: primary CD4+ T cells isolated from seven viremic antiretroviral therapy (ART)-naive infected individuals were reactivated with PHA\IL-2 and virus replication monitored by intracellular p24 staining. **(F)** Six days post-reactivation cluster A epitope exposure was evaluated as described in (A). Error bars indicate mean ± SEM and statistical significance was tested using **(A,B,C)** a one-way ANOVAs, **(D)** a paired student t test or **(F)** a paired one-way ANOVA (** $P$ <0.01, ***$P$ <0.001, **** $P$ < 0.0001, ns, not significant).

**Supplemental Information**

Supplemental information includes 7 figures and 2 tables

**Supplemental Material and Methods:**

**Immunoprecipitation of envelope glycoproteins**

For pulse-labeling experiments, $3X10^5$ 293T cells were co-transfected by the calcium phosphate method with the pcDNA3.1-JRFL vector expressing wild-type or mutant envelope glycoproteins. One day after transfection, cells were metabolically labeled for 5 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 and then chased over-night with complete DMEM medium containing excess of methionine and cysteine. Cells were subsequently lysed in RIPA buffer (140 mM NaCl, 8 mM $Na_2HPO_4$, 2 mM $NaH_2PO_4$, 1% NP40, 0.05% sodium dodecyl sulfate (SDS)). Precipitation of radiolabeled HIV-$1_{JRFL}$ envelope glycoproteins from cell lysates or medium was performed with a mixture of sera from HIV-1-infected individuals for 1 hour at 4°C in the presence of 50 µl of 10% Protein A-Sepharose (American BioSciences).

Processing and association indices were determined by precipitation of radiolabeled cell lysates and supernatants with mixtures of sera from HIV-1-infected individuals. The association index is a measure of the ability of the mutant gp120 molecule to remain associated with the Env trimer complex on the expressing cell, relative to that of the wild-type Env trimers. The association index is calculated as follows: association

index = ([mutant gp120]$_{cell}$ × [wild-type gp120]$_{supernatant}$)/ ([mutant gp120]$_{supernatant}$ × [wild-type gp120]$_{cell}$). The processing index is a measure of the conversion of the mutant gp160 Env precursor to mature gp120, relative to that of the wild-type Env trimers. The processing index was calculated by the formula: processing index = ([total gp120]$_{mutant}$ × [gp160]$_{wild\text{-}type}$)/ ([gp160]$_{mutant}$ × [total gp120]$_{wild\text{-}type}$).

**Viral Neutralization Assay**

The HIV-1 Env pseudovirus was generated by transfecting 293T cells with *rev/env* expression plasmid and an *env*-deficient HIV-1 backbone (pSG3Δenv) using Fugene 6 transfection reagents (Invitrogen). Each batch of Env pseudovirus was titrated using a single round infection of TZM-bl cells, and the viral neutralization was measured as described (Seaman et al., 2010; Wu et al., 2009) using the same assay system. Briefly, 40 µl of virus was incubated at 37°C for 30 min with 10 µl of serially diluted serum in duplicate wells before the addition of TZM-bl cells. To keep assay conditions constant, sham medium was used in place of serum in control wells. Infection levels were determined after two days with Bright-Glo luciferase assay system (Promega, Madison, WI, USA). Neutralization curves were fit by nonlinear regression using a 5-parameter hill slope equation built in Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The serum reciprocal dilutions required to inhibit infection by 50% were reported as ID50 titers.

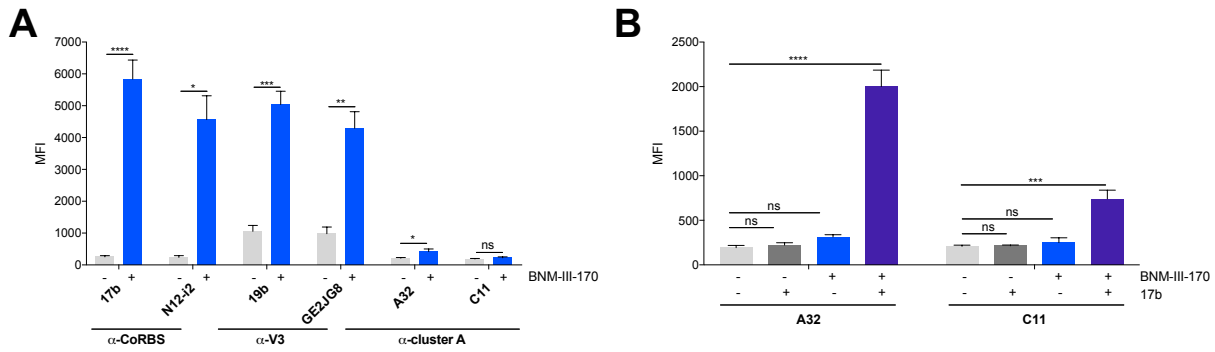


**Figure 4.8 (Figure S1 in Article). CD4mc fail to enhance recognition of full-length HIV-1 Env by anti-Cluster A Abs in the absence of CoRBS Abs (Related to Figure 1).** 293T cells were transfected with a plasmid expressing the HIV-$1_{JRFL}$ gp160 Env. **(A)** Env recognition by CD4i Abs against the CoRBS, the V3 and cluster A was evaluated in the presence or absence of the CD4mc BNM-III-170. **(B)** The exposure of cluster A epitopes was assessed with the Alexa-Fluor 647 (AF647)-conjugated A32 or C11 Abs in the presence of the CoRBS 17b antibody with or without BNM-III-170, as described in Material and Methods. Data are presented as means and SEM of the mean fluorescence intensity (MFI). Data are the averages from at least three independent experiments. Statistical significance was tested using **(A)** multiple-t tests; correcting for multiple comparisons using the Holm-Sidak method and **(B)** one-way ANOVAs or Kruskal-Wallis test (*P<0,05, ** *P* <0.01, ***$P$ <0.001, **** $P$ < 0.0001, ns, not significant).

