



HAL
open science

Rôle des polymorphismes dans la région C-Terminale de l'enveloppe du VIH dans l'infection de cellules primaires

Eveline Santos da Silva

► To cite this version:

Eveline Santos da Silva. Rôle des polymorphismes dans la région C-Terminale de l'enveloppe du VIH dans l'infection de cellules primaires. Médecine humaine et pathologie. Université de Lorraine, 2013. Français. NNT : 2013LORR0279 . tel-01750624

HAL Id: tel-01750624

<https://hal.univ-lorraine.fr/tel-01750624>

Submitted on 29 Mar 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



AVERTISSEMENT

Ce document est le fruit d'un long travail approuvé par le jury de soutenance et mis à disposition de l'ensemble de la communauté universitaire élargie.

Il est soumis à la propriété intellectuelle de l'auteur. Ceci implique une obligation de citation et de référencement lors de l'utilisation de ce document.

D'autre part, toute contrefaçon, plagiat, reproduction illicite encourt une poursuite pénale.

Contact : ddoc-theses-contact@univ-lorraine.fr

LIENS

Code de la Propriété Intellectuelle. articles L 122. 4

Code de la Propriété Intellectuelle. articles L 335.2- L 335.10

http://www.cfcopies.com/V2/leg/leg_droi.php

<http://www.culture.gouv.fr/culture/infos-pratiques/droits/protection.htm>

Ecole Doctorale BioSE (Biologie-Santé-Environnement)

Thèse

Présentée et soutenue publiquement pour l'obtention du titre de

DOCTEUR DE L'UNIVERSITE DE LORRAINE

Mention : « Sciences de la Vie et de la Santé »

par Eveline SANTOS DA SILVA

**Rôle des polymorphismes dans la région C-Terminale de
l'enveloppe du VIH dans l'infection de cellules primaires**

12 Décembre 2013

Membres du jury :

Rapporteurs :	Mme. Clarisse BERLIOZ-TORRENT	Directeur de Recherche, Institut Cochin, Université Paris Descartes, Paris
	Mr. Jean RUELLE	Chargé de Recherche, Université Catholique de Louvain, Bruxelles
Examineurs :	Mr. Marcelo DE CARVALHO BITTENCOURT	Professeur, Faculté de Médecine, Université de Lorraine, Nancy
	Mme. Joelle FRITZ	Docteur, Luxembourg Centre for Systems Biomedicine, Luxembourg
	Mr. Gilbert FAURE	Professeur, Faculté de Médecine, Université de Lorraine, Nancy, Directeur de thèse
	Mme. Carole DEVAUX	Docteur, Laboratoire de Rétrovirologie, CRP- Santé, Luxembourg, Co-Directeur de thèse
Invités :	Mr. Christian RABAUD	Professeur, Faculté de Médecine, Université de Lorraine, Nancy

Remerciements

Je n'aurais jamais pu réaliser ce travail de thèse sans le soutien d'un grand nombre de personnes. Ce travail a pu voir le jour grâce à vous.

Je voudrais en premier lieu remercier le Professeur Gilbert Faure, mon directeur de thèse, de m'avoir permis d'entreprendre une thèse de doctorat. Un grand merci aussi au Dr Carole Devaux, mon co-Directeur de thèse, pour avoir supervisé ce travail de thèse. Je tiens aussi à remercier le Dr Jean-Claude Schmit de m'avoir accueilli au sein du Laboratoire de Rétrovirologie du CRP-Santé du Luxembourg.

Je tiens à exprimer ma plus grande reconnaissance au Dr Danielle Perez Bercoff pour son encadrement tout au long de ce travail de thèse, pour sa patience, pour les discussions intéressantes, pour les multiples conseils, pour son enthousiasme et pour toutes les heures qu'elle a consacrées à ce travail.

Je tiens aussi à remercier en particulier les collègues qui ont contribué à la réussite de ce projet de thèse : Dr Martin Mulinge, Morgane Lemaire, Cécile Masquelier, Arek Rybicki et Jean-Yves Servais. Je n'y serais certainement pas arrivé sans eux.

Je souhaiterais exprimer ma gratitude au Dr Andy Chevigné et au Dr Sylvie Delhalle pour m'avoir accordé de leur temps et pour leurs conseils judicieux.

Ma gratitude va également à tous les membres du Laboratoire de Rétrovirologie qui m'ont chaleureusement accueilli. Et je tiens à remercier en particulier Manu, Gilles, Charlène, Nadia et Julie que j'ai eu un grand plaisir à côtoyer tous les jours sous les hottes. Merci pour vos encouragements, pour vos conseils, pour les discussions et pour les fous rires.

Merci aux anciens doctorants Martin, Alain et Franky pour leurs sages conseils, sans oublier les autres doctorants avec qui j'ai pu partager un bureau : Martyna, Pierre-Arnaud et Cyprien.

Je désire en outre remercier à tous les autres collègues du CRP-Santé qui m'ont aidé tout au long de mon parcours : Karthik, Daniel, Marwan, Aurélie, René, Béné, ...

Je suis particulièrement reconnaissante envers le Fonds National de la Recherche du Luxembourg (FNR) pour la contribution financière à la réalisation de ce travail.

Merci à mes amis pour leur soutien sans lesquels ce travail n'aurait pas pu se faire, surtout à Sonia, Sergio, Russel, Silvia et Susi.

Un grand merci également à mes parents et à ma sœur Nadia pour leur soutien, leur confiance et surtout leur patience tout au long de ce travail.

A tous ceux, amis et collègues de travail, qui m'ont activement ou passivement aidé à compléter cette expérience inoubliable un GRAND MERCI !

Table des matières

Remerciements	i
Tables des matières	iii
Liste des abréviations	vii
Résumé de thèse	ix
Résumé de thèse anglais	xii
INTRODUCTION.....	1
1. GENERALITES	3
1.1. Découverte du virus de l'immunodéficience humaine.....	3
1.2. Pandémie et origine de l'infection.....	4
1.2.1. Pandémie	4
1.2.2. Origine du VIH	5
1.2.3. Classification du VIH	6
2. LE VIRUS DE L'IMMUNODEFICIENCE HUMAINE (VIH).....	8
2.1. Le Génome	8
2.2. Structure du virus	12
2.3. Les protéines virales	13
2.3.1. Les protéines de structure	13
2.3.2. Les protéines enzymatiques	14
2.3.3. Les protéines régulatrices.....	15
2.3.4. Les protéines auxiliaires	16
3. LE CYCLE VIRAL.....	18
3.1. Attachement et entrée	19
3.2. Reverse transcription.....	20
3.3. Intégration.....	22
3.4. Transcription virale et synthèse de protéines virales.....	23
3.5. Assemblage.....	25
3.6. Maturation	27

4. PATHOGENESE	28
4.1. Transmission virale	28
4.2. Evolution de l'infection virale.....	28
4.2.1. La phase aiguë.....	29
4.2.2. La phase asymptomatique.....	31
4.2.3. SIDA	33
4.3. Réponse immunitaire.....	33
4.3.1. La réponse innée	33
4.3.2. La réponse adaptative	34
4.3.2.1. La réponse cellulaire	34
4.3.2.2. La réponse humorale	34
4.3.3. Les facteurs de restriction	35
4.4. Traitements et échappatoires du virus.....	36
5. CELLULES CIBLES DU VIH.....	38
5.1. Sous-populations lymphocytaires T CD4+ et infection par le VIH.....	38
5.2. Macrophages	40
5.3. Cellules dendritiques	42
5.4. Latence et réservoirs viraux	44
6. ENVELOPPE DU VIH	44
6.1. La protéine de surface (gp120)	45
6.2. La protéine transmembranaire (gp41)	47
6.3. La queue intracytoplasmique (gp41CT)	48
6.3.1. Structure	48
6.3.2. Topologie	50
6.3.3. Cellules permissives et cellules non permissives	51
6.3.4. Fonctions.....	52
6.3.4.1. Incorporation de Env dans le virion	52
6.3.4.2. Infectivité.....	54
6.3.4.3. Contrôle de l'infectivité.....	54
6.3.4.4. Endocytose et trafic intracellulaire.....	55
6.3.4.5. Activation cellulaire	56
6.3.4.6. Interaction avec des protéines virales et cellulaires	57
<u>OBJECTIFS</u>	<u>59</u>
<u>RESULTATS</u>	<u>61</u>

1. The frantic play of the concealed HIV cytoplasmic tail.....	63
1.1. Résumé.....	63
1.2. Article (revue).....	(1-24)
2. Polymorphisms in the gp41 cytoplasmic tail of HIV-1 subtype C viruses impair replication capacity in CD4+ T lymphocytes but not in macrophages	65
2.1. Résumé.....	65
2.2. Article	(1-47)
<u>DISCUSSION.....</u>	<u>69</u>
<u>CONCLUSIONS</u>	<u>81</u>
<u>PERSPECTIVES.....</u>	<u>85</u>
<u>BIBLIOGRAPHIE.....</u>	<u>89</u>
<u>ANNEXE</u>	<u>109</u>

Liste des abréviations

3'PPT	« 3' Polypurine tract »
6-HB	six hélices α
ADN	acide désoxyribonucléique
ALIX	« Alg2-interacting protein X »
APC	cellules présentatrices d'antigènes
APOBEC	« apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like »
ARN	acide ribonucléique
ARN _m	acide ribonucléique messenger
ARN _t	acide ribonucléique de transfert
ARV	Antirétroviraux
BST-2	tetherine
CA ou p24	capside
CCR5	« CC Chemokine Receptor 5 »
CMH	complexe majeur d'histocompatibilité
cPPT	« central polypurine tract »
CRF	forme recombinante circulante
CTL	lymphocytes T CD8 cytotoxiques
CV	charge virale
CXCR4	« CXC Chemokine Receptor 4 »
DC	cellules dendritiques
DC-SIGN	« dendritic cell-specific ICAM-grabbing non-integrin »
DIS	« Dimerization Initiation Site »
dNTP	désoxynucléosides triphosphates
<i>env</i>	enveloppe (gène)
Env	complexe enveloppe (protéine)
ESCRT	complexe de tri endosomal requis pour le transport
<i>gag</i>	« group specific antigen » (gène)
Gag ou Pr55 ^{Gag}	précurseur Gag
GALT	tissu lymphoïde associé au tube digestif
gp160	précurseur de l'enveloppe
gp41CT	queue intracytoplasmique de l'enveloppe
HAART	thérapies antirétrovirales hautement actives
HEK	« human embryonic kidney cells »
HR	« Heptad Repeat »
HTLV	virus T-lymphotropique humain
IFN	interféron
IL	interleukine
IN	intégrase
kb	kilo base
kDa	kilo Dalton
LPS	lipopolysaccharide

LT CD4+	lymphocytes T CD4+
LTR	« long terminal repeats »
MA ou p17	matrice
MDDC	cellules dendritiques dérivées des monocytes
MDM	macrophages dérivés des monocytes
MIP-1 α	« macrophage inflammatory protein alpha »
MPER	« membrane-proximal external region »
NC	nucléocapside
<i>nef</i>	facteur négatif (gène)
Nef	facteur négatif (protéine)
NES	Nuclear Export Signal
NFAT	facteur nucléaire des lymphocytes T activés
NK	cellules naturelles tueuses
NNRTI	inhibiteurs non nucléosidiques de la transcriptase inverse
NRTI	inhibiteurs nucléosidiques de la transcriptase inverse
p17 ou MA	matrice
p24 ou CA	capside
PAMP	motifs moléculaires associés aux pathogènes
PBMC	« peripheral blood mononuclear cell »
PBS	« Primer Binding Site »
PIC	complexe de préintégration
<i>pol</i>	polymérase (gène)
PPT	« Poly Purine Tract »
PR	protéase
Pr55 ^{Gag} ou Gag	précurseur de Gag
Pr160 ^{GagPol}	précurseur de GagPol
PRA1	« prenylated Rab acceptor 1 »
R5	virus à tropisme CCR5
RAN	« Ras-related Nuclear protein »
RANTES	« regulated upon activation normal T-cell expressed and secreted »
Rev	« regulation of virion expression »
RRE	« rev responsive element »
RT	transcriptase inverse
SAMHD1	« Sterile alpha motif domain and Histidine-Aspartic domain-containing protein 1 »
SIDA	syndrome de l'immunodéficience acquise
SIV	virus de l'immunodéficience simienne
Sp1	« Spacer 1 »
Tat	« transactivator of transcription »
TAR	« transactivation-responsive region »
TGF- β	facteur de croissance de transformation β
URF	forme recombinante unique
Vif	« viral infectivity factor »
VIH	virus de l'immunodéficience humaine
Vpr	« viral protein R »
Vpu	« viral protein U »
X4	virus à tropisme CXCR4

Résumé de thèse

Le virus de l'immunodéficience humaine (VIH) est l'agent étiologique du syndrome d'immunodéficience acquise (SIDA). Il cible des cellules du système immunitaire, principalement les lymphocytes T CD4+ (LT CD4+) mais aussi les macrophages (MDM) et les cellules dendritiques, abolissant les défenses de l'organisme. En raison de son importante variabilité, le VIH a été classé en types (1 et 2), eux-mêmes subdivisés en sous-types. Le VIH-1 sous-type B est le plus étudié, bien qu'il ne soit pas le plus répandu. La glycoprotéine virale de l'enveloppe (Env) est primordiale pour le virus car elle permet au virus de pénétrer dans les cellules cibles grâce à l'ectodomaine de Env et d'initier un nouveau cycle infectieux. Le gène *env* est le plus variable du VIH. Cette variabilité lui permet entre autres d'éluder la reconnaissance par le système immunitaire, et a donc été amplement étudiée. Néanmoins la queue intracytoplasmique de l'enveloppe (gp41CT) a été considérablement moins étudiée que son ectodomaine et de nombreuses zones d'ombres persistent concernant ses fonctions. La gp41CT est principalement impliquée dans l'incorporation de l'enveloppe dans les virions bourgeonnants et elle est responsable du trafic intracellulaire de Env grâce à divers motifs distribués le long de sa séquence et grâce à de nombreuses interactions avec des protéines cellulaires. La délétion de gp41CT inhibe la réplication virale dans les cibles primaires du virus, ainsi que dans de nombreuses lignées lymphocytaires T, mais ce phénotype varie en fonction des lignées cellules utilisées. A ce jour, quasiment toutes les études sur gp41CT sont basées sur la souche de référence NL4.3, et des mutants ponctuels ou des mutants de délétion. Ces études ne prennent donc pas en considération la variabilité et les polymorphismes dans cette région.

Lors de ce travail de thèse, nous nous sommes intéressés aux polymorphismes dans gp41CT inhérents aux divers sous-types de VIH et avons étudié leur impact sur l'infectivité et la réplication virale dans des cellules primaires, cibles naturelles du virus. Afin d'éviter de tronquer gp41CT, nous avons utilisé des particules virales recombinantes ayant une structure constante NL4.3 et exprimant des Env de patients. De ce fait, les particules virales recombinantes diffèrent uniquement par leur gp41CT. Les paires de virus recombinant construites expriment soit l'enveloppe entière issue du patient (« recombinants Env »), soit

l'ectodomaine de la même souche virale fusionné à gp41CT du clone de référence NL4.3, générant une protéine Env chimérique (« recombinants EnvEC »). Dix enveloppes de sous-type viral B et douze enveloppes de sous-type C (responsable de la majorité des infections VIH dans le monde) ont été testées. Des LT CD4+ et des macrophages ont été infectés en parallèle avec ces couples de virus.