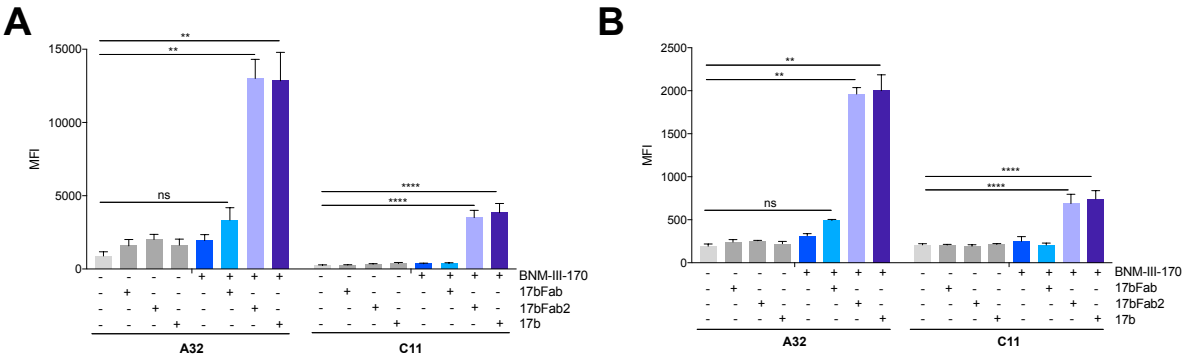


**Figure 4.9 (Figure S2 in Article). CoRBS interaction enables recognition of HIV-1-Env expressing cells by anti-cluster A Abs (Related to Figure 1).** Cell surface staining of 293T cells expressing either **(A)** the HIV-$1_{JRFL}$ ΔCT Env or **(B)** the HIV-$1_{JRFL}$ gp160 Env

with AF-647-conjugated A32 or C11, in the presence of the CoRBS 17b full antibody, its Fab, or Fab'2 fragment, alone or in combination with 50 μM of BNM-III-170, as described in STAR Methods. Data are presented as means and SEM of the mean fluorescence intensity (MFI) divided by the MFI detected by mock-transfected. Data are the averages from at least three independent experiments. Statistical significance was evaluated using one-way ANOVAs or Kruskal-Wallis test, ** $P$ <0.01, **** $P$ < 0.0001; ns, not significant.

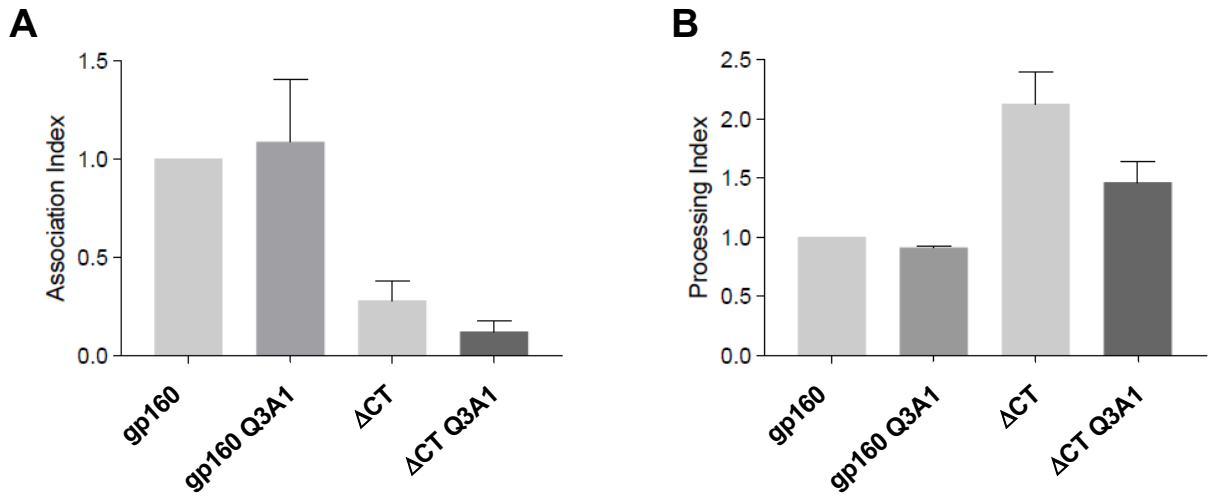


**Figure 4.10 (Figure S3 in Article). Processing and subunit association of HIV-1 $_{JRFL}$ Envs (Related to Figure 2).** Cell lysates and supernatants from $^{35}$S-labeled cells transiently expressing the indicated HIV-1$_{JRFL}$ Env variants were precipitated with a mixture of sera from HIV-1 infected individuals. The precipitated proteins were loaded onto SDS-polyacrylamide gels and analyzed by autoradiography and densitometry to calculate their **(A)** association and **(B)** processing indexes, as described in supplemental material and methods. Mean and SEM derived from at least three independent experiments are shown.

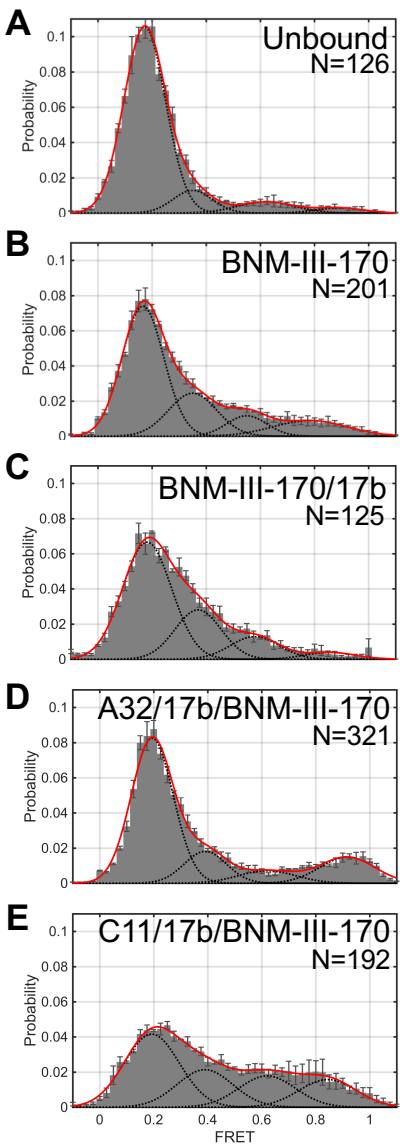


**Figure 4.11 (Figure S4 in Article). CD4mc in combination with anti-cluster A and CoRBS Abs stabilize a new Env conformation (Related to Figure 2).** Histograms of FRET values observed for HIV-$1_{JR-FL}$ Env ΔCT **(A)** in the absence of bound ligands, **(B)** in the presence of the CD4mc BNM-III-170 (100 $\mu$M), **(C)** in the presence of both BNM-III-170 (100 $\mu$M) and the CD4i Ab 17b (5$\mu$g/ml), **(D)** in the presence of BNM-III-170 (100 $\mu$M), 17b (5$\mu$g/ml), and the anti-cluster A Ab A32 (5$\mu$g/ml) or **(E)** C11. In all cases the histograms were formed by compiling the indicated number (N) of smFRET traces. Overlaid on the FRET histograms is the sum of four Gaussian distributions with means and standard deviations determined by HMM analysis (0.18 ± 0.06, 0.38 ± 0.07, 0.65 ±

0.08, 0.90 ± 0.08). Error bars reflect the standard deviation in the number of data points per histogram bin determined from three independent groups of smFRET traces.