Dans les LT CD4+, la réplication des virus recombinants de sous-type C était significativement plus faible que celle des sous-types B. La réplication des recombinants Env de sous-type C était également significativement diminuée par rapport à celle des virus recombinants EnvEC, alors qu'il n'y avait pas de différence entre les couples Env et EnvEC de sous-type B. Ce phénotype particulier n'a néanmoins pas été observé dans les MDM, où les réplifications virales étaient similaires tant pour les recombinants sous-types B que pour les C. L'ajout de déoxynucléosides lors de l'infection des MDM a permis d'augmenter l'infection mais il n'y avait toujours pas de différence entre les recombinants Env et EnvEC. Lorsque les couples de virus recombinants de sous-type C étaient présentés par des cellules dendritiques aux LT CD4+, la réplication des recombinants Env restait amoindrie. Des expériences de microscopie confocale ont montré que la faible infectivité des virus recombinants de sous-type C n'était pas due à un défaut de localisation des enveloppes dans les LT CD4+ mais à un défaut d'agrégation et de polarisation des protéines de structure Gag au site d'assemblage à la membrane cellulaire. A l'inverse, aucun défaut d'agrégation ou de polarisation de Gag n'était observé dans les LT CD4+ infectés avec les virus EnvEC équivalents, indiquant que le défaut était bien lié à gp41CT. Aucune différence n'a été observée pour les sous-types B dans les LT CD4+. Dans les MDMs, des points de colocalisation Env/Gag étaient observables aussi bien les virus de sous-type B que les virus de sous-type C, et ce indépendamment de gp41CT. La quantification des enveloppes incorporées dans les virions EnvEC et dans leur équivalent Env a montré des niveaux d'enveloppe similaires. Donc la faible réplication des virus Env de sous-type C n'est pas due à un défaut d'incorporation de Env dans le bourgeon, mais à une altération de l'agrégation et de la polarisation de Gag dépendant de gp41CT. Cela suggère l'implication d'une autre protéine intermédiaire qui serait présente dans les LT CD4+ mais pas dans les MDMs.

L'analyse des séquences protéiques des enveloppes a permis de constater que la majorité des motifs impliqués dans le trafic intracellulaire était conservés dans toutes les enveloppes. Néanmoins certains motifs récurrents dans les recombinants Env peu

infectieux ont pu être mis en évidence et leur rôle a été testé par mutagenèse : LLL₇₇₆→FIL et une insertion de 7 acides aminés (AA) dans LLP-2 et LL₈₅₆→LQ à l'extrémité C-terminale de LLP-1. Ces motifs ont été remplacés dans deux enveloppes primaires de patients (préalablement clonées) par les motifs présents dans la référence NL4.3 et dans les sous-types B. Lorsque ces mutations sont introduites seules, leur impact est dépendant du contexte génétique de Env. En revanche les doubles et les triples mutants comportant une ou deux mutations dans LLP-2 et la mutation LQ₈₅₆→LL dans LLP-1 répliquent comme les virus avec l'Env primaire ou mieux que chacun des simples mutants, suggérant que les polymorphismes dans ces deux α-hélices sont liés.

L'ensemble de nos résultats montre que la présence de polymorphismes dans gp41CT contribue à la faible capacité répliquative des virus de sous-type C, dans les LT CD4+ mais pas dans les MDMs. La conservation des signaux impliqués dans le trafic intracellulaire de Env se traduit par une distribution normale de Env dans les LT CD4+, mais les polymorphismes dans les α-hélices LLP-2/3 et LLP-1 semblent liés, suggérant des contraintes évolutives. L'agrégation des protéines Gag, en revanche, est altérée ou empêchée, suggérant l'implication d'un facteur cellulaire dans l'interaction Env/Gag dans les LT CD4+ mais pas dans les macrophages.

Résumé de thèse anglais

Entry of the human immunodeficiency virus (HIV) into target cells is mediated by the viral envelope (Env) glycoprotein ectodomain and initiates a new infectious cycle. It has thus been the focus of extensive studies. The cytoplasmic tail of Env (gp41CT) is mainly involved in Env incorporation into budding virions. It also regulates Env intracellular trafficking and interacts with a number of cellular factors. Truncations of gp41CT result in replication incompetent viruses in primary target cells and in several, but not all, T cell lines. However gp41CT has been less studied and most studies are based on the reference strain NL4.3 and mutants thereof, neglecting viral variability.

This work focuses on the impact of subtype-dependent polymorphisms within gp41CT on viral replication in primary target cells, CD4⁺ T cells (CD4⁺ TL) and monocyte-derived macrophages (MDMs). In order to avoid truncations, we used NL4.3-derived viral recombinant particles harboring patient-derived full Envs (gp160, “Env recombinant”), or that same patient-derived Env ectodomain (gp140) fused to the gp41CT of the reference strain NL4.3 resulting in a chimeric Env (“EnvEC recombinant”). Ten subtype B and twelve subtype C patient-derived Env (most widespread worldwide) were used to infect CD4⁺ TL and MDMs.

Replication of subtype C recombinant viruses was significantly lower in CD4⁺ TL compared to subtype B. Furthermore Env recombinants replicated less than their EnvEC counterpart whereas no difference was observed for subtype B Env and EnvEC pairs. In contrast, replication of subtype B and subtype C Env and EnvEC recombinants viruses was very similar in MDMs, regardless of subtype and of gp41CT. Similar differences in replication were again assessed when pairs of subtype C recombinant viruses were presented to CD4⁺ TL by dendritic cells.

Confocal microscopy revealed that low viral replication of subtype C recombinant viruses was not caused by defects in Env localization in CD4⁺ TL but resulted from altered clustering and polarization of the structural protein Gag. Infection of CD4⁺ TL with the EnvEC recombinant counterparts did not show defects in Gag clustering or polarization, highlighting that the alteration was linked to the gp41CT. Likewise, Env and Gag clusters

were readily detected for subtype B Env and EnvEC recombinants. Env-Gag colocalization spots were readily detected in MDMs infected with subtype B and subtype C recombinants, independently of the gp41CT. Env incorporation into EnvEC and Env recombinant virions was comparable. Thus low replication of subtype C Env recombinants is not due to impaired Env incorporation but to a gp41CT-dependent defect of Gag clustering and polarization, suggesting the involvement of a cellular protein which is present in LT CD4+ but not in MDMs.

Most motifs involved in intracellular trafficking were highly conserved in all Envs, but some motifs seemed to be associated with replication incompetent Envs: LLL₇₇₆ → FIL and a 7 amino acid (AA) in LLP-2 and an LL₈₅₆ → LQ motif at the C-terminal end of LLP-1. These motifs were reverted to the motifs found in subtype B gp41CTs in two subtype C patient Envs. Single mutations had a background-dependent impact on viral replication. However double or triple mutants harboring one or two mutations in LLP-2 and the LQ₈₅₆ → LL mutation in LLP-1 showed replication levels similar to the wild-type or higher levels than each single mutation suggesting that polymorphisms in these two α -helix are linked.

Our results indicate that polymorphisms in gp41CT contribute to low viral replication of subtype C Envs in CD4+ TL but not in MDMs. Preservation of intracellular trafficking motifs in Env translated into normal Env distribution in CD4+ TL, however polymorphisms in α -helix LLP-2/3 and LLP-1 seem linked, suggesting evolutionary constraints. The cell-type specific alteration of Gag clustering suggests the involvement of a cellular factor present in CD4+ TL but not in MDMs.

INTRODUCTION

1. Généralités

1.1. Découverte du virus de l'immunodéficience humaine

En juin 1981 aux Etats-Unis, le médecin Michael Gottlieb a constaté 5 cas de pneumonie à *Pneumocystis carinii* et de candidose buccale chez de jeunes patients homosexuels (1). L'apparition simultanée de maladies opportunistes, éliminées normalement de l'organisme par le système immunitaire, a conduit le médecin à la conclusion qu'un germe commun devait être responsable du développement de ces pathologies.

Dans un premier temps, l'hypothèse qui circulait parmi la population scientifique suggérait que l'agent étiologique du syndrome d'immunodéficience acquise (SIDA) devait être un rétrovirus. Or les seuls rétrovirus connus à l'époque étaient les virus T-lymphotropiques humains type 1 et 2 (HTLV-I et -II) découverts par Robert Gallo en 1980 et 1982. La découverte du virus du SIDA revient en 1983 à l'équipe de chercheurs français de l'institut Pasteur à Paris dirigé par le docteur Luc Montagnier qui furent les premiers à isoler le virus et à l'identifier en tant que lentirétrovirus (2). L'année suivante plusieurs articles confirmant l'implication de ce rétrovirus dans les cas de SIDA ont été publiés (3-6). Jusqu'en 1986, le virus étiologique du SIDA était connu sous plusieurs noms : virus T-lymphotropique humain type 3 (Lai ou HTLV-III), virus associé à la lymphoadenopathie (LAV) et accessoirement B.R.U. d'après les initiales du premier patient à partir duquel le virus a été isolé. Finalement, il a été décidé d'un commun accord que l'agent étiologique du SIDA serait qualifié de VIH pour virus de l'immunodéficience humaine.

1.2. Pandémie et origine de l'infection

1.2.1. Pandémie

En 2011, le nombre total d'infections par le VIH depuis le début de l'épidémie a été estimé à plus de 60 millions. Le nombre de d'individus vivant avec le VIH est en progression constante, atteignant 34 millions (31.4 millions – 35.9 millions) en 2011 (7)) et 1.7 millions (1.5 millions - 1.9 millions) de décès liés au SIDA ont été recensés en 2011 (7)). En trente ans, depuis les premiers cas de SIDA jusqu'à nos jours, le regard qui est porté sur cette pathologie a peu à peu évolué. En effet, après sa découverte, la pathologie était considérée comme une maladie mortelle avec une espérance de vie de 2 ans (8) tandis que maintenant grâce à l'émergence des thérapies antirétrovirales efficaces, l'infection par le VIH est entrée dans les esprits comme étant une maladie chronique, toujours mortelle, mais partiellement contrôlable. Les patients vivent plus longtemps grâce aux antirétroviraux, dans de meilleures conditions et bien que le nombre d'individus nouvellement infectés ne diminue pas, de plus en plus de patients ont accès à des traitements et le nombre de décès liés au SIDA diminue. En effet, en 2011, 8 millions d'individus avaient accès à une thérapie antirétroviral, ce qui correspond à 20 fois plus d'individus qu'en 2003. Il est aussi encourageant de voir que le nombre de nouvelles infections dans le monde décline graduellement (diminution de 20% entre 2001 et 2011 passant à 2.5 millions (2.2 millions - 2.8 millions) nouvelles infections en 2011 (7)) [FIGURE 1]. Plus de deux tiers des individus infectés par le VIH se trouvent sur le sol africain, continent d'où est originaire le VIH.

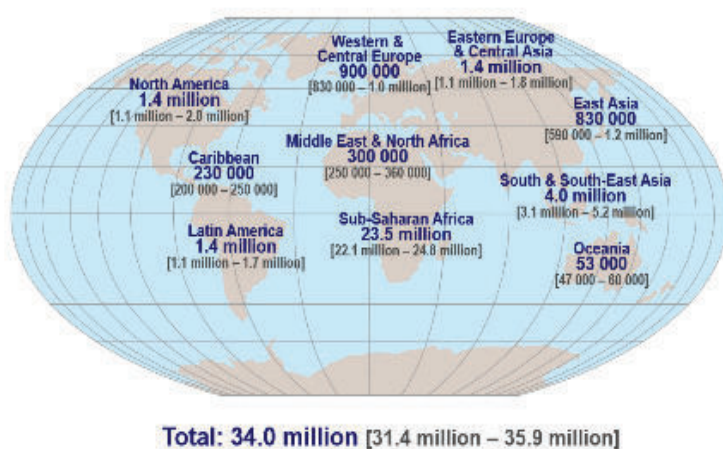


Figure 1. Estimation du nombre d'adultes et enfants infectés par le VIH en 2011 (Organisation mondiale de la santé).

1.2.2. Origine du VIH

En 1986, des scientifiques ont isolé une souche de lentivirus chez un patient de l'ouest de l'Afrique qui présentait des symptômes similaires au SIDA (9). Ce nouveau virus possédait 40% d'homologie avec le VIH (10) et a été qualifié de VIH de type 2 (VIH-2), tandis que la première souche de VIH isolée a été classifiée comme type 1 (VIH-1) ou plus communément VIH. Des études phylogénétiques ont permis de constater que le VIH-1 était plus étroitement lié au virus zoonotique de l'immunodéficience simienne (SIV) responsable de l'infection de chimpanzés (la sous-espèce de chimpanzé *Pan troglodytes troglodytes*) (11, 12) alors que le VIH-2 était apparenté au SIV infectant le singe vert mangabey (*Cercocebus atys*) (13, 14). Malgré les mécanismes de protection contre les infections inter-espèces, le virus zoonotique SIVcpz aurait été transmis du chimpanzé à l'homme et serait à l'origine du VIH-1 et le virus SIVsmm du singe vert mangabey serait l'ancêtre du VIH-2 [FIGURE 2].

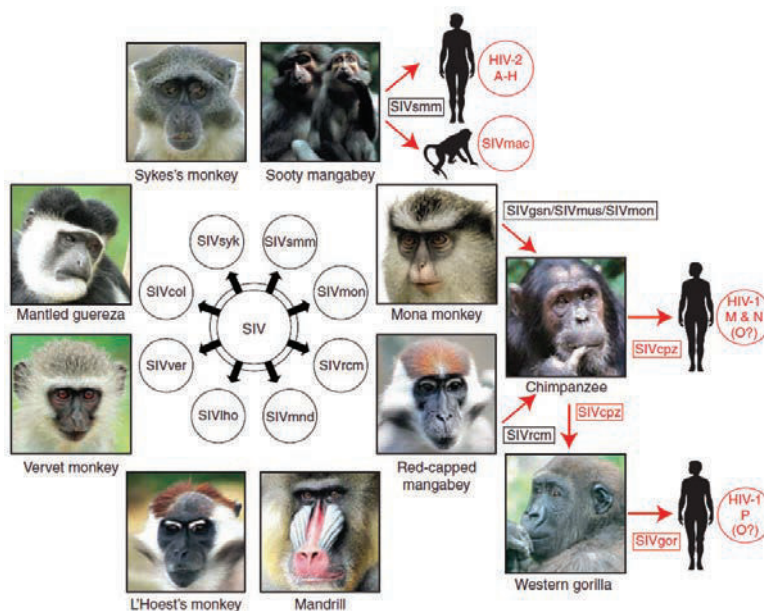


Figure 2. Origines du VIH (15).

Les données disponibles indiquent que trois événements distincts et indépendants de transmission inter-espèce ont résulté en la ramification du VIH-1 en 3 groupes : groupe M (pour Main en anglais), le groupe O (pour Outlier) et le groupe N (pour Non-M, Non-O)

(16, 17). Le groupe M représente la majorité des infections mondiales. Toutefois la variabilité génomique du VIH, et en l'occurrence du groupe M, est extrêmement importante et peut être imputée à plusieurs facteurs : le taux de réplication virale très élevé (10^{10} particules virales sont produites par jour) (18) associé à la fidélité médiocre de la polymérase virale, la transcriptase inverse (RT) qui introduit 10^{-4} à 10^{-5} erreurs par base par cycle (19) ainsi qu'à l'absence de relecture (proof-reading) (20). De plus, de nombreux événements de recombinaison génomique viennent incrémenter la variabilité du virus (21, 22). Cette hétérogénéité ne fait qu'accroître suite aux flux migratoires.

1.2.3. Classification du VIH

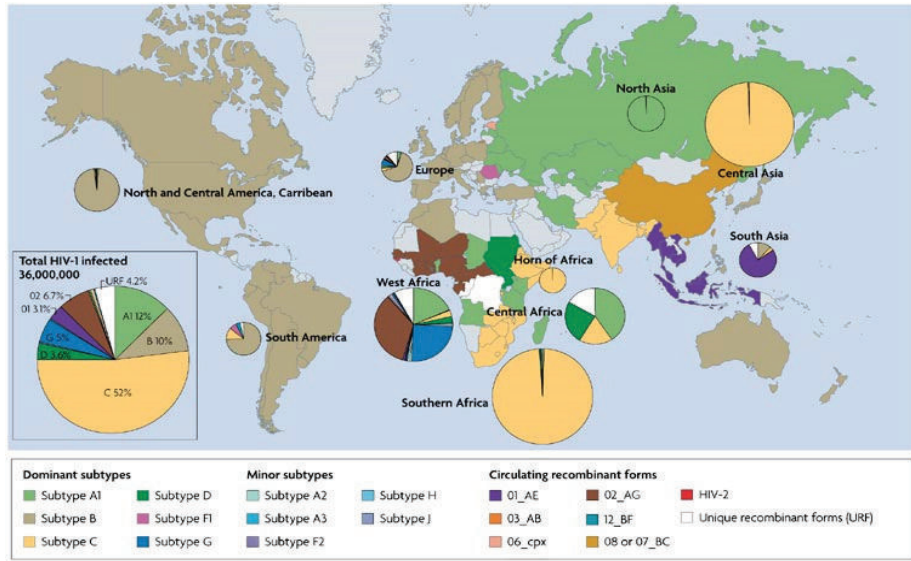
De façon générale, le VIH est répertorié comme suit : type, groupe, sous-type, sous-sous-type et formes recombinantes circulantes ou uniques. Il y a deux types de VIH : VIH-1 et VIH-2, toutefois, le VIH-2 est confiné à l'Afrique de l'Ouest alors que le VIH-1 est majoritairement répandu mondialement et représente 95% des infections (23). Pour des raisons de classification et d'épidémiologie, le VIH-1 est encore subdivisé en groupes : M (pour Major), O (pour Outlier) et N (pour Non-M, Non-O). Les virus du groupe M sont responsables de la majorité des infections dans le monde, et est à son tour subdivisé en 9 sous-types: A, B, C, D, F, G, H, J et K. Au fil du temps et de nouveaux séquençages, certains sous-types ont même été répartis en sous-sous-types: A1, A2, A3, A4, F1 et F2. Finalement des recombinaisons entre des sous-types de virus ont aussi été observées et répertoriées comme formes recombinantes circulantes (CRF) si elles étaient identifiées dans au moins 3 individus sans lien épidémiologique, sans quoi on parle de formes recombinantes uniques (URF).

Les variations génétiques entre les sous-types sont de l'ordre de 25 à 35% entre sous-types (24-26). Au sein d'un même sous-type, la diversité de la séquence des protéines virales est de 15% au niveau du gène *gag*, 10% au niveau du gène *pol*, tandis que le gène de l'enveloppe virale (*env*) présente le plus de variabilité atteignant 24% (27). Au niveau d'un même organisme, on retrouve aussi de grandes différences génétiques entre les virus, l'on parle alors de quasi-espèces.

L'hétérogénéité se retrouve aussi au niveau de la distribution géographique du VIH et elle est maximale en Afrique Centrale, épicode de l'épidémie [FIGURE 3]. Le sous-type B correspond à la première souche de VIH isolée et il s'agit du sous-type le plus répandu en Amérique du Nord, en Europe de l'Ouest et en Australie. La majorité des connaissances acquises sur le VIH ont été obtenues à partir d'études sur des souches de référence de sous-type B. Néanmoins, on considère à l'heure actuelle qu'environ 90% des infections VIH mondiales sont causées par des sous-types non-B et des formes recombinantes. C'est le sous-type C qui est le plus répandu (environ 48% des infections mondiales)(28). Il est prédominant en divers pays africains (Afrique du Sud, Namibie, Botswana, Zambie, Zimbabwe, Mozambique, Burundi, Tanzanie, Ethiopie, Somalie et dans une partie du Soudan) ainsi qu'en Inde. Le sous-type A représente 12% des infections et sévit en Afrique Centrale-Est (Rwanda, Kenya, Uganda, Tanzanie). Le sous-type D est répandu en Afrique Centrale et en Afrique de l'Est. Le sous-type F est majoritaire en Europe de l'Est comme la Roumanie mais est aussi retrouvé en Amérique du Sud. Les sous-types G, H, J et K ont été décrits au Burkina Faso, au Mali, au Niger, en Côte d'Ivoire, au Gabon et en République Démocratique du Congo par où ils se sont répandus en Europe du Sud ainsi qu'en Asie.

22% des infections mondiales au VIH sont dues aux formes recombinantes (CRF) du virus (28). Les CRF les plus répandus sont le CRF01_AE (l'ancien sous-type E) et le CRF02_AG étant respectivement présent chez 5% et 8% de la population mondiale infectée au VIH-1. Le CRF_01AE est prédominant en Thaïlande et au Vietnam tandis que le CRF02_AG est très fréquent en Afrique de l'ouest (29).

L'épidémie provoquée par le VIH-2 est restreinte à l'Afrique de l'Ouest. Depuis les premiers cas diagnostiqués en 1978, huit sous-types (A-H) du virus VIH-2 ont été décrits parmi lesquels les groupes A et B sont les plus prévalents (30).



Nature Reviews | Microbiology

Figure 3. Distribution géographique mondiale des différents sous-types de VIH (31).

2. Le virus de l'immunodéficience humaine (VIH)

2.1. Le Génome

Le génome du VIH est constitué de deux copies d'ARN simple brin de polarité positive d'une longueur approximative de 9 kb. Contrairement aux rétrovirus simples qui ne possèdent que trois gènes principaux (*gag*, *pol* et *env*), le VIH est constitué de neuf cadres de lecture qui lui permettent de coder 15 protéines virales (32) [FIGURE 4].

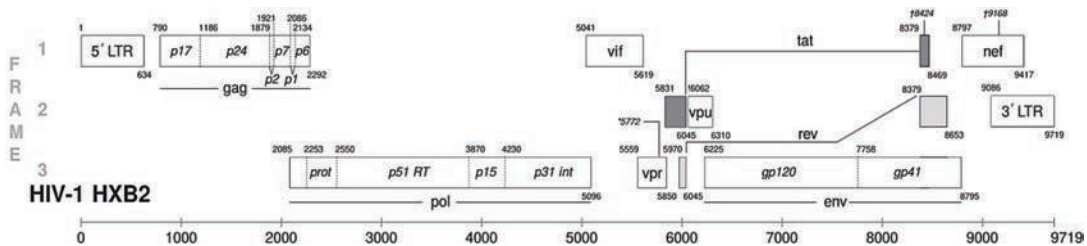


Figure 4. Représentation du génome viral VIH-1.

Les gènes viraux

Les protéines structurelles et enzymatiques sont codées par les trois gènes principaux.

- Le gène **gag** (« group specific antigen ») code le précurseur polypeptidique Pr55^{Gag} qui est clivé par la protéase virale (PR), générant les protéines de structure de la matrice (p17 ou MA), la capsid (p24 ou CA), les protéines p7, p6, ainsi que les peptides séparateurs (« spacer ») Sp2 et Sp1.
- Le gène **pol** (« polymerase ») code les protéines enzymatiques du virus sous forme d'un long précurseur polyprotéique Pr160^{GagPol} issu d'un décalage de -1 du cadre de lecture au niveau du ribosome. Ce décalage survient dans environ 5% des événements de traduction de Pr55^{Gag}, générant environ 20 précurseurs Pr55^{Gag} pour un précurseur Pr160^{GagPol} (33). Le clivage du précurseur par la protéase virale est un processus hautement ordonné et réglé (34). Il libère la protéase par un mécanisme d'autocatalyse, ainsi que l'intégrase (IN) et la transcriptase inverse (RT).
- Le gène **env** (« envelope ») code le précurseur polyprotéique glycosylé gp160, qui est clivé par la protéine cellulaire furine en deux sous-unités gp120 et gp41. La sous-unité de surface hautement glycosylée gp120 est liée de façon non covalente à gp41, qui est ancrée dans la membrane plasmique. Le complexe gp120-gp41 est exprimé à la surface du virion et des cellules infectées sous forme trimérique.

Le VIH possède aussi deux gènes régulateurs (*tat* et *rev*), ainsi que des gènes codant des protéines auxiliaires (*nef*, *vif*, *vpr* et *vpu*).

- Le gène **tat** (« transactivator of transcription ») code la protéine Tat à partir de deux exons situés dans deux cadres de lecture différents. Le premier exon code les acides aminés 1 à 72 et le deuxième exon code les acides aminés 73 à 101.
- Le gène **rev** (« regulation of virion expression ») code la phosphoprotéine virale Rev à partir de deux exons chevauchant les exons de *tat* mais débutant dans un autre cadre de lecture.
- Les gènes **vif** (« viral infectivity factor »), **vpr** (« viral protein R ») et **vpu** (« viral protein U ») sont situés entre les gènes *pol* et *env*, et exploitent d'autres cadres de lectures, alors que le gène **nef** (« negative factor ») débute à la fin de *env*. Les protéines auxiliaires codées par ces gènes ne sont pas indispensables à la réplication virale mais

favorisent la réplication et l'infectivité virales par plusieurs mécanismes et interviennent dans l'échappement au système immunitaire en contrant des facteurs de restriction intrinsèques (**voir section 4.3.3**).

Les séquences LTR

De part et d'autre du génome viral sont situées des séquences non codantes identiques appelées « long terminal repeats » (LTR) qui permettent l'intégration du génome viral dans le génome cellulaire. Les LTRs ont une longueur d'environ 640 nucléotides (nt) et sont constitués de trois régions : la séquence U3 en 3', la séquence R et la séquence U5 en 5' [FIGURE 5]. Le LTR 5' sert également de promoteur viral régulant la transcription des gènes viraux.

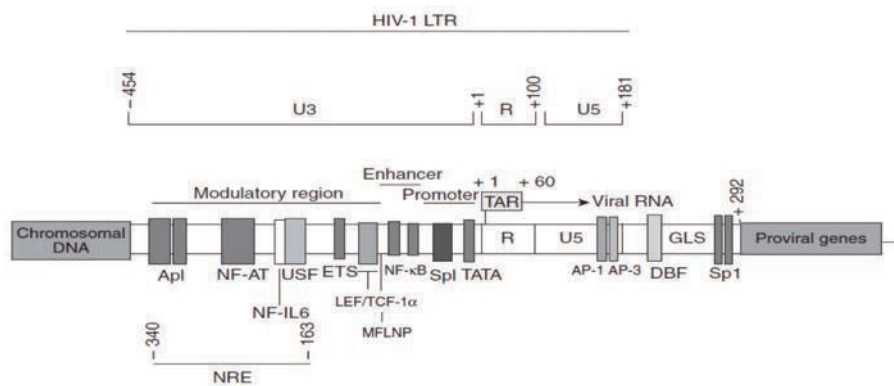


Figure 5. Représentation du LTR 5' du VIH sous-type B. Mise en évidence des 3 séquences du LTR 5' et indication des sites de liaison aux facteurs de transcription (35).

La **séquence U3** est composée de trois régions : la région promotrice (nt -78 à -1), la région enhancer (nt -105 à -79) et la région modulatrice. La région promotrice contient une TATAA-box et trois sites Sp1, nécessaires à l'initiation de la transcription. La région enhancer contient deux sites NF-κB (un troisième site NF-κB est présent dans les LTRs des virus de sous-type C, alors que le sous-type E ne présente qu'un site NF-κB (36, 37)). La région modulatrice permet la liaison de plusieurs facteurs de transcription cellulaires (tels que AP1 et NF-AT) (38).

La transcription du génome viral débute au niveau de la **séquence R**. Cette dernière adopte des structures secondaires en épingle à cheveux qui permettent de réguler la

transcription. Ainsi, la présence de l'élément TAR (« Transactivation-responsive element ») situé entre les nt +1 à +60 permet l'activation de la transcription du génome viral grâce à l'interaction avec la protéine virale transactivatrice Tat, ainsi que d'autres facteurs de transcription cellulaires (39). Enfin, il y a aussi un signal de polyadénylation (AAUAAA) qui signale la fin de la transcription dans le LTR3'.

La **séquence U5** présente aussi des structures secondaires en forme d'épingle à cheveux: la région PBS (« Primer binding site ») qui permet la fixation de l'ARN de transfert lysine (ARN^{t_{Lys,3}}) servant d'amorce lors de la transcription inverse de l'ARN en ADN viral complémentaire (+) (40), et les structures DIS (« Dimerization initiation site »), SD et PSI (ψ) sont impliquées respectivement dans la dimérisation de l'ARN, l'épissage et l'encapsidation des brins d'ARN viral dans le virion bourgeonnant [FIGURE 6]. Le rôle de ces sites et structures sera abordé plus en détail dans le chapitre détaillant le cycle de réplication du virus.

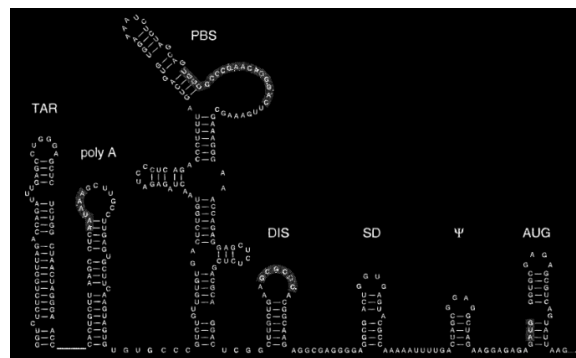


Figure 6. Représentation de la séquence R et de la région 5' non-traduites. Les structures TAR, poly A, PBS, DIS, SD et PSI sont indiquées. Image modifiée à partir de (41).

Autres séquences régulant la réplication virale

Le génome viral contient également d'autres signaux importants parmi lesquels les séquences 3'PPT (« 3' Polypurine tract ») située dans le LTR3' et cPPT (« central polypurine tract ») situé dans le gène *pol*. Ces séquences sont riches en purines et interviennent lors de la transcription inverse. Suite à la synthèse du brin d'ADN(-) par la transcriptase inverse, l'activité RNaseH de la transcriptase inverse dégrade l'ARN viral pour ne laisser que les séquences 3'PPT et cPPT qui serviront d'amorce à la synthèse du brin d'ADN(+) (42).

Une autre séquence importante dans le génome viral est la séquence RRE (« Rev responsive element ») située au niveau du gène *env*. Elle permet l'interaction avec la protéine virale Rev afin d'exporter l'ARNm viral nouvellement synthétisé du noyau jusqu'au cytoplasme où a lieu la traduction (43). Etant synthétisée très tôt lors du cycle viral, la protéine Rev peut réguler l'expression de protéines virales précoces et tardives.

2.2. Structure du virus

Le VIH est une particule de forme sphérique (44) d'un diamètre d'environ 100 à 120 nm. Le virion est entouré d'une membrane phospholipidique provenant de la membrane cellulaire de la cellule d'où le virion a bourgeonné. La glycoprotéine d'enveloppe virale transmembranaire gp41 est ancrée dans la membrane et lie de façon non-covalente la sous-unité de surface gp120. Une couche protéique constituée de protéines de matrice (MA ou p17) borde la membrane et entoure la capsid (CA ou p24). Cette dernière est de forme conique et renferme les deux molécules d'ARN génomique viral associées à la nucléocapside (NC ou p7) ainsi que des enzymes virales (transcriptase inverse, protéase, intégrase) [FIGURE 7].