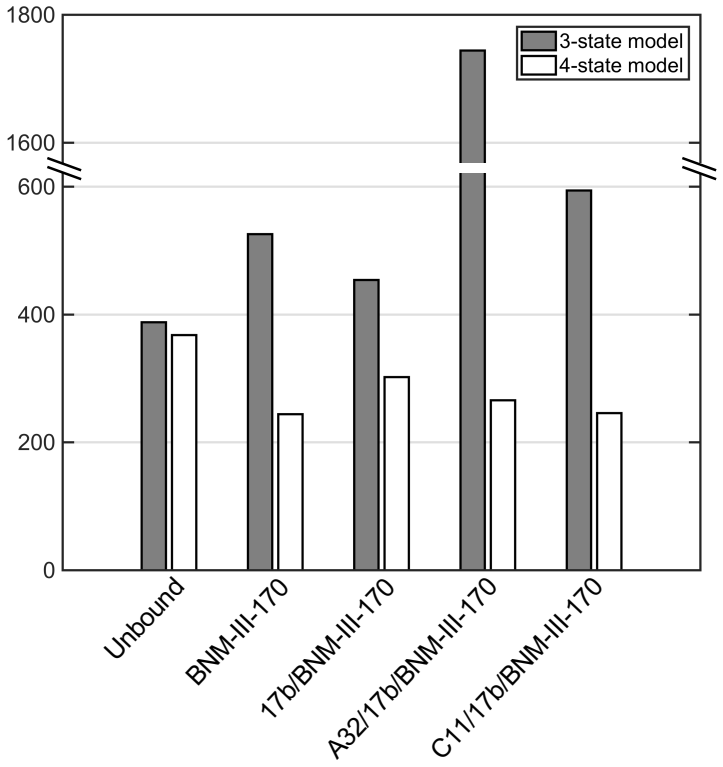


**Figure 4.12 (Figure S5 in Article). Model selection using HMM analysis of smFRET trajectories (Related to Figure 2 in Article).** smFRET trajectories acquired from Env ΔCT, under the 5 conditions shown in Figure 2, were to 3- and 4-state models using the Baum-Welch algorithm. The maximized likelihood estimated during fitting was corrected for different numbers of model parameters using the Akaike Information Criterium (AIC). Reduction in AIC indicates improved model fitness. Little reduction in AIC is seen for unbound Env ΔCT since only minimal observation of State 2A was made. Under conditions in which State 2A is more prominent, the improvement of model fitness is greater upon addition of the fourth model state.

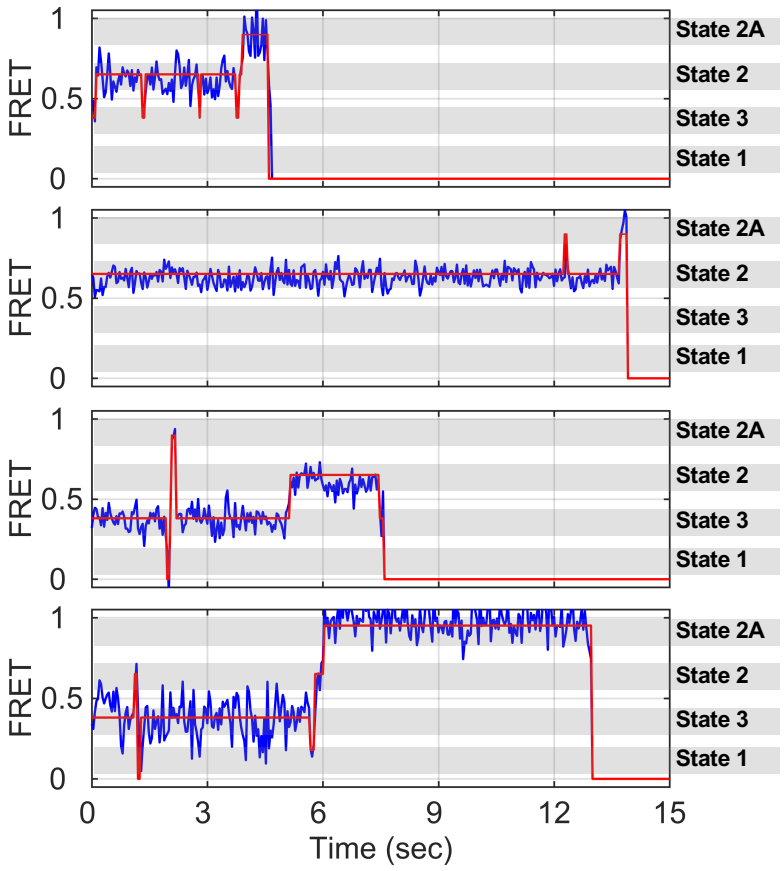


**Figure 4.13 (Figure S6 in Article). Example smFRET trajectories showing transitions to State 2A (Related to Figure 2).** FRET trajectories acquired from individual Env DCT molecules (blue) with idealization generated through HMM analysis overlaid in red. Shaded bars and state labels indicate approximately positions of the FRET values identified. Most, though not all, transitions to State 2A occur via State 2.