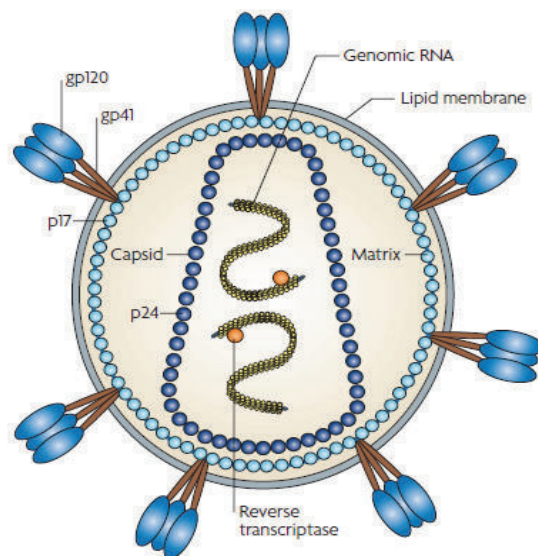


Figure 7. Représentation schématique de la structure du VIH (45).

2.3. Les protéines virales

2.3.1. Les protéines de structure

Parmi les protéines de structure, on compte la matrice, la capside et la nucléocapside toutes issues du précurseur Pr55^{Gag}, ainsi que le complexe glycoprotéique de l'enveloppe. Le virion peut incorporer également de façon sélective et spécifique des protéines d'origine cellulaire, parmi lesquelles les molécules du complexe majeur d'histocompatibilité (CMH) et les molécules d'adhésion ICAM-1, ainsi que plusieurs autres protéines cellulaires (46).

La matrice (MA ou p17) : La matrice correspond à la région N-terminale du précurseur Pr55^{Gag}. Dans le virion immature, Pr55^{Gag} est associé à la face interne de la membrane virale par l'intermédiaire du groupement myristoyl de la matrice (47-52). Lors de l'assemblage de nouveaux virions à la membrane cellulaire, la matrice est responsable d'acheminer les précurseurs Pr55^{Gag} et Pr160^{Gag-Pol} jusqu'au site d'assemblage dans les radeaux lipidiques à la membrane cellulaire (53, 54) et recrute l'enveloppe virale en interagissant avec la portion intracytoplasmique de la sous-unité gp41 et permet ainsi son incorporation dans le virion (55-65). Le clivage du précurseur par la protéase virale lors de la maturation du virion résulte en la séparation de la protéine de 17 kDa qui reste associée à la membrane. Cette séparation confère au virion sa plasticité, et par là, son pouvoir infectieux (66-69). Dans la cellule nouvellement infectée, la matrice semble aussi faciliter le transport de l'ADN proviral dans le noyau conjointement avec l'intégrase (70).

La capside (CA ou p24): Située en aval de la matrice dans Pr55^{Gag}, la capside est aussi libérée lors du clivage par la protéase virale dans le virion en cours de maturation. La matrice s'assemble en hexamères de trimères (71-73). La capside est constituée de deux domaines, le domaine N-terminal et le domaine C-terminal, qui interviennent dans l'oligomérisation de Pr55^{Gag} ainsi que dans l'assemblage du virion (74, 75). Dans le virion mature, la capside forme une structure conique constituée de 1000 à 2000 monomères de capside associées en forme de treillis. Cette structure renferme l'ARN viral associé à la nucléocapside, ainsi que quelques molécules de transcriptase inverse et d'intégrase. Ainsi la dissociation de la capside après la fusion des membranes virale et cellulaire lors de l'entrée virale permet le relargage dans le cytoplasme du génome viral et des enzymes virales nécessaires à un nouveau cycle de réplication (76).

La nucléocapside (NC ou p7) : La nucléocapside se situe en aval de la capsid sur Pr55^{Gag} et est libérée lors de la maturation du virion. Il s'agit d'une petite protéine chaperone possédant deux domaines en doigt de zinc permettant de lier spécifiquement l'ARN viral non-épissé au niveau de la séquence PSI (ψ) du LTR viral (77). Cette interaction stabilise la dimérisation des ARN viraux et assure la sélectivité des ARN lors de l'assemblage (78, 79) et la condensation facilitant l'empaquetage de l'ARN génomique (80). La nucléocapside est également impliquée dans le transfert du second brin lors de la reverse transcription permettant la liaison avec l'ARN_t^{Lys,3} (79, 81).

La protéine p6 : La protéine p6 correspond aux 51 acides aminés C-terminaux de Gag. Elle intervient dans le mécanisme d'encapsidation de la protéine accessoire Vpr (82) ainsi que dans l'exocytose de la particule virale en recrutant la machinerie cellulaire lors du bourgeonnement.

L'enveloppe (Env) : L'enveloppe est un complexe trimérique hautement glycosylé ancré dans la membrane phospholipidique des virions ou des cellules infectées. Chaque virion exprime 7 à 14 enveloppes à sa surface (83-85). Chaque monomère est composé d'une glycoprotéine de surface (gp120) liée de façon non-covalente à une glycoprotéine transmembranaire (gp41). Elle permet l'entrée du virus dans les cellules cibles. L'enveloppe constituant le sujet principal de ce travail, sa structure et ses fonctions seront détaillées dans la **section 6**.

2.3.2. Les protéines enzymatiques

Toutes les protéines enzymatiques du VIH sont codées par le gène *pol* sous forme de précurseur Pr160^{GagPol}.

La protéase (PR) : La protéase est un homodimère dont le site catalytique se trouve à la jonction entre les deux monomères (86, 87). La protéase joue un rôle primordial dans la maturation et l'infectivité du virion. En effet, le virion bourgeonnant immature est très peu infectieux (66, 67). Le clivage protéolytique des précurseurs Pr55^{Gag} et Pr160^{GagPol} de virion immature par la protéase confère le caractère infectieux au virus (66, 67). Ce processus engendre la génération de nouvelles protéines de protéase par un mécanisme d'autocatalyse (88).

La transcriptase inverse (RT) : La transcriptase inverse est un hétérodimère constitué des sous-unités p66 et p51 (89). Lors du processus de maturation du virion, la protéase virale clive Pr160^{GagPol} générant p66, qui est à son tour clivée pour engendrer p51. Ces sous-unités s'assemblent pour former la transcriptase inverse. La RT catalyse la synthèse d'ADN complémentaire à partir de l'ARN viral génomique ainsi que la synthèse du brin d'ADN(+) à partir du brin complémentaire (90, 91) générant un ADN double brin. La sous-unité p66 assure également l'activité RNaseH qui dégrade l'ARN viral en 3' au fur et à mesure de la transcription inverse (92). Au niveau de l'hétérodimère, p66 adopte une conformation « ouverte » qui facilite la liaison entre l'ARN génomique et les ARN de transfert, tandis que la conformation « fermée » de p51 stabilise le complexe hétérodimère acide nucléique (93).

L'intégrase (IN) : L'intégrase est la troisième enzyme libérée lors du clivage de Pr160^{GagPol}. Elle joue un rôle clé dans la répllication du VIH en transportant l'ADN viral dans le noyau de la cellule cible et catalysant l'intégration de l'ADN viral double brin dans le génome de l'hôte (94). L'intégrase est constituée de quatre sous-unités identiques de 32 kDa. La protéine subit un grand nombre de modifications post-traductionnelles (ubiquitination, SUMOylation, acétylation et phosphorylation) (95, 96) et interagit avec un grand nombre de protéines cellulaires pour former le complexe de pré-intégration. Trois domaines structurels distincts sont décrits au niveau de l'intégrase : le domaine N-terminal (résidus 1-50) fixant le zinc, domaine central catalytique et le domaine C-terminal (résidus 212-288). Le domaine N-terminal participe à la stabilité du tétramère par l'intermédiaire de la liaison au zinc (97). Le domaine central catalyse l'intégration du génome viral dans le génome de la cellule hôte (98). Enfin, le domaine C-terminal peut lier l'ADN de façon non-spécifique améliorant la stabilité du complexe intégrase-ADN (99).

2.3.3. Les protéines régulatrices

Les premières ARN viraux synthétisés dans la cellule infectée après l'intégration du génome viral sont les ARN codant les protéines régulatrices Tat et Rev, ainsi que Nef.

La protéine Tat : La protéine fait 14 kDa à 16 kDa et est localisée dans le noyau où elle accomplit sa tâche de transactivateur transcriptionnel des gènes viraux, mais aussi

cellulaires dérégulant l'expression de certaines protéines (100, 101). Lors de la première étape de la transactivation, Tat se fixe sur la séquence TAR située sur le promoteur LTR du génome viral (102) [FIGURE 5] et stabilise le complexe de facteurs de transcription cellulaires autour de l'ARN polymérase II (101, 103). La protéine Tat peut aussi être relarguée par la cellule infectée et pénétrer dans de nouvelles cellules où le facteur de transcription peut exercer son activité de transactivateur et perturber ainsi l'équilibre des protéines cellulaires (104).

La protéine Rev : La protéine Rev est une phosphoprotéine de 18 kDa qui est principalement localisée dans le noyau cellulaire, mais est capable de faire la navette entre le noyau et le compartiment cytoplasmique. Seuls les ARN épissés sortent du noyau à l'aide de la machinerie cellulaire, les ARN non-épissés ou partiellement épissés sont préservés de la dégradation dans le noyau grâce à la fixation de la protéine Rev sur l'élément RRE situé dans *env*. Cette interaction permettant l'exportation des complexes Rev-ARN vers le cytoplasme (105). La région N-terminale de la protéine est riche en arginines, elle abrite le signal de localisation nucléaire et lie la séquence RRE des ARN viraux. De part et d'autre du signal de localisation nucléaire sont des régions hydrophobes responsables de la multimérisation de Rev (106). La région C-terminale de Rev est riche en leucines et contient le signal d'exportation nucléaire (107).

2.3.4. Les protéines auxiliaires

Les protéines auxiliaires contribuent par plusieurs mécanismes à créer un environnement le plus favorable possible à la réplication virale. Le VIH possède quatre protéines auxiliaires : Vif, Vpr, Vpu et Nef qui interagissent avec une multitude de protéines virales et cellulaires.

La protéine Vif : Cette protéine joue un rôle essentiel dans l'infectivité du virus. Chaque virion contient environ 30 à 100 protéines Vif qui sont libérées dans le cytoplasme de la cellule nouvellement infectée (108). Vif peut ainsi contrer l'activité antivirale du facteur de restriction cellulaire APOBEC3G en induisant sa polyubiquitination suivi de sa dégradation par le protéasome (109). Vif semble aussi être impliqué dans la structure de la capsid lors de l'assemblage et/ou de la maturation (110).

La protéine Vpr : La protéine virale Vpr est introduite dans le virion bourgeonnant grâce à son interaction avec l'extrémité C-terminale de Pr55^{Gag}. Cette protéine de 14 kDa est impliquée dans le transport du complexe de pré-intégration dans le noyau de cellules ne proliférant pas (111). De plus, Vpr peut interagir avec la protéine virale Tat et ainsi moduler la transcription (112). Enfin, Vpr bloque le cycle cellulaire en phase G2 (113) et possède une activité pro-apoptotique dans la cellule infectée (114).

La protéine Vpu : Cette protéine de 16 kDa est constituée d'un domaine N-terminal transmembranaire et d'un domaine C-terminal cytoplasmique. Vpu induit la dégradation du récepteur CD4 par le protéasome en ubiquitinant la partie C-terminale du récepteur, diminuant de cette façon l'interaction entre le récepteur et le précurseur gp160 au niveau du réticulum endoplasmique (115-117). Vpu joue également un rôle primordial dans le bourgeonnement du virion en contrant le facteur de restriction tetherin (BST-2) (118-124). Contrairement aux autres protéines auxiliaires, Vpu n'est pas incorporée dans le virion.

La protéine Nef : La protéine Nef est incorporée dans le virion et fait partie des premières protéines virales synthétisées lors d'une nouvelle infection. Il s'agit d'un homodimère myristoylé de 27 kDa sans activité enzymatique et possède un domaine central riche en prolines. Nef est impliquée dans la pathogénèse du VIH (125). Nef interagit avec plusieurs partenaires cellulaires et arbore toute une palette de fonctions cruciales pour la prolifération du virus. Cette protéine est responsable de la régulation négative des molécules du complexe majeur d'histocompatibilité de classe 1 (CMH-I) dans les cellules infectées, ce qui permet d'échapper au système immunitaire et favorise la propagation du virus (126, 127). De plus, Nef est impliquée dans la régulation négative des récepteurs CD4 à la surface de la cellule, empêchant la fixation de virions néo-synthétisés à la surface de la cellule productrice (128). Nef diminue également l'expression d'autres protéines cellulaires telles que le récepteur CD3, le récepteur CD28, CTLA-4 et les CMH-II (129-131). L'absence de Nef réduit considérablement l'infektivité du virus. Il est intéressant de constater que les individus infectés par un virus défectueux au niveau du gène Nef ne progressent pas ou progressent lentement vers le SIDA (132, 133).

3. Le cycle viral

Le cycle de réplication du VIH peut être schématiquement divisé en 6 étapes [FIGURE 8] : l'attachement du virus à la cellule cible et l'entrée du virus, la décapsidation et la transcription inverse du génome viral, le transport nucléaire de ce dernier et son intégration dans le génome de la cellule, la production de protéines virales par la machinerie cellulaire et l'assemblage à la membrane de la cellule, le bourgeonnement, et enfin, une dernière étape de maturation transformant les virions immatures non infectieux en virions infectieux capables d'infecter de nouvelles cellules et de recommencer le cycle.

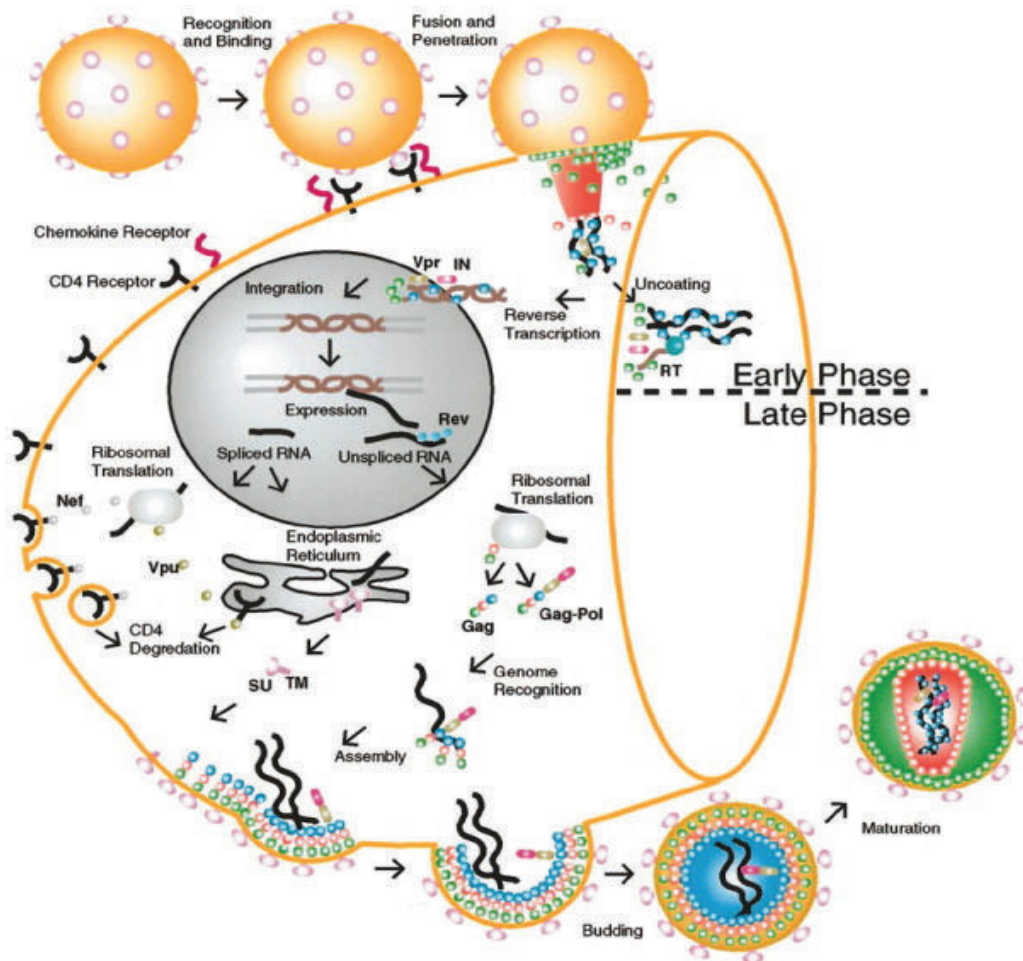


Figure 8. Représentation schématique de toutes les étapes du cycle viral du VIH (134).