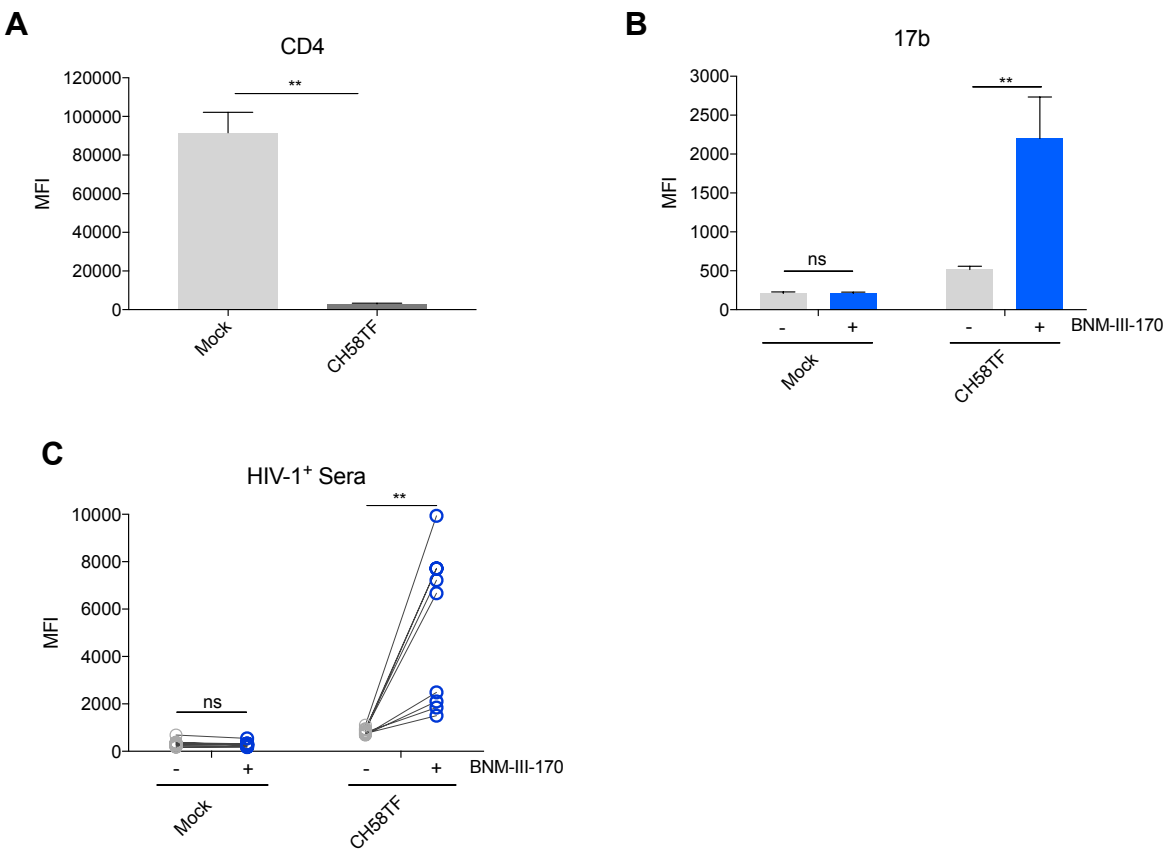


**Figure 4. 14 (Figure S7 in Article). Cell surface staining of HIV-1 infected cells (Related to Figure 7 in Article).** Primary CD4-T cells were mock infected or infected with CH58 T/F virus and stained 48 h later for **(A)** CD4, **(B)** 17b antibody, or **(C)** HIV-1$^+$ sera. Results shown are representative staining of infected (p24+) or mock-infected cells obtained in at least three independent experiments. Error bars indicate mean ± SEM. Statistical significance was tested using an unpaired student t test, or multiple-t tests; correcting for multiple comparisons using the Holm-Sidak method, ** $P$ <0.01; ns, not significant.

| | Q23.17 (A) | Q842.d12 (A) | AC10.29 (B) | AD17 (B) | JR-FL (B) | Yu2 (B) | Du156.12 (C) | ZM109.4 (C) |
|---|---|---|---|---|---|---|---|---|
| **#1 (MT1015)** | 192 | 389 | 169 | 1104 | 256 | 137 | 100 | <25 |
| **#2 (MT0163)** | 62 | 118 | 38 | 46 | 68 | <25 | 115 | 30 |
| **#3 (MT6007)** | <25 | <25 | <25 | <25 | 51 | 46 | <25 | 49 |
| **#4 (MT6017)** | <25 | <25 | <25 | 254 | 107 | <25 | <25 | <25 |
| **#5 (MT1008)** | 35 | <25 | <25 | <25 | 36 | 25 | <25 | 64 |
| **#6 (MT6021)** | 2157 | <25 | 91 | <25 | 126 | <25 | <25 | 50 |
| **#7 (MT1003)** | <25 | 50 | <25 | <25 | <25 | <25 | <25 | 46 |
| **#8 (MT1012)** | <25 | <25 | <25 | 99 | <25 | <25 | <25 | <25 |
| **#9 (MT1010)** | 27 | 74 | <25 | <25 | 186 | 33 | 77 | 27 |

| ID50 color code | <25 | 25-99 | 100-999 | >1000 |
|---|---|---|---|---|

**Table 4.1 (Table S1 in Article). Neutralization ID50 titers of 5 HIV-1+ sera against 8 Env isolates.**

The neutralization ID50 titers are shown as the serum reciprocal dilutions required to inhibit the viral infection by 50%. The tested Env isolates are indicated at the top row, with clade information specified in parentheses.

| | Occupancy (%) | | | |
|---|---|---|---|---|
| | **State 1 (0.18 FRET)** | **State 2 (0.65 FRET)** | **State 2A (0.90 FRET)** | **State 3 (0.38 FRET)** |
| **WT** | | | | |
| unbound | 79 ± 3 | 10 ± 2 | 4.8 ± 0.9 | 6.3 ± 0.9 |
| CD4mc | 64 ± 3 | 15 ± 2 | 13 ± 2 | 7 ± 1 |
| CD4mc/17b | 61 ± 4 | 20 ± 3 | 11 ± 1 | 8 ± 1 |
| CD4mc/17b/A32 | 65 ± 2 | 10 ± 2 | 20 ± 2 | 6 ± 1 |
| CD4mc/17b/C11 | 41 ± 3 | 24 ± 2 | 20 ± 3 | 15 ± 2 |
| **ΔCT** | | | | |
| unbound | 71 ± 3 | 17 ± 3 | 1.0 ± 0.4 | 8.0 ± 0.9 |
| CD4mc | 58 ± 3 | 19 ± 1 | 19 ± 3 | 8.0 ± 0.9 |
| CD4mc/17b | 54 ± 3 | 21 ± 2 | 15 ± 3 | 10 ± 2 |
| CD4mc/17b/A32 | 29 ± 3 | 20 ± 3 | 39 ± 4 | 12 ± 2 |
| CD4mc/17b/C11 | 37 ± 3 | 27± 2 | 21 ± 2 | 15 ± 2 |
| **ΔNef** | | | | |
| Unbound | 79 ± 3 | 12 ± 2 | 4.4 ± 2 | 3.9 ± 0.9 |
| CD4 | 40 ± 3 | 27 ± 3 | 20 ± 3 | 13 ± 2 |
| **CL-** | | | | |
| Unbound | 54 ± 5 | 29 ± 4 | 9.0 ± 0.9 | 11 ± 3 |
| CD4mc/17b/A32 | 25 ± 3 | 24 ± 3 | 34 ± 3 | 17 ± 2 |
| CD4mc/17b/C11 | 26 ± 3 | 30 ± 3 | 29 ± 4 | 15 ± 2 |
| **Serum** | | | | |
| HIV- | 77 ± 3 | 11 ± 2 | 4.0 ± 0.9 | 7.8 ± 0.9 |
| #1 | 68 ± 3 | 13 ± 2 | 11 ± 2 | 7.9 ± 0.9 |
| #2 | 78 ± 3 | 10 ± 2 | 8.9 ± 0.9 | 3.2 ± 0.9 |
| #3 | 51 ± 3 | 17 ± 2 | 18 ± 3 | 14 ± 2 |
| #4 | 64 ± 4 | 16 ± 3 | 16 ± 4 | 3.9 ± 0.9 |
| #5 | 46 ± 3 | 23 ± 2 | 16 ± 3 | 15 ± 2 |
| #6 | 51 ± 4 | 21 ± 3 | 13 ± 3 | 15 ± 3 |
| #7 | 64 ± 4 | 17 ± 3 | 15 ± 3 | 4.2 ± 0.9 |
| #8 | 67 ± 4 | 18 ± 3 | 6.3 ± 0.9 | 8.6 ± 0.9 |
| #9 | 50 ± 2 | 19 ± 1 | 20 ± 2 | 11 ± 1 |
| **Serum + CD4mc** | | | | |
| HIV- | 66 ± 3 | 12 ± 2 | 10 ± 2 | 12 ± 2 |
| #1 | 77 ± 3 | 10 ± 2 | 7.0 ± 0.9 | 5.2 ± 0.9 |
| #2 | 47 ± 3 | 20 ± 2 | 20 ± 2 | 13 ± 2 |
| #3 | 51 ± 3 | 19 ± 2 | 18 ± 3 | 13 ± 2 |
| #4 | 40 ± 4 | 24 ± 3 | 23 ± 4 | 14 ± 3 |
| #5 | 37 ± 4 | 16 ± 3 | 34 ± 4 | 12 ± 2 |
| #6 | 25 ± 3 | 13 ± 2 | 48 ± 3 | 13 ± 2 |
| #7 | 26 ± 2 | 13 ± 2 | 48 ± 3 | 13 ± 2 |
| #8 | 28 ± 2 | 13 ± 1 | 49 ± 2 | 10 ± 1 |
| #9 | 26 ± 3 | 2.0 ± 0.9 | 68 ± 3 | 4.0 ± 0.9 |

**Table 4.2 (Table S2 in Article). FRET state occupancies determined through HMM analysis.**

**Highlights**

- HIV-1 Env is highly dynamic and is known to sample three different conformations
- Here we report the characterization of a new Env conformation: State 2A
- State 2A is difficult to trigger and is susceptible to antibody attack

**eTOC Blurb**

HIV-1 envelope glycoproteins (Env) are very flexible and are known to sample at least three different conformational States. Here we report the characterization of a fourth Env State which is efficiently recognized by antibodies capable of mediating potent antibody-dependent cellular toxicity.

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# CHAPTER V

## DISCUSSION AND PERSPECTIVES

### 5.1 Contribution to current knowledge

The studies presented in this thesis provide new insights into the pathogenesis of HIV-1 infection. My PhD work was the first demonstrating how Nef clones isolated from two different categories of infected patients (EC or CP) control Env-conformation and exposure of CD4i epitopes. We showed how the differential exposure of these epitopes impacts ADCC responses. We also demonstrated that Nef isolates from EC fail to prevent ADCC responses through their inability to fully downregulate CD4 from the cell surface, resulting in enhanced susceptibility of infected cells to ADCC compare to Nef from CP. We further showed that the ability of HIV-1 Nef protein to prevent NK cells activation, via reducing the expression of NKG2D ligands [508], is weakened in Nef clones from EC. Importantly, the high levels of NKG2D ligands on the surface of cells infected with viruses coding for Nef from EC have positively correlated with increased susceptibility to ADCC. The biological significance of such an association was then corroborated by the evaluation of antibody-mediated blockade of NKG2D, which resulted in a significant decrease in the susceptibility of HIV-1-infected cells to ADCC. Indeed, our work highlighted the involvement of NKG2D/NKG2D ligand interactions in the enhanced susceptibility to ADCC responses of HIV-1-infected cells carrying Nef from EC.

In this thesis, we also uncovered a new Env conformation that is recognized by CD4i-Abs and is highly susceptible to ADCC responses. Since Env represents the only antigen exposed on the surface of viral particles or infected cells, it is obligatory to study Env-conformational dynamics to develop better vaccine and therapeutic approaches. The functional and mature Env-trimer exists in a closed-unliganded conformation (state 1) that can't be recognized by CD4i non-neutralizing Abs. Nevertheless, binding to CD4 receptor during viral entry drives its transition from the closed "state 1" to CD4-bound "state 2 and 3" conformations [560]. These downstream conformations can be recognized by several CD4i Abs targeting the CoRBS and the V3 loop, but the cluster A Abs, which have a strong ADCC activity, do not bind to states 2 and 3. By exploring the Env-conformation dynamics in association with different classes of CD4i Abs, we identified a fourth conformation, named State 2A, vulnerable to Abs attack.

Taken together, the finding presented in this thesis underlines the potential role of ADCC in the durable suppression of virus replication and plasma viremia in this rare subset of HIV-1 infected individuals. This control of viral infection is the result of a complex interaction of host immune and viral factors. We focused on the attenuated functions of HIV-1 Nef accessory protein, in particular the downmodulation of CD4 and NKG2D ligands, which represents intrinsic mechanisms regulating Env-conformation and NK-cells activation, respectively. Indeed, our data suggest that the ability of Nef to limit CD4 expression at the surface of infected cell prevents Env-CD4 interaction and thus, it might be an immune evasion mechanism aimed to avoid the exposure of Env in this antibody vulnerable State 2A conformation, which is susceptible to ADCC activity. Thereby, we

can hypothesize that cells infected with viruses coding for Nef from EC might represent Env sampling State 2A conformation due to its inability to efficiently remove surface CD4. It would be important to confirm this hypothesis in future experiments.