3.1. Attachement et entrée

L'infection par le VIH est initiée par l'interaction entre la sous-unité de surface de l'enveloppe, gp120, et le récepteur cellulaire CD4 à la surface des cellules cibles (135, 136). Cette interaction induit un changement de conformation au niveau de gp120 exposant la boucle hypervariable V3. Cette dernière peut alors interagir avec un co-récepteur cellulaire. Les principaux co-récepteurs sont CCR5 et CXCR4, des protéines à 7 domaines transmembranaires couplés aux protéines G (137-141). Le choix du co-récepteur, et donc la séquence de la boucle V3 qui interagit avec le co-récepteur, définit le tropisme viral : ainsi les virus qui utilisent le co-récepteur CCR5 sont nommés « R5 » et les virus qui utilisent CXCR4 sont nommés « X4 ». Certains virus peuvent aussi utiliser les deux co-récepteurs (virus dual R5X4) (138, 142). L'interaction avec le co-récepteur induit une nouvelle modification conformationnelle au niveau de la sous-unité transmembranaire gp41 qui expose le peptide de fusion hydrophobe situé à l'extrémité N-terminale (143, 144). Le peptide de fusion peut alors s'insérer dans la membrane de la cellule cible dont il perturbe la stabilité (145). En aval du peptide de fusion se trouvent les deux régions hydrophobes HR1 et HR2 (Heptad Repeat) (146, 147). Ces structures forment des hélices α connectées par un pont disulfure au sein d'une boucle hydrophobe. Les trois domaines HR des trimers Env s'assemblent pour former une structure en faisceau. Lors de la fusion, les hélices HR2 se replient de façon antiparallèle le long des sillons hydrophobes formés par le faisceau d'hélices HR1. Ce processus résulte en une structure extrêmement stable formée de six hélices (six helix bundle ou 6-HB) (146, 147) [FIGURE 9]. Cette interaction conduit au rapprochement et à la fusion des membranes et au relargage de la capsid avec le matériel génomique viral dans le cytoplasme de la cellule. Une étude menée par Miyauchi *et al.* a mis en évidence que l'entrée du VIH serait dépendante d'une étape d'endocytose du virus impliquant la glycoprotéine d'enveloppe et la dynamine, alors que la fusion des membranes virale et cellulaire à la membrane plasmique reste abortive (148).

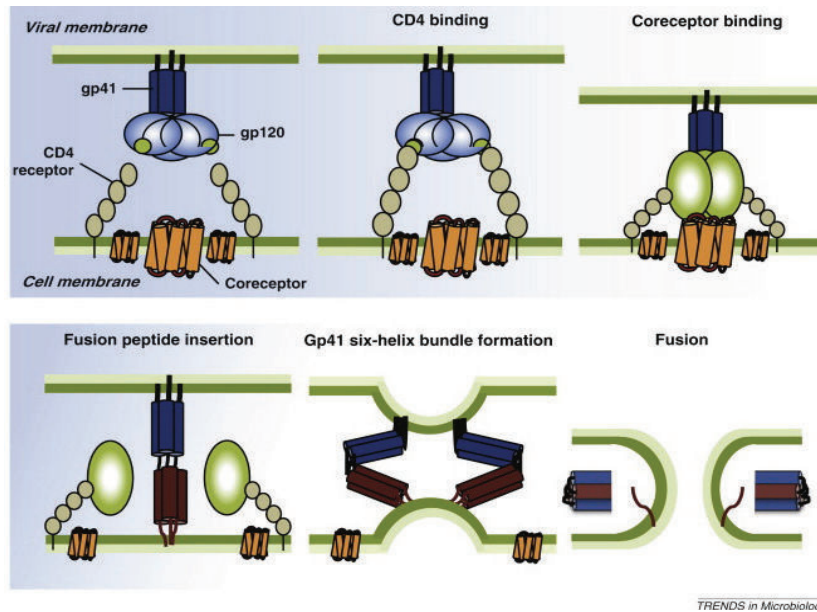


Figure 9. Représentation schématique du processus d'attachement et de fusion du virus (149).

3.2. Reverse transcription

Dans le cytoplasme, la capside se désagrège en migrant le long du cytosquelette d'actine et la transcription inverse débute simultanément (150). Un $\text{ARN}_t^{\text{Lys},3}$ incorporé dans le virion se fixe à l'ARN viral au niveau du site PBS dans la séquence LTR du génome (151, 152). Cet $\text{ARN}_t^{\text{Lys},3}$ sert d'amorce à la RT pour initier la synthèse du brin d'ADN complémentaire (-) à partir de l'ARN viral (40) [FIGURE 10]. La transcription inverse débute avec l'assemblage du complexe de transcription inverse (« reverse transcription complex », RTC), comprenant l'ARN viral associé à l' $\text{ARN}_t^{\text{Lys},3}$, la transcriptase inverse et d'autres protéines virales dont la nucléocapside, l'intégrase et des protéines cellulaires (81, 153-155). Les séquences U5 et R situées en amont du site PBS sont synthétisées en premier. Simultanément, l'activité RNaseH de la RT dégrade l'ARN viral ayant servi de matrice pour la synthèse du brin d'ADN(-) contenant les séquences U5 et R. Seule la séquence d'ARN viral PBS lié à l' $\text{ARN}_t^{\text{Lys},3}$ échappe à la dégradation. La séquence R du brin d'ADN(-) néosynthétisé s'hybride à la séquence R complémentaire de la région 3' de l'ARN viral (156-158) et permet de continuer l'élongation du brin d'ADN(-) jusqu'en 5' (157). Entre temps, la RNaseH continue de dégrader l'ARN viral au sein des complexes ARN-ADN à l'exception

des séquences d'ARN riches en purines (séquences PPT). Le génome viral contient deux séquences PPT, l'une en 3' au niveau de la région U3 (3'PPT) et l'autre dans *pol* (cPPT). Ces séquences servent d'amorce à la synthèse du brin d'ADN(+) (159, 160). La RT commence la synthèse du brin d'ADN(+) au niveau du 3'PPT jusqu'à l'ARN_t^{Lys3} synthétisant la séquence PBS. La synthèse du brin (+) à partir de cPPT débute par après et s'étend jusqu'à l'extrémité 3'. Cette élongation a pour conséquence le déplacement du brin (+) néosynthétisé qui vient s'hybrider sur l'extrémité 5' du brin d'ADN(-) ce qui permet la synthèse de la partie manquante du brin d'ADN(+). Lorsque la synthèse de l'ADN proviral double brin est complétée, le RTC se convertit en complexe de préintégration (« preintegration complex », PIC) qui est intervenant dans l'intégration de l'ADN proviral dans le génome de la cellule infectée.

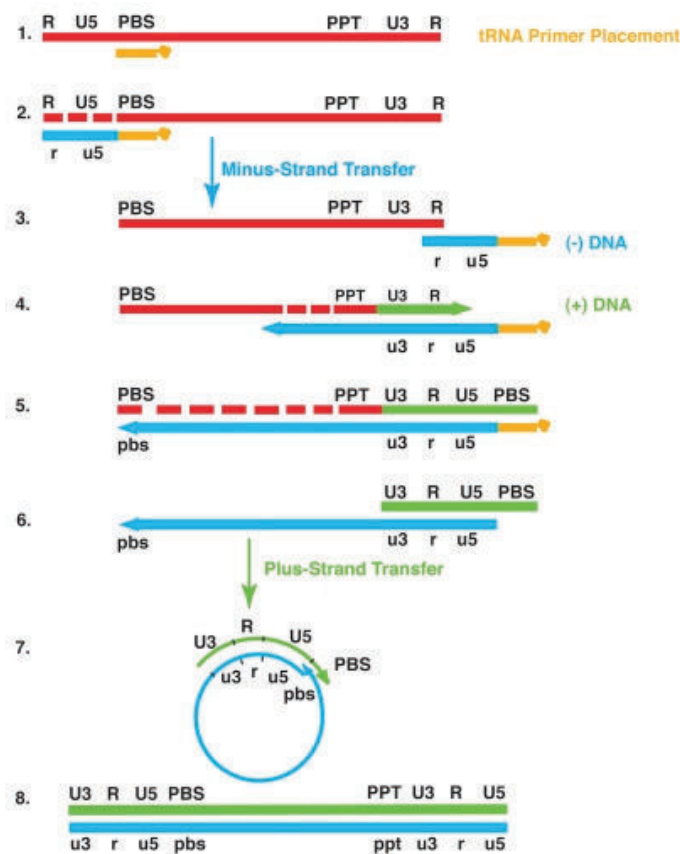


Figure 10. Illustration schématique de l'étape de transcription inverse (161).

3.3. Intégration

L'intégrase est la protéine clé de l'étape d'intégration. Elle forme le PIC et lie les extrémités LTR de l'ADN proviral néosynthétisé. Avant de pouvoir insérer l'ADN proviral dans le génome humain, l'intégrase doit cliver les dinucléotides GT aux extrémités 3' des brins d'ADN proviral. De part et d'autre de l'ADN proviral sont ainsi exposés les dinucléotides CA avec des groupements hydroxyles indispensables à l'intégration dans le génome de la cellule (162).

Une fois cette étape effectuée, le PIC peut quitter le cytoplasme et migrer vers le noyau. Les protéines virales Vpr, matrice et intégrase incluses dans le PIC contiennent des signaux d'importation nucléaire favorisant le processus de transport à travers les pores nucléaires qui est effectué par la machinerie cellulaire. Le cPPT favorise également l'import nucléaire du PIC (163-168).

Arrivé dans le noyau, l'intégrase interagit avec le facteur de croissance dérivé de l'épithélium du cristallin (LEDGF) permettant l'attachement à l'ADN génomique (169) et catalyse la réaction d'intégration de l'ADN proviral dans l'ADN cellulaire (170). Les groupements hydroxyles situés aux extrémités de l'ADN proviral orchestrent la destruction des liaisons phosphodiester du génome permettant à l'ADN proviral de s'insérer grâce à ses extrémités 3'. Les dinucléotides CA non-appariés en 5' qui persistent après l'insertion sont éliminés par les enzymes cellulaires, tandis que des régions monobrin de 5 nucléotides de chaque côté de l'insertion sont réparées par des enzymes cellulaires (171, 172) **[FIGURE 11]**. L'intégration de l'ADN proviral survient préférentiellement au sein de gènes actifs au niveau de l'euchromatine où les gènes sont plus accessibles (173). Par la suite, le provirus détourne la machinerie cellulaire pour exprimer les gènes viraux et produire les protéines virales.

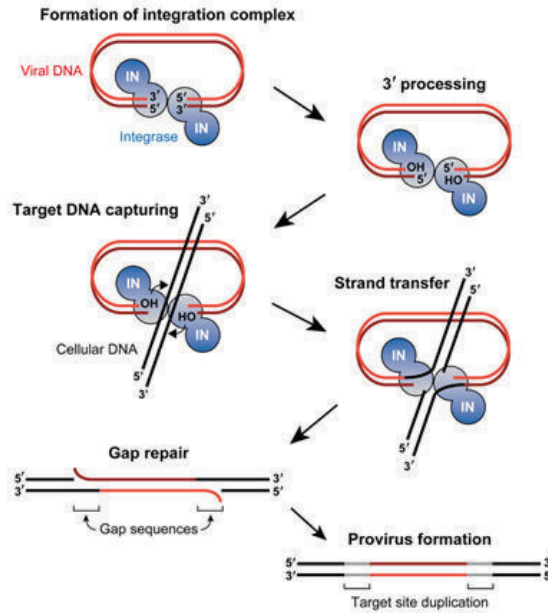


Figure 11. Schéma de l'intégration du matériel génomique virale dans le génome de la cellule infectée (174).

3.4. Transcription et synthèse de protéines virales

La transcription virale est effectuée entièrement par l'ARN polymérase II cellulaire, toutefois la régulation de l'expression des gènes viraux dépend de plusieurs facteurs cellulaires et viraux. L'environnement génétique ainsi que les modifications épigénétiques de l'ADN proviral influencent l'expression des gènes viraux (103, 175, 176). Le niveau d'activation cellulaire de la cellule infectée a aussi un impact sur le niveau de transcription (177), en effet les facteurs de transcription cellulaires NF- κ B, NF-AT et AP-1 peuvent activer la transcription virale à partir du promoteur LTR5' (178). Deux sites de liaison pour NF- κ B sont présents dans la région enhancer du LTR5', ces mêmes sites peuvent aussi lier NF-AT, toutefois les sites Sp1 situés dans le LTR permettent d'augmenter l'activité transcriptionnelle de NF- κ B (179). Bloquer le facteur de transcription NF- κ B réduit de la synthèse de protéines virales (180).

L'élément clé de la régulation est toutefois la protéine régulatrice Tat qui intervient dans un premier temps au niveau du remodelage de la chromatine facilitant l'accessibilité

au LTR5' et augmentant ainsi la transcription (181). D'autre part, Tat fixe la séquence TAR située dans la séquence R de l'ARN viral (102) et interagit avec les protéines cellulaires cycline T1 et kinase CDK9 pour hyperphosphoryler l'ARN polymérase II, ce qui favorise l'élongation de l'ARNm (39, 101). L'ARNm néosynthétisé correspond au génome viral dans son entièreté. Des mécanismes d'épissage alternatifs engendrent l'apparition de divers ARNm. Les ARNm complètement épissés sont les premiers à être produits. Ils correspondent aux protéines de régulation Tat et Rev ainsi qu'à la protéine Nef. L'exportation de ces ARNm hors du noyau vers le cytoplasme permet la synthèse des protéines virales correspondantes. Les ARNm partiellement épissés codent les protéines accessoires Vpr, Vpu et Vif, ainsi que le précurseur de l'enveloppe. Enfin, les ARNm non épissés codent les précurseurs Pr55^{Gag} et Pr160^{GagPol} et servent également de génome viral pour les nouveaux virus bourgeonnants.

Les ARNm partiellement épissés ou non épissés sont normalement retenus dans le noyau jusqu'à leur épissage complet ou leur dégradation. C'est la protéine virale Rev qui permet l'exportation de ces ARNm vers le cytoplasme. En effet, Rev reconnaît la séquence RRE présente sur les ARNm partiellement épissés ou non épissés. La liaison de Rev à la séquence RRE entraîne l'oligomérisation de plusieurs protéines Rev (182). Un certain seuil d'oligomérisation de Rev doit être atteint pour que survienne l'exportation de l'ARNm grâce au signal d'exportation nucléaire (NES) situé dans la région C-terminal de Rev (181). L'association du complexe ARNm-Rev avec les protéines cellulaires CRM-1 et Ran-GTP permet la migration à travers les pores nucléaires (107). Une fois dans le cytoplasme, l'hydrolyse du Ran-GTP en Ran-GDP catalyse la dissociation du complexe. Rev peut alors interagir avec la protéine importine- β (183) et retourner dans le noyau pour continuer son rôle de navette des ARNm non épissés ou partiellement épissés [FIGURE 12].

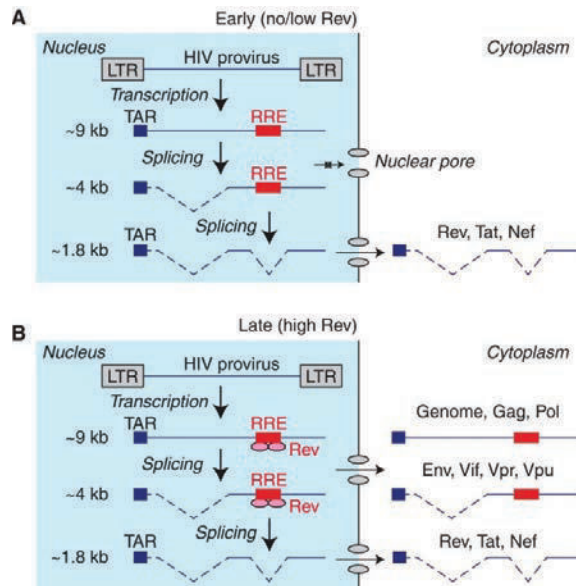


Figure 12. Illustration des phases précoces et tardives de l'expression de l'ARNm viral. Dans la phase précoce, seuls les ARNm complètement épissés peuvent sortir du noyau (A). Il y a production des protéines virales précoces Rev, Nef et Tat. Lors de la phase tardive, un certain seuil de Rev est atteint qui s'associe à l'élément RRE et permet la sortie des ARNm partiellement épissés (B) (184).

3.5. Assemblage

L'assemblage des particules virales est constitué de deux étapes : le rassemblement des divers constituants à la membrane et le bourgeonnement des virions immatures. La polyprotéine Pr55^{Gag} joue un rôle primordial dans l'assemblage et orchestre les deux étapes.

Suite à la synthèse des précurseurs Pr55^{Gag} et Pr160^{GagPol} dans le cytoplasme, les polyprotéines errent jusqu'à leur arrimage à la membrane cellulaire par l'intermédiaire de la matrice située sur la partie N-terminale des précurseurs. Le groupement myristoyl de la matrice interagit spécifiquement avec les phospholipides phosphatidylinositol 4,5-biphosphate contenus dans la membrane (47, 54), ce qui permet l'ancrage au niveau du feuillet interne de la membrane. La partie centrale de Pr55^{Gag} correspondant à la capsidite permet l'oligomérisation des précurseurs Pr55^{Gag} et initie la formation de particules sphériques (185). L'oligomérisation et la formation de particules virales se fait préférentiellement au niveau des radeaux lipidiques riches en cholestérols, sphingolipides

et gangliosides, que l'on retrouve en grande quantité dans les virions matures (186) [FIGURE 13]. L'interaction de la partie C-terminale de Pr55^{Gag} contenant la nucléocapside retient l'ARN viral génomique par la liaison du signal PSI (ψ) dans l'ARN et la structure en doigts de zinc dans la nucléocapside (77). Cette interaction a pour conséquence de favoriser l'étape d'oligomérisation des précurseurs et permet le compactage et l'incorporation de l'ARN viral dans le virion en formation (79, 80). De plus, les précurseurs Pr55^{Gag} et Pr160^{GagPol} interagissent aussi ensemble pendant l'assemblage viral (187, 188).

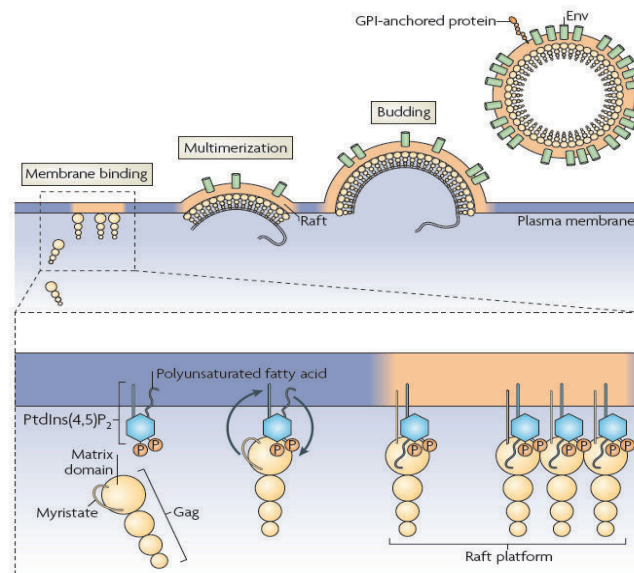


Figure 13. Représentation de l'ancrage des précurseurs Pr55^{Gag} à la membrane cellulaire et bourgeonnement d'un virion (189).

La matrice intervient dans l'incorporation de l'enveloppe dans le virion en interagissant de façon directe ou indirecte avec la queue intracytoplasmique de l'enveloppe (gp41CT) (55-65, 190). Les protéines virales Vif et Vpr sont amenées dans le virion en formation par l'intermédiaire de leur interaction avec Gag (191, 192), tandis que l'ARN_t^{Lys,3} se lie à la séquence PBS de l'ARN viral et est encapsidé (151, 152, 193).

La libération du virion bourgeonnant est orchestrée par la machinerie cellulaire, dont le complexe de tri endosomal requis pour le transport (« endosomal sorting complex required for transport », ESCRT) ainsi que la protéine cellulaire ALIX interagissent avec Gag dans le processus de bourgeonnement (194).

3.6. Maturation

Le virion bourgeonnant de la cellule infectée doit subir une dernière étape de maturation pour passer de l'état de virion immature non-infectieux à celui de virion mature infectieux. Le processus de maturation est catalysé par la protéase virale. Cette dernière résulte de l'interaction entre deux précurseurs Pr160^{GagPol} ce qui permet la dimérisation de la protéase et l'autoclavage de la polyprotéine (195, 196). S'en suit le clivage des précurseurs Pr55^{Gag} et Pr160^{GagPol} dont l'ordre de clivage dépend de l'affinité de la protéase pour le site de clivage (34, 197), ainsi on observe des différences d'efficacité de clivage pouvant aller jusqu'à 400 fois entre le clivage rapide Sp1-NC et le clivage plus lent CA-Sp1 (198). Les clivages des précurseurs, et surtout la séparation MA-CA (68) modifient la morphologie du virion [FIGURE 14] : alors que le virion immature présentait une rigidité prononcée visible comme une couche dense en microscopie électronique, le virion mature est beaucoup moins rigide (66, 67) et la capsid apparaît comme une structure conique séparée de l'enveloppe. La souplesse de la particule virale lui confère son pouvoir infectieux, le virion immature étant 14 fois moins infectieux que le virus mature (66, 67, 199-201).

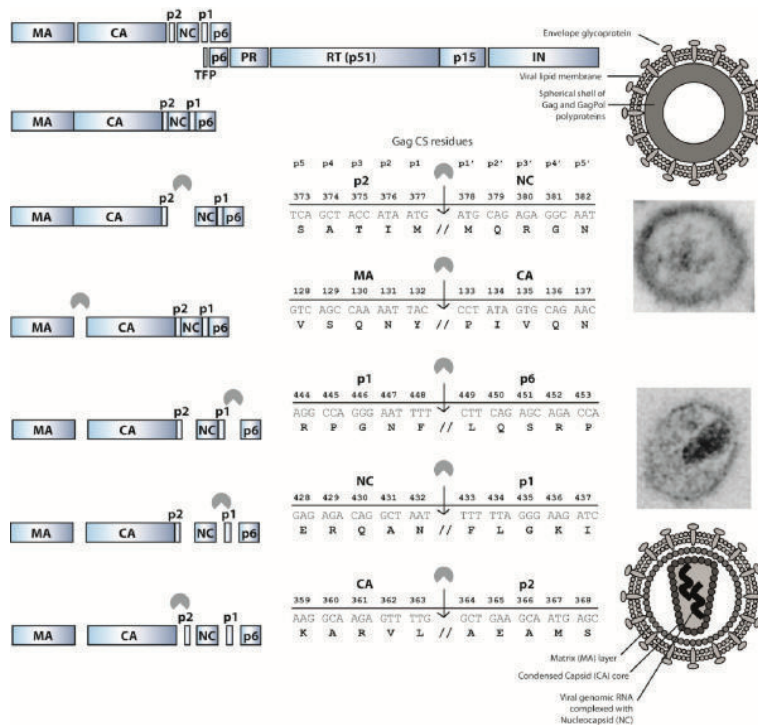


Figure 14. Représentation schématique du clivage des précurseurs Pr55^{Gag} et Pr160^{GagPol} (202).

4. Pathogénèse

4.1. Transmission virale

Le VIH est transmis principalement par voie sexuelle au niveau des muqueuses (90% des infections recensées dans le monde) (203). Le contact direct avec du sang contaminé par le VIH (partage de seringues contaminées, transfusion de sang contaminé) et la transmission verticale (au moment de l'accouchement ou par allaitement) constituent les autres voies d'accès du virus dans l'organisme hôte.

Le risque de transmission est extrêmement faible lorsque l'inoculum de départ contient moins de 3500 copies d'ARN viral par millilitre (204, 205). Malgré la quantité importante de virions présents dans un inoculum, au final il n'y a qu'un seul, voire deux virus qui parviennent à introduire leur génome viral dans une cellule cible et à propager efficacement l'infection (206). Ce ou ces premiers virus sont qualifiés de virus transmis fondateurs. Plusieurs facteurs déterminants de l'hôte peuvent influencer l'efficacité de la transmission du virus fondateur, et notamment l'intégrité des barrières muqueuses et des sécrétions de mucus (207), la disponibilité de cellules cibles à proximité du site d'exposition (208), le niveau d'activation du système immunitaire (209) ainsi que le niveau d'inflammation des tissus génitaux (210).

4.2. Evolution de l'infection virale

L'infection peut évoluer de façon très différente en fonction de facteurs génétiques liés à l'hôte ou au virus. De ce fait, certains individus peuvent vivre jusqu'à une vingtaine d'années avec le virus sans traitement antirétroviral et sans développer de symptômes d'immunodéficience, alors que d'autres manifestent des symptômes endéans quelques années et succombent en quelques mois. Malgré ces divergences de progression de l'infection par le VIH, cette dernière suit trois phases:

- (i) la phase aiguë qui correspond au début de l'infection par le VIH et dure quelques semaines,
- (ii) la phase asymptomatique qui peut durer plusieurs années,
- (iii) la phase SIDA.

4.2.1. La phase aiguë

L'infection aiguë ou primo-infection est caractérisée par un pic de réplication virale (avec des charges virales (CV) supérieures à 10^5 - 10^6 copies d'ARN viral/ml), de ce fait, un individu nouvellement infecté est plus susceptible de transmettre le virus (211, 212), d'autant qu'il ne connaît souvent pas son statut. Par ailleurs, les concentrations exorbitantes de virus engendrent une activation généralisée du système immunitaire qui favorise la réplication virale. Le virus étant cytopathogène, la forte réplication virale résulte en une chute transitoire des lymphocytes T CD4+ (LT CD4+) [FIGURE 15]. Le pic de CV est contrôlé par le système immunitaire environ 6 semaines après l'infection (213) et se stabilise entre 10^3 et 10^5 copies d'ARN viral/ml. Le taux de LT CD4+ circulants est quasiment restauré, mais le taux de LT CD4+ du système gastro-intestinal est, lui, irréversiblement compromis (214).

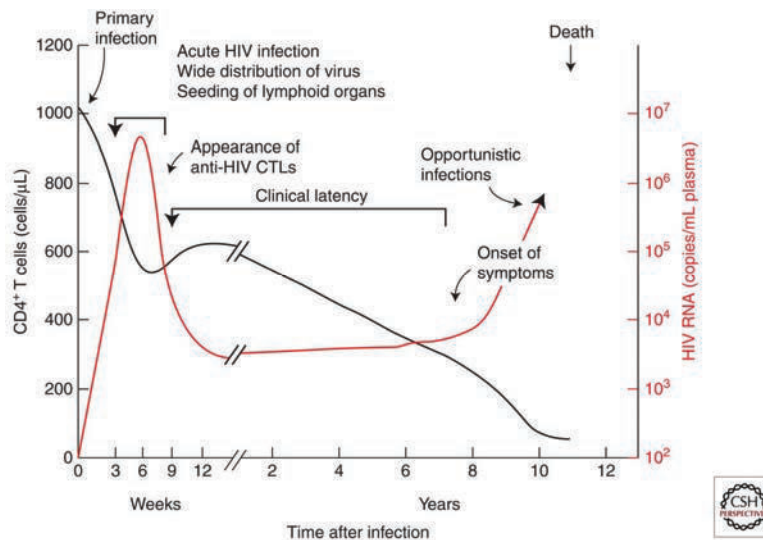


Figure 15. Evolution du taux de lymphocytes T CD4+ (en noir) et de la charge virale (en rouge) au cours de l'infection par le VIH (215).

Au niveau du site d'introduction, le virus rencontre plusieurs types cellulaires différents parmi lesquels des LT CD4+, des macrophages et des cellules dendritiques telles que des cellules de Langerhans. Toutes ces cellules sont des cibles potentielles du virus, toutefois les LT CD4+ mémoire effecteurs longeant les muqueuses sont une cible de choix

et permettent une première amplification du virus fondateur (216-218). La prolifération virale en début d'infection est favorisée par les cellules dendritiques immatures distribuées le long des muqueuses au lieu d'infection (219). En temps normal, ces cellules capturent les pathogènes, les dégradent, tout en migrant vers les ganglions lymphoïdes pour les présenter aux lymphocytes T et ainsi déclencher une réponse adaptative (220). Or le virus parvient à détourner ce mécanisme de défense pour l'utiliser à son avantage (219). En effet, les virus capturés par les cellules dendritiques ne sont pas totalement dégradés (221) et utilisent les cellules dendritiques comme moyen de transport jusqu'aux ganglions lymphoïdes où sont concentrés les LT CD4+, cibles primaires du VIH (222). Dans la majorité des cas, les virus transmis et présents en début d'infection sont des virus R5, probablement parce que les virus R5 infectent préférentiellement les LT CD4+ CCR5+ mémoire, les macrophages et les cellules dendritiques présents en abondance dans les muqueuses et ganglions lymphatiques. Une fois dans les ganglions, le virus rencontre une densité très importante de LT CD4+ auxquels il est transmis et où il se réplique très efficacement. De nouveaux virions ainsi que des cellules infectées sont ensuite disséminés dans tout l'organisme via les organes lymphoïdes. La réplication exponentielle continue lorsque le virus parvient à atteindre et proliférer dans le tissu lymphoïde associé au tube digestif (gut-associated lymphoid tissue, GALT), engendrant une destruction massive et définitive des LT CD4+ résidants dans le GALT (214, 223) [FIGURE 16]. Cette prolifération virale effrénée se reflète dans le pic d'ARN viral et stimule l'activation du système immunitaire, réapprovisionnant le virus en cibles potentielles (224, 225). Le déclin de la CV coïncide avec l'apparition de lymphocytes CD8 spécifiques au VIH (226, 227) qui vont exercer une pression de sélection sur le virus entraînant l'émergence de virions génétiquement différents mais toujours apparentés au(x) virus fondateur(s) de départ (228). Ces nouveaux virus sont plus aptes à échapper au système immunitaire, favorisant ainsi la prolifération virale. Enfin, 3 à 12 semaines après le début de l'infection, apparaissent des anticorps dirigés contre le virus (séroconversion) marquant le début de la phase asymptomatique.