The importance of ADCC in disease progression remains controversial among the scientific community for the last 30 years [639]. Indeed, the first reports demonstrating the presence of antibodies that are capable of eliciting an ADCC response against HIV-1 date to the late 1980s [640-642]. While some studies have positively supported the involvement of ADCC activity during disease progression [531, 532, 535, 643], others have reported diametrically opposed findings to those publications [545, 644]. In fact, the ability of HIV-1 to defend itself from such recognition and elimination by the immune response, as the one described in this thesis by Nef, raises possible therapeutic approaches. Our findings support the use of Nef inhibitors in therapeutic approaches aimed to boost immune responses, normally interrupted by Nef, to eliminate virally infected cells, including ADCC. We could speculate that if efficient Nef inhibitors are developed, they could potentially help all HIV-1-infected individuals develop an EC phenotype, i.e. control viral replication in absence of ART (see below 5.2).

## 5.2 Studying Elite Controller's phenotype

A small group of infected individuals, known as elite controllers, display tight and permanent control of viral replication and clinical progression in the absence of retroviral therapy. These infected individuals have been the object of numerous studies that aimed to identify the elements responsible for this clinical phenotype. Several host genetic characteristics have also been associated to the non-progressor phenotype of these individuals, principally HLA-B genotypes (HLA-B57/B58 or B27) [645], and HLA-C [646]. For instance, the highly effective HLA class I-restricted T cell responses that target conserved viral peptide epitopes is considered a major factor regulating the durable control of HIV-1 infection on EC subset [647]. Additionally, the presence of non-canonical HLA escape mutation in EC patients used in our studies showed to affect the activity of several viral proteins, including Nef [637]. Multiple functions of Nef protein, including downregulation of HLA class I and CD4, enhancement of viral infectivity and replication, significantly impaired in EC Nef clones in comparison to those isolated from CP [637, 648, 649]. Further analysis of these Nef sequences from ECs and CPs used in our studies was previously conducted showing no common residue signatures involved in decreased CD4 downregulation [637]. Nevertheless, this impairment of Nef's activities was reported to be influenced by the HLA-I B*57 associated polymorphism [637]. This reduced ability of Nef to decrease surface expression of CD4 has resulted in increased exposure of CD4i and enhanced susceptibility of these cells to ADCC (Chapter II). Independently of its impaired capacity to mediate CD4 downregulation, we showed that Nef from these ECs also inefficiently reduced the expression of NKG2D ligands on infected cells. This observation is highly important in regard to NK cell activation and in demonstrating the role of NKG2D

to act as a co-receptor for anti-HIV-1 ADCC in EC (Chapter III). Since the mechanism and the motif(s) used by Nef to downregulate NKG2D ligands are still unknown, it would be important to identify the naturally occurring mutations in Nef that might impact NKG2D ligands downregulation. It would be also desirable to evaluate whether the impaired NKG2D-mediated downregulation of Nef protein is influenced by the HLA-I*B57 or other immune-induced pressures.

Taken together, we believe that studying the phenotype of HIV-1 controllers is a useful strategy to understand anti-HIV immune responses. The combination of viral impairment, shown in this thesis regarding Nef, along with cellular and humoral immunity could shape this durable suppression of viral replication and disease progression without antiretrovirals. Studies presented in this thesis (Chapter II and Chapter III) showed that several parameters of ADCC responses are targeted by HIV-1 defences. First, Env is a major target for functional antibody responses and its conformation is indirectly modulated by Nef through its ability to downregulate CD4. Second, NK cells are considered one of the major effector cells for ADCC responses and their activation is affected by Nef-mediating NKG2D ligands downmodulation. Therefore, targeting Nef's functions developing potent Nef-specific inhibitors could improve the immune recognition and elimination of infected cells. Of note, whether Vpu's functions are also attenuated in EC and whether this could play a role in ADCC is not known and would be interesting to evaluate in future studies.

## 5.3 ADCC responses: the adaptation of HIV-1 to its host

The hallmark of HIV-1 is its capacity to adapt and overcome host immune defences. The presence of an antibody class capable of inducing ADCC against cells exposing Env in its CD4-bound (open) conformation appears to be widespread in both natural infection and vaccine trials [345, 564]. It is quite remarkable that these Env-epitopes represent highly conserved regions that are important in Env functionality and susceptibility to ADCC. The ability of HIV-1 to limit the exposure of these epitopes through its accessory proteins Nef and Vpu [562] seems to be efficient to protect the virus. Despite the cooperative effects of these two accessory proteins to reduce the overall exposure of CD4i epitopes at the surface of infected cells [562, 564, 570, 571, 650], it is yet unknown which one has the higher impact. It is thought that viruses coding Env variants apt to spontaneously sample the CD4-bound rearrangement, such as HIV-1 X4-tropic or lab-adapted strains [651-653], would rely more on the anti-tetherin activity of Vpu to control Env-accumulation and ADCC responses. While cells infected with viruses encoding Env-sampling a closed conformation [654, 655] might depend on both Nef and Vpu-mediated CD4 downregulation functions to limit the surface levels of CD4 to avoid the adaptation of Env-CD4 conformation and ADCC response. Independently of which accessory proteins has the most marked effect on ADCC responses, our results strongly suggest that targeting them with small inhibitors may provide a new strategy to eliminate HIV-1 infected cells.