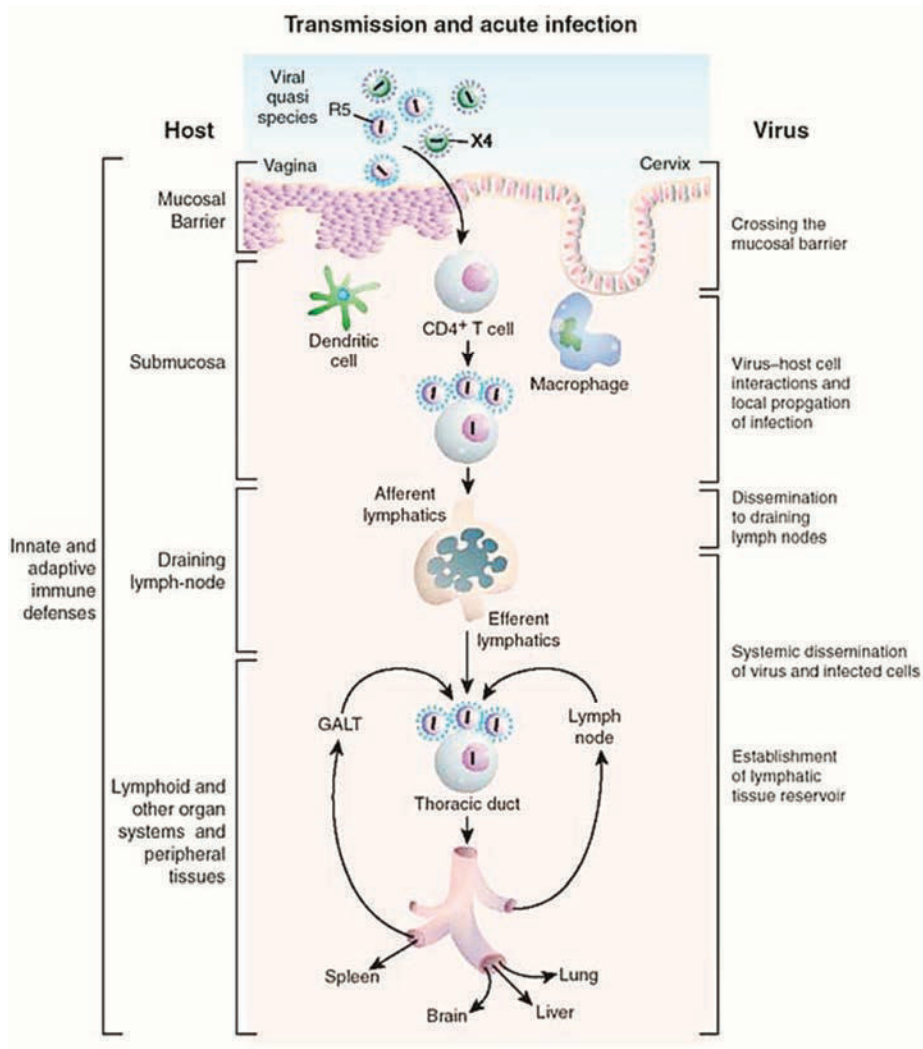


Figure 16. Progression de l'infection depuis l'entrée du virus dans l'organisme jusqu'à la dissémination virale dans l'organisme (215).

4.2.2. La phase asymptomatique

La phase asymptomatique peut être de durée variable (2 à 8 ans en absence de traitement). Cette phase est caractérisée par l'absence de symptômes liés à l'infection par le VIH et une CV stable. Durant cette phase, 10^{10} nouvelles particules virales sont produites par jour (18, 229, 230), le système immunitaire contrôle l'infection mais les LT CD4+ sont progressivement détruits (231). Certains individus progressent plus lentement et leur phase asymptomatique est plus longue (Long Term Non Progressors, LTNP), et certains,

bien que peu nombreux, maintiennent une charge virale faible et un taux de LT CD4+ supérieur à 500/ml en absence de traitement antirétroviral. Ces individus, appelés Elite Controllers, sont très étudiés pour comprendre les mécanismes de contrôle de l'infection.

Les mécanismes de défense mis en place par l'organisme pour contrer l'infection induisent une activation prolongée et soutenue du système immunitaire. La destruction des lymphocytes dans le GALT lors de la primo-infection altère considérablement la barrière intestinale ouvrant des brèches par lesquelles des antigènes tels que les lipopolysaccharides (LPS) pénètrent dans l'organisme. Ces vagues d'antigènes contribuent à maintenir un environnement pro-inflammatoire ainsi que la stimulation du système immunitaire (232). Cet état d'activation est propice à la prolifération et à l'activation de nouveaux LT CD4+ qui constituent de nouvelles cibles pour le virus et favorisent la réplication virale (224, 233). On observe également une dérégulation généralisée du système immunitaire avec activation et prolifération de lymphocytes B et production d'anticorps non-spécifiques pouvant induire des maladies auto-immunes (234, 235).

Chez plus de la moitié des personnes infectées par le VIH, la population virale issue d'un virus fondateur se diversifie profondément au cours de l'infection et aboutit à terme à un élargissement ou un changement de tropisme viral, les virus R5 évoluant vers l'utilisation de CXCR4. Ce changement de tropisme se produit 5 à 7 ans après le début de l'infection et a des conséquences catastrophiques sur l'évolution de l'infection (236). En effet, les virus X4 répliquent plus rapidement que les R5 et ont des effets cytopathogènes prononcés. Dès lors, l'apparition de ces virus est souvent associée à une progression plus rapide de la maladie vers la phase du SIDA. En outre, un changement de tropisme fournit au virus un répertoire de cellules cibles plus vaste, dès lors qu'il n'est plus restreint aux cellules exprimant CCR5, mais peut infecter tous les nouveaux LT CD4+ naïfs qui expriment le CXCR4.

La réplication virale dans les cellules activées est très efficace avec 10^{10} particules virales produites par jour, engendrant des effets cytopathogènes importants et la mort rapide des cellules infectées (18, 229). Malgré cela, la perte de LT CD4+ n'est pas seulement liée à la mort des cellules infectées (237), mais aussi à un effet d'apoptose des cellules T CD4+ avoisinantes (« bystander apoptosis ») (238). Ces effets sont en grande partie responsables pour la chute progressive des LT CD4+ pendant la phase asymptomatique.

Les mécanismes contrôlant ce processus ne sont pas encore bien connus, mais il semblerait que l'enveloppe virale du VIH intervienne dans ce processus (238, 239).

4.2.3. Le SIDA

Le stade de syndrome d'immunodéficience humaine (SIDA) défini par l'apparition de certains cancers, infections opportunistes et maladies sévères associés à une détérioration du système immunitaire. Les traitements combinant plusieurs inhibiteurs antirétroviraux, communément qualifiés de HAART (Highly active antiretroviral therapy), permettent de repousser le début de la phase SIDA de plusieurs années, voire décennies. Sans traitement, les patients atteignant la phase SIDA succombent dans les années qui suivent suite à l'émergence de maladies opportunistes facilement éliminées dans un organisme immunocompétent (240). Les infections opportunistes les plus courantes sont des candidoses œsophagiennes, les pneumonies à *pneumocystis carinii*, des toxoplasmoses cérébrales ou encore des néoplasies malignes tel que des lymphomes non-hodgkiniens ou des sarcomes de Kaposi (1).

4.3. Réponse immunitaire

Les deux axes de la réponse immunitaire sont sollicités pour combattre l'infection par le VIH, la réponse innée avec les cellules dendritiques et cellules naturelles tueuses (NK) et la réponse adaptative avec les lymphocytes T CD8+ conjointement avec les LT CD4+ et la réponse humorale.

4.3.1. La réponse innée

La réponse innée se déclenche dès la détection de motifs associés aux parasites, bactéries ou virus et absents des cellules autologues. Lors de l'infection aiguë par le VIH, les cellules dendritiques détectent le virus et sont les premières à libérer des cytokines antivirales (241, 242). Néanmoins cela a aussi comme conséquence d'accroître le niveau d'activation du système immunitaire augmentant ainsi le nombre de LT CD4+ susceptibles

d'être infectés. Les cellules NK interagissent avec le complexe majeur d'histocompatibilité de classe 1 (CMH-1) exprimé sur les cellules infectées et sécrètent des cytokines, des chimiokines ainsi que d'autres molécules antivirales, telles que les granzymes. Ce mécanisme induit la destruction des cellules infectées et permet de restreindre la réplication virale (243).

4.3.2. La réponse adaptative

4.3.2.1. La réponse cellulaire

Les lymphocytes CD8+ spécifiques du VIH apparaissent très tôt dans l'infection et sont à l'origine du déclin du pic viral pendant la phase aiguë de l'infection. Il est intéressant de constater que l'intensité de la réponse des lymphocytes CD8+ cytotoxiques (CTL) est corrélée à la CV (244) alors que l'efficacité de la réponse est associée à la « qualité » des lymphocytes (245). Plusieurs mécanismes de défense sont à la disposition des CTL. Ils peuvent relarguer des protéases et des molécules de perforine pour lyser directement les cellules infectées (246), ainsi qu'induire l'apoptose par l'intermédiaire du ligand FasL exprimé à leur surface. Les lymphocytes CD8+ sont aussi capables de sécréter des chimiokines (RANTES, MIP-1 α et MIP-1 β) et des α -défensines qui empêchent l'entrée du virus dans de nouvelles cellules (247). Certains LT CD4+ sont spécifiques au VIH et possèdent une activité cytolytique qui leur permet de combattre le virus sans toutefois l'éliminer (248). Par ailleurs, chez les Elite Controllers, le spectre d'épitopes reconnus par les CTL est plus large que chez les progressseurs normaux, et le phénotype HLA-DR+, CD38- traduit probablement un potentiel de prolifération plus important (249).

4.3.2.2. La réponse humorale

L'infection induit également une réponse humorale, mais une faible proportion d'individus infectés par le VIH produisent des anticorps avec une activité neutralisante (250, 251). La réponse humorale induite est dirigée essentiellement contre Env, seule protéine virale exprimée à la surface des cellules infectées et des virions. Toutefois,

l'extrême variabilité de Env, conjointement aux nombreuses glycosylations arborées par cette dernière, rendent le développement d'anticorps neutralisants particulièrement difficile et permettent au virus d'échapper au système immunitaire (252). La variabilité de Env induit un décalage entre les épitopes reconnus par les anticorps induits et les virus circulants. La glycosylation permet de masquer les épitopes ou les régions essentielles à la réplication virale. Ainsi, il est intéressant de noter que les enveloppes tardives (présentes durant la phase SIDA), sont souvent moins glycosylées que les enveloppes des virus présents durant les phases aiguë et asymptomatique de l'infection. Il a été suggéré qu'au stade SIDA, le système immunitaire est tellement compromis qu'il n'est plus nécessaire de masquer les épitopes ciblés par les anticorps neutralisants (253, 254). Ceci pourrait expliquer que des anticorps neutralisants peuvent être détectés plusieurs années après le début de l'infection (255).

4.3.3. Les facteurs de restriction

Outre les réponses immunes cellulaire et humorale, l'organisme possède aussi des mécanismes antiviraux intracellulaires qui protègent les cellules contre l'intrusion de virus. Ces facteurs intrinsèques s'apparentent à l'immunité innée dans la mesure où ils n'agissent pas spécifiquement contre le VIH mais sont communs à plusieurs virus ou familles de virus. Toutefois, le VIH parvient à contrer certains de ces facteurs de restriction grâce aux protéines accessoires virales (**section 2.3.4**). A l'heure actuelle, cinq facteurs de restriction ayant une activité antirétrovirale ont été identifiés :

La protéine SAMHD1 : SAMHD1 est actif au niveau de la lignée myéloïde (spécialement au niveau des cellules dendritiques) et des LT CD4+ quiescents (256, 257) où il parvient à contrôler l'infection par le VIH. SAMHD1 possède une activité triphosphohydrolase qui convertit les désoxynucléosides triphosphates (dNTP) en désoxynucléosides (dN) et triphosphates (258-260). SAMHD1 est ainsi capable de réguler la quantité de dNTP disponibles dans la cellule et de ce fait bloquer la transcription inverse en asséchant le réservoir de dNTP.

La protéine APOBEC3G (aussi nommée CEM15) (261) : Il s'agit d'une cytidine déaminase qui a pour rôle d'éliminer un groupement aminé des cytidines les convertissant

en uridine. Lors de la synthèse du brin (-) d'ADN viral par la transcriptase inverse, APOBEC3G convertit les nucléosides de cytosine en uracile, ce qui résulte en l'accumulation de transitions G→A dans le brin d'ADN (+) (262). APOBEC3G est incorporé dans les virions en formation grâce à l'interaction avec la région nucléocapside du précurseur Pr55^{Gag}. L'activité d'APOBEC3G est toutefois compromise par la protéine accessoire Vif. En effet, cette dernière induit l'ubiquitination du facteur de restriction et sa dégradation (263).

La protéine BST-2 ou tetherine : La tetherine est induite par les interférons de type I (264) et cible les virus enveloppés (119). Insérée au niveau de la membrane cellulaire, la tetherine permet de retenir le virus bourgeonnant à la surface de la cellule. Ces virus peuvent ensuite être internalisés et dégradés dans les endosomes. Néanmoins le VIH parvient encore une fois à échapper à ce mécanisme de défense par l'intermédiaire de la protéine virale Vpu qui induit la dégradation du facteur de restriction (118-124).

La protéine MX2 (myxovirus resistance 2) : Il s'agit d'un inhibiteur du VIH induit par l'interféron alpha. L'expression de MX2 permet de réduire l'infection par divers sous-types de VIH. La protéine agit en altérant l'import nucléaire de l'ADN viral. Il a aussi été montré que des mutations dans Gag interférant avec l'import nucléaire de l'ARN circulaire 2LTR diminuent l'efficacité de MX2 (265) (266).

La protéine TRIM5-α : Cette protéine diffère légèrement entre l'homme et le singe. Seule la protéine TRIM5-α du primate possède une activité antivirale, tandis que TRIM5-α humain est incapable de bloquer l'infection. La protéine du primate agit après l'entrée virale au niveau de la capsid et induit une diminution de la synthèse d'ADN viral (267-269).

4.4. Traitements antirétroviraux et échappement du virus

Le développement de molécules antirétrovirales administrées en combinaison a permis d'allonger considérablement l'espérance de vie des individus atteints par le VIH. Le premier médicament introduit en clinique a été la Zidovudine (AZT), un inhibiteur de la transcriptase inverse (270). Au fil des années, de nouveaux médicaments ont été développés. Il existe plusieurs classes de médicaments, ciblant diverses étapes du cycle de réplication virale :

(1) les inhibiteurs nucléosidiques de la transcriptase inverse (NRTI), il s'agit de nucléosides dépourvus du groupement 3'-hydroxyle, qui agissent par compétition avec les nucléosides cellulaires et terminent prématurément l'élongation de l'ADN viral,

(2) les inhibiteurs non-nucléosidiques de la transcriptase inverse (NNRTI), qui interagissent avec une région proche du site catalytique de l'enzyme et induisent un changement de conformation bloquant l'activité enzymatique (271),

(3) les inhibiteurs de protéase inhibant l'activité enzymatique de la protéase virale, ce qui résulte en la production de virions immatures et non-infectieux (272),

(4) les inhibiteurs d'intégrase, qui empêchent l'étape d'intégration de l'ADN viral dans le génome de la cellule hôte (273) et enfin

(5) les inhibiteurs d'entrée et de fusion, qui peuvent soit agir par compétition et lier le co-récepteur CCR5 ou empêcher le repliement de la gp41 et bloque ainsi l'étape d'entrée ou de fusion (274) (**Tableau 1**).