## 5.4 Implications on the viral biology and pathogenesis

### 5.4.1 The importance of Nef as a virulence factor

Nef-mediated CD4 downregulation:

CD4 downregulation by Nef plays a crucial role in HIV-1 infection that was first postulated as a mechanism to prevent viral superinfection [656]. Nef-mediated CD4 removal has been also linked to T cell activation through its ability to promote phosphorylation of various substrates [657]. Nef enhances viral infectivity through both CD4-dependent and independent mechanisms. When Nef is present on producing cells, Nef can enhance viral infectivity through downregulation of CD4, leading to more efficient release of virions and more efficient Env engagement to CD4 receptors on the surface of subsequent target cells [489, 490]. Indeed, the presence of Nef protein is required to prevent the incorporation of CD4 into the nascent HIV-1 particles [489]. Thus, viruses produced in the absence of Nef incorporate fewer Env and more CD4 molecules, resulting in less infectious viruses. It has been also reported that the presence of high levels of CD4 leads to the accumulation of the viral structural proteins in the host cells, inducing a reduction of viral particle release [490]. A study has shown that an important physiological function of CD4 downregulation by Nef is its ability to prevent the exposure of CD4i epitopes in HIV-1 Env that essentially renders infected cells susceptible to ADCC [562]. Our work presented in Chapter II showed an incomplete downregulation of CD4 molecules by Nef isolated from EC. The residual levels of CD4 at the surface of infected cells were sufficient to interact with Env, leading to increased exposure of Env CD4i epitopes and enhanced sensitivity of infected cells to ADCC. Our study suggests that targeting Nef-mediated CD4 downmodulation would be crucial for therapeutic strategies

in HIV-1 infected patients. Notably, mutations within the dimerization interface of Nef protein have showed to significantly abolish Nef-mediated CD4 downregulation. Since this dimerization is required for Nef's interaction with CD4 and AP-2, it represents a potential target site for inhibitors or antiviral drugs against Nef [467, 658]. We believe that screening for Nef-small molecules aimed to abolish Nef dimerization and therefore its ability to downregulate CD4 would assist the host's immune responses in overcoming virus escape and initiation of a continuous immune control.

Nef-mediated NKG2D ligands downregulation:

Since NK cells represent a major component of the host immune defences against pathogens, viruses including HIV-1 have developed strategies to escape their responses. Indeed, Nef's ability to mediate reduction of NKG2D ligands expression enables HIV-1 infected cells to more efficiently evade cytotoxic NK-cell recognition and lysis. Nef downmodulates the surface expression of NKG2D ligands MICA, ULBP1, and ULBP2 through an unknown mechanism and domains that differ from those required for CD4 downregulation [508]. The removal of NKG2D ligands from the surface of infected cells prevent their interaction with activating receptor NKG2D on NK cells, which otherwise leads to the activation of NK cell effector function causing the elimination of target cells. Interestingly, the activating NKG2D receptor can not only mediate direct killing of target cells expressing its ligands, but was also shown to act in conjunction with CD16 receptor to act as a co-receptor for anti-HIV-1 ADCC [524]. In this context, studying the effect of Nef-mediated NKG2D ligands downregulation in EC is highly relevant for understanding viral mechanisms involved in susceptibility and evasion of ADCC

response. Studies have exhibited unique phenotypic features of NK cells among HIV controllers and LTNPs that include high levels of IFN-γ production, activation markers, and cytolytic activity [659, 660], Therefore, future research should also investigate whether this unique NK cells phenotype could result in better autologous effector function of cells obtained from HIV-1 ECs versus other groups, in particular the role of NKG2D in ADCC responses. While several factors in which NKG2D is contributing to ADCC response remains a subject for investigation, our observation confirm the importance of the cumulative activating and inhibitory signals in the functionality of NK cells [574]. The capacity of HIV-1 Nef to manipulate several cellular receptors that are able to send both activating and inhibitory signals to NK cells, makes it intriguing to further investigate strength of the different manipulations among different clades and categories of infected individuals. Finally, it is important to further explore whether ligands for other activating or co-activating receptors presented on HIV-1 infected cells could participate to this accumulative activating signal and ADCC response.

Alternative approaches aimed to directly target HIV-1 Nef accessory protein are emerging in the field. Bouchet and colleagues have reported a single-domain antibody (sdAb) construct that has high affinity toward HIV-1 Nef, known as sAb19 or Neffin [661]. The sdAb was isolated from llama immunized with a recombinant Nef protein and has showed to have anti-Nef activity, when expressed intracellularly. The intracellular expression of sdAb19 has resulted in inhibition of the Nef-mediated CD4 downmodulation. Therefore, we believe that expressing sdAb19 in HIV-1 infected cells could result in increased formation of Env-CD4 complexes and thus exposure of CD4i

epitopes at the cell surface, potentially triggering recognition and elimination of infected cells through ADCC response. However, despite the future promising of gene therapy technologies for HIV-1 treatment, targeting infected cells with a safe and efficient vector to express recombinant protein such as sdAb19 is still very challenging, but we believe it deserves to be explored.

Among the multitude effects that HIV-1 Nef displays on infected cells, it is still unclear to what extent each individual activity affects the pathogenesis *in vivo*. Indeed, many evidences have supported the indispensable role of Nef in the progression to AIDS, as this protein is a master regulator of CD4 T cells signalling pathways and certain surface proteins. Vaccination of rhesus macaques with attenuated SIVmac239ΔNef has showed to result in low viral load and durable suppression of disease progression [157, 662]. Certainly, the Sydney Blood bank Cohort represents one of the best examples that highlights the association between the absence of Nef functions and delayed progression to AIDS *in vivo*. For more than 29 years of infection, recipients of a blood transfusion from a donor infected with virus encoding a large deletion in *nef* and the U3 region of LTR remain LTNPs with undetectable viremia and normal CD4 T cells counts [159]. Similarly, we demonstrated that attenuated Nef's activities in rare subset of controllers (ECs) might contribute to their disease outcome *in vivo*. Comparing Nef clones isolated from EC versus CP have showed that a slight, yet significant, differences in Nef's downregulation of CD4 and NKG2D ligands were surprisingly sufficient to induce a potent immune response. This observation suggests that targeting and attenuating Nef could be sufficient to impact its immune evasion mechanisms that helps HIV-1 replicate *in vivo*.

### 5.4.2 Masking HIV-1 Env Glycoprotein Conformation

The native closed conformation of HIV-1 Env is tightly controlled by complex mechanisms that include several parts of the glycoproteins. To prevent the exposure of epitopes targeted by CD4i Abs, Env works on limiting its transition to downstream State 2 and State 3 conformation. The topological layers in the gp120 inner domain play a critical role in orchestrating the CD4-binding induced conformational changes [265, 293, 295]. The arrangement of the variable regions on HIV-1 Env could also delay the spontaneous transition to the CD4-bound state, causing decreased exposure of CD4i-epitopes [278, 281, 663]. Likely, filling the highly conserved Phe43 cavity with a hydrophobic residue could allow the spontaneously sampling of State 2 and State 3 conformations [566, 567]. The exposure of CD4i epitopes is especially important to allow the recognition and elimination of Env-expressing infected cells by ADCC. Thus, it is well established that Env undergoes a series of conformations rearrangement that transit between either unliganded State 1 or in response to CD4 binding, transits between State 2 and State 3 [560, 561, 664]. These conformational changes require the formation of Env-CD4 complex that are targeted by CD4i-Abs attack [562]. Since these ADCC-mediating CD4i Abs are largely prevalent in the sera of infected individuals [564], they may provide a base to target HIV-1 Env epitopes during infection. The use of CD4mc could represent a great strategy to facilitate Env-conformational changes on infected cells, despite Nef function. CD4mc have been shown to initiate the opening of HIV-1 Env trimer to allow the binding of CoRBS Abs, which then facilitate exposure of anti-cluster A epitopes. Thus, sensitizing HIV-1 infected cells to ADCC [374]. This approach has proven to sensitize endogenously infected *ex vivo* amplified primary CD4+ T cells to ADCC

mediated by autologous sera and effector cells [374]. Therefore, it represents a promising tool to unmask the conformational state of HIV-1 Env that is vulnerable to attack by easy-to-elicit anti-cluster A Abs.

## 5.5 Design of vaccine approaches

While the development of an effective vaccine against HIV remains to be achieved, our findings provide new scientific knowledge that could advance the efforts toward a new concept of vaccine. Currently, efforts are focused on the design and the utility of antigens aimed at eliciting the production of neutralizing antibodies that target a wide range of viral isolates. However, the generation of these antibodies are hard to induce due to their unusual high levels of somatic mutations and thus require sustained progressive antigenic stimulation [665]. It has been also suggested that germline parental B cells of broad-spectrum neutralizing antibodies are minimally stimulated by antigens recognized by the mature form of antibodies [665]. Nevertheless, it is important to mention that non-neutralizing antibodies that target Env with the CD4-bound “open” conformation are often induced in vaccine trials in both human and non-human primates [349, 666, 667]. However, the work presented in this thesis suggests limited action of these antibodies *in vivo* due to the protective and escape mechanisms developed by HIV-1 that include Nef-mediated CD4 downregulation. Since small CD4mc have been shown to dramatically enhance the vaccine efficacy of gp120-immunized rhesus macaques against Tier 2 SHIV challenges [375], it would be desirable to determine whether the induction of an open Env-conformation by CD4mc could facilitate the establishment of protective immunity in individuals at high risk of infection.

Despite the ability of HIV-1 to escape ADCC response, it has been shown that this response has played an essential protective role in the RV144 vaccine trial. It is important to mention that the RV144 vaccine trial was conducted in Thailand, where the majority of circulating strains belong to the recombinant circulating form CRF01-AE [668, 669]. Sequence analysis of these viruses revealed the presence of bigger amino acid (histidine instead of serine) at position 375. The presence of such an amino acid causes the Env to adopt a more open conformation that is sufficient to induce better recognition and elimination of infected cells by CD4i antibodies and sera of infected patients [266, 567]. This suggests that immunogen design should highly consider the conformation of Env of circulating strains in any given geographical location in order to be effective.

## 5.6 Design of therapeutic approaches

Therapeutic approaches are always linked to the eradication of latent HIV-1 in resting CD4+ T cells from infected individuals. To do so, the “shock and kill” strategy aims to reactivate the latent virus and induce its destruction by the immune system. While the idea of reactivating and eliminating latently infected cells via ADCC is intriguing, it is still unclear how this will be done *in vivo*. Various strategies have been put forward to reactivate the latent virus through the use of small molecules known as latency-reversing agents. These molecules aim to activate viral transcription [670, 671]. In vitro reactivation of infected cells from HIV-1 infected patients has shown that it is possible to induce increased recognition of infected cells through the use of CD4mc compounds. Compelling evidence supports the potential use of CD4mc to eliminate HIV-1-infected cells [373]. We

now know that this is achieved through the stabilization of a new Env conformation, State 2A. We showed, that this new and vulnerable conformation can be stabilized by the CD4 receptor (if a Nef- virus is used) or by a “cocktail” comprising CD4mc, CoRBS and anti-cluster A Abs (Chapter IV). However, it is still unclear whether the level of Env present in latently infected cells is sufficient for the use of CD4mc. Therefore, the use of CD4mc could be pursued in combination type I IFNs or IL-27 since they have been shown to increase Env accumulation at the surface of infected cells therefore enhancing ADCC response in the presence of CD4mc [572].

Additionally, the generation of bispecific Abs (bsAbs) could be an appealing strategy to target reactivated cells. These molecules are essentially formed of two antibody fragments, one targeting Env and the other binding to a cellular receptor [672-674]. Alternatively, the generation of bispecific proteins (bsps) with the capacity to target different Env epitopes such as CD4BS and CoRBS has been described and has showed to generate potent ADCC activities against HIV-1 infected cells [674-676]. The potent activity of bsAbs and bsps appears to be linked to their IgG Fc portion that enhances their stability, half-life, and most importantly the recruitment of FcR-bearing effector cells. In this context, it would be relevant to investigate the generation of other combinations of psbs proteins, such as CoRBS and anti-Cluster A and their potency in ADCC response in presence of CD4mc. This could further influence the development of passive administration of antibodies both in therapeutic and vaccination approaches.

## CONCLUSION

The work presented in this thesis has demonstrated that ADCC might be contributing to the phenotype of HIV-1 elite controllers. We have elucidated that the inability of HIV-1 Nef to fully downregulate surface CD4 levels and NKG2D ligands from the cell surface can positively influence the outcome of ADCC responses in this cohort. The residual amounts of CD4 presented at the surface of infected cells interact with Env, thus triggering conformational changes that expose CD4i epitopes and enhance susceptibility of infected cells to ADCC by Cluster A Abs and HIV-1+ sera. We also showed that the impairment of Nef mediated NKG2D ligands downregulation provides a better activating signal for NK cells that results in enhancing its effector function. Finally, we have showed for the first time that upon interaction with cellular CD4 or CD4mc in presence of CoRBS Abs the HIV-1 Env samples a new conformational state which is accessible by ADCC mediating Abs. Taken together, these observations suggest that ADCC responses against cells exposing Env CD4i epitopes might be a critical component of the immune response in EC. This thesis brings new information that could put us one step closer to stopping HIV-1.

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