NRTI	NNRTI	Inhibiteurs de protéase	Inhibiteurs d'intégrase	Inhibiteurs de fusion ou entrée
Zidovudine (AZT)	Nevirapine (NVP)	Saquinavir (SQV)	Raltegravir (RAL)	Enfurvirtide
Didanosine (ddI)	Delavirdine (DLV)	Ritonavir (RTV)	Elvitegravir (ELV)	Maraviroc
Zalcitabine (ddC)	Efavirenz (EFV)	Indinavir (IDV)		Vicriviroc
Lamivudine (3TC)	Etravirine (ETR)	Atazanavir (ATV)		
Stavudine (D4T)	Rilpivirine (RPV)	Darunavir (DRV)		
Abacavir (ABC)		Fosamprenavir (FPV)		
Tenofovir (TDF)		Lopinavir (LPV)		
Emtricitabin (FTC)		Tipranavir (TPV)		

Tableau 1. Récapitulatif des médicaments antirétroviraux approuvés par l'agence américaine des médicaments (FDA).

Malgré les résultats encourageants obtenus par l'administration de trithérapies combinant plusieurs molécules antirétrovirales, généralement 2 NRTIs et un NNRTI ou un IP, leur efficacité peut être compromise par des doses thérapeutiques sub-optimales, résultant d'une mauvaise compliance du patient, d'une pharmacocinétique particulière du patient et/ou d'une interaction avec d'autres substances ou médicaments. L'échec se traduit par un échec virologique (augmentation de la CV) suivi d'un échec immunologique (diminution des LT CD4+) et est généralement accompagné de l'émergence de virus portant des mutations de résistance. Dès l'apparition de mutations de résistance à une classe de médicaments, le patient doit changer de régime de médicaments. Au fil du temps,

plus un patient a reçu de traitements, plus il risque d'accumuler des mutations de résistance aux diverses classes de médicaments et plus les options thérapeutiques sont compromises. De plus, même lors d'un traitement efficace, la réplication virale persiste à des taux très bas mais constants (275). Par ailleurs, les traitements ciblent des étapes du cycle viral nécessitant une réplication active, et ne peuvent donc atteindre le virus latent dans des cellules mémoire au repos (176).

5. Cellules cibles du VIH

Le principal récepteur du VIH étant la molécule CD4, le VIH infecte donc les cellules exprimant ce récepteur : les lymphocytes T CD4+, les macrophages et les cellules dendritiques [FIGURE 17].

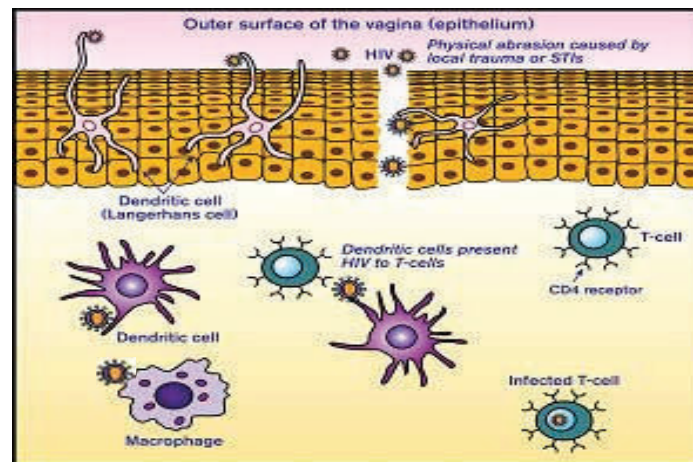


Figure 17. Cellules cibles du VIH. Image modifiée à partir de (276).

5.1. Sous-populations lymphocytaires T CD4+ (LT CD4+) et infection par le VIH

Les LT CD4+ (ou lymphocytes auxiliaires) orchestrent l'activation et la prolifération des lymphocytes CD8+, des cellules NK et des lymphocytes B. Selon l'environnement cytokinique, les LT CD4+ naïfs activés sont polarisés en sous-populations effectrices Th1, Th2, Th17 ou des lymphocytes T régulateurs induits (iTreg) (277) sécrétant différents types

de cytokines [FIGURE 18]. Des stimuli interleukine 12 (IL-12) et interféron γ (IFN γ) induisent une polarisation inflammatoire de type Th1 qui stimule une réponse immune cellulaire (lymphocytes T CD8+, macrophages) (278). L'IL-4 polarise les LT CD4+ naïfs en Th2, qui interviennent dans les réponses immunitaires dirigées contre les parasites extracellulaires et produisent plusieurs cytokines anti-inflammatoires (IL-4, IL-5, IL-9, IL-10, IL-13 et IL-25) et stimulent le bras humoral de la réponse immune (278). Le facteur de croissance de transformation β (TGF- β) conjointement à l'IL-6 induit la différenciation en Th17, une sous-population sécrétrice d'IL-17, impliquée dans les maladies auto-immunes et qui dirige les réponses immunitaires contre des bactéries extracellulaires et des champignons (279). La stimulation TGF- β , acide rétinoïque et IL-2 entraine la différenciation en iTreg qui sont impliqués dans la régulation de la réponse immune (280). Les LT CD4+ mémoire, et en particulier les Th17 et les iTreg, seraient préférentiellement infectées par le virus (281, 282). La destruction massive de ces cellules dans le GALT est irréversible et engendre des perturbations dans l'équilibre Th17/iTreg.

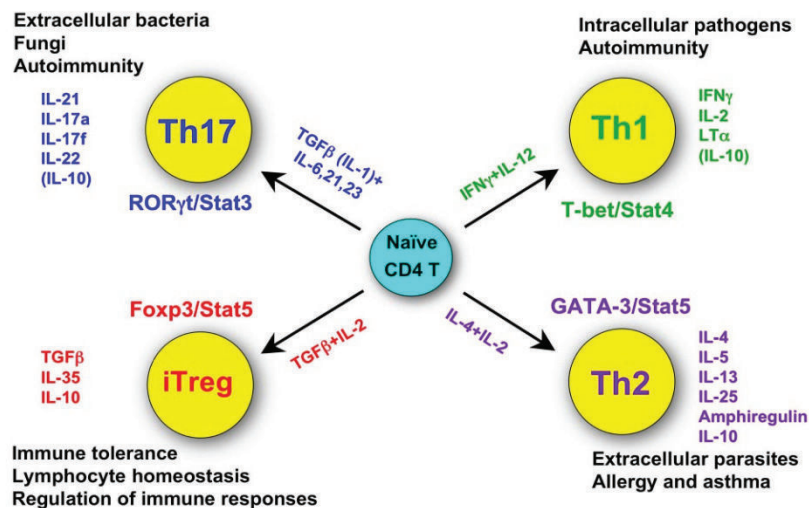


Figure 18. Sous-populations de lymphocytes T CD4+ et leur fonctions (277).

La grande majorité de ces cellules effectrices ont une durée de vie très courte, seule une minorité survit et retourne à un état de repos en tant que LT CD4+ mémoires. Cette population est à son tour subdivisée en deux sous-populations : les lymphocytes T

mémoires centraux (T_{CM}) situés dans les organes lymphoïdes secondaires où ils peuvent interagir avec des cellules dendritiques, et les lymphocytes T mémoires effecteurs (T_{EM}) circulant dans les tissus périphériques où ils peuvent rapidement agir comme cellules effectrices. Lors de l'infection, les LT CD4+ mémoire ou les DC migrent des muqueuses vers les organes lymphoïdes secondaires où la densité de LT CD4+ naïfs et de T_{CM} constitue un environnement privilégié pour la transmission de la souche fondatrice et l'expansion de l'infection (215, 283) (**section 4.1**).

Les LT CD4+ activés sont extrêmement susceptibles au VIH (284). Différentes sous-populations de LT CD4+ peuvent être infectées en fonction du tropisme viral (R5 ou X4), de l'état d'activation de la cellule cible et de l'expression de co-récepteurs. Les LT CD4+ mémoires dans le sang et les tissus périphériques expriment des taux élevés de CCR5 et moins de CXCR4, tandis que les LT CD4+ naïfs expriment davantage de CXCR4 et beaucoup moins de CCR5 (285). Les virus fondateurs étant majoritairement R5, il n'est pas étonnant que les LT CD4+ mémoires soient les premières cellules cibles du virus lors de l'infection par le VIH (286, 287).

5.2. Macrophages

Les macrophages sont impliqués dans la première ligne de défense de l'organisme face aux microorganismes étrangers. Les macrophages ont deux fonctions bien précises : la phagocytose de pathogènes ou débris cellulaires et la présentation d'antigènes par les molécules du CMH. Les macrophages sont issus de monocytes circulant dans le sang qui se différencient en macrophages lorsqu'ils arrivent dans les tissus (288). En fonction des divers tissus, les macrophages portent différents noms (il s'agit de cellules de la microglie dans le cerveau, de cellules de Kupffer dans le foie et de macrophages alvéolaires dans les poumons). Comme pour les lymphocytes, l'environnement cytokinique influence la fonction et la physiologie des macrophages, qui peuvent être polarisés en M1 (impliqués dans les réponses antibactériennes, antivirales et anti-tumorales et qui restreignent les dommages tissulaires) et en macrophages M2 (possèdent une fonction anti-inflammatoire et favorisent le processus de cicatrisation) (288). Il a récemment été suggéré que les

macrophages polarisés M1 étaient plus résistants à l'infection par le VIH (289-291), alors que les M2 seraient plus capables de transmettre l'infection.

Les macrophages peuvent être infectés par le VIH, mais ils sont beaucoup moins susceptibles à l'infection par rapport aux LT CD4+ (292). La production de virus dans les macrophages est aussi beaucoup plus lente que dans les lignées lymphoïdes (293), en raison de la triphosphohydrolase SAMHD1 qui restreint considérablement le processus de transcription inverse (294) (**section 4.3.3**). L'infection par le VIH semble réduire la capacité phagocytaire des macrophages (295, 296). Une caractéristique importante des macrophages est leur résistance aux effets cytotoxiques induits par le virus (296). Ces cellules ont une durée de vie très longue et constituent de ce fait un réservoir important pour le virus (297). De plus, ils peuvent produire et accumuler le virus dans de larges vacuoles intracellulaires reliées à la surface (« virus-containing compartments » VCC) (297, 298). Le virus peut ainsi être stocké pendant de longues périodes de temps à l'abri des anticorps neutralisants (299) et des traitements antirétroviraux [FIGURE 19]. Enfin, ces cellules patrouillent dans tout l'organisme et représentent donc un véhicule de premier choix pour la dissémination du VIH dans l'individu infecté.

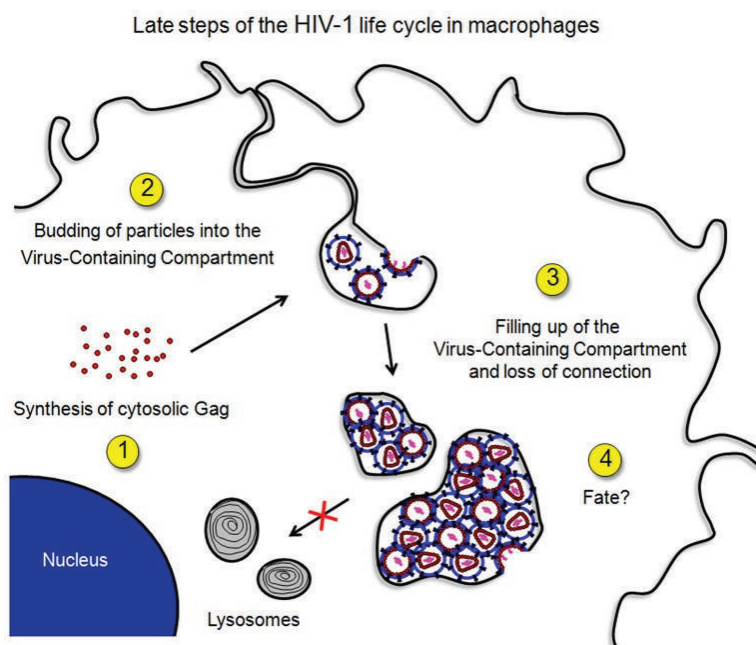


Figure 19. Production et stockage de particules virales dans les macrophages. Les virions s'assemblent et bourgeonnent au niveau des VCC puis y sont stockés (300).

5.3. Cellules dendritiques (DCs)

Positionnées le long des muqueuses, les DCs sont des sentinelles ayant pour but de capturer des particules étrangères, les dégrader et présenter des antigènes aux LT CD4+ afin d'activer ces derniers et d'induire une réponse immunitaire adaptée et spécifique.

Les microorganismes étrangers phagocytés au site d'entrée, induisent la maturation et la migration des DCs vers les ganglions lymphoïdes. C'est dans ce compartiment rempli de lymphocytes, que les DCs présentent les antigènes étrangers sur leur CMH-II aux lymphocytes (219). Ces cellules sont très rares dans le sang (0.5 à 2%) (301) et sont donc majoritairement étudiés sous la forme de DCs dérivés de monocytes (« monocyte-derived dendritic cells » ou MDCC) qui présentent un phénotype similaire aux DCs matures (302).

Lorsque des virions VIH pénètrent l'organisme au niveau des muqueuses, les particules virales sont capturées par les DCs puis transportées vers les ganglions lymphoïdes où elles devraient être présentées aux LT CD4+ pour initier une réponse immunitaire. Or le virus contourne ce mécanisme de défense et l'utilise pour canaliser l'infection des LT CD4+. Les DCs capturent le VIH grâce à plusieurs récepteurs aux lectines de type C, qui lient l'enveloppe virale. Parmi les récepteurs les plus connus, on compte les « dendritic cell-specific ICAM-grabbing non-integrin » (DC-SIGN) (303, 304), les immunorécepteurs des DCs (DCIR) (305) et des récepteurs à mannose (306-308). Ces récepteurs lient les oligosaccharides constituant les glycosylations exprimées à la surface de la sous-unité d'enveloppe virale gp120 (309). Etant donné que les DCs expriment les récepteurs usuels pour le VIH, qui sont le CD4 et le CCR5, ces cellules sont également susceptibles à l'infection par des virus R5 (306, 310), toutefois, l'infection est particulièrement inefficace (10 à 100 fois moins d'infection par rapport aux LT CD4+) (311). L'infection productive des DCs ou *cis*-infection est fortement restreinte à cause tout d'abord du taux réduit de récepteurs CD4 et CCR5 à sa surface (310) ainsi que la présence de plusieurs récepteurs reconnaissant les motifs associés aux pathogènes (PAMPs) déclenchant des mécanismes antiviraux (312) [FIGURE 20]. De plus, les DCs possèdent aussi le facteur de restriction cellulaire SAMHD1 qui restreint considérablement l'étape de transcription inverse en réduisant le réservoir de dNTP disponibles (294). Néanmoins les liaisons de l'enveloppe virale avec les récepteurs aux lectines de type C permettent au virus

de glisser le long de la cellule jusqu'à des invaginations où peuvent se former des synapses virologiques (313) [FIGURE 20]. Le mécanisme de *trans*-infection permet l'infection des LT CD4+ par l'intermédiaire des DCs sans infection productive de ces dernières [FIGURE 20]. Lors de ce processus, le virus est endocyté par les DCs, ce qui lui permet d'échapper aux PAMPs à la surface des DCs et d'être transporté jusqu'aux LT CD4+. Le transfert de particules virales se fait par contact des DC et LT CD4+ (formation d'une synapse virale) (314, 315). Globalement, la capacité migratoire, l'interaction avec des LT CD4+ et le transfert de virus suggèrent que les DCs sont étroitement liées à la dissémination du virus lors du début de l'infection.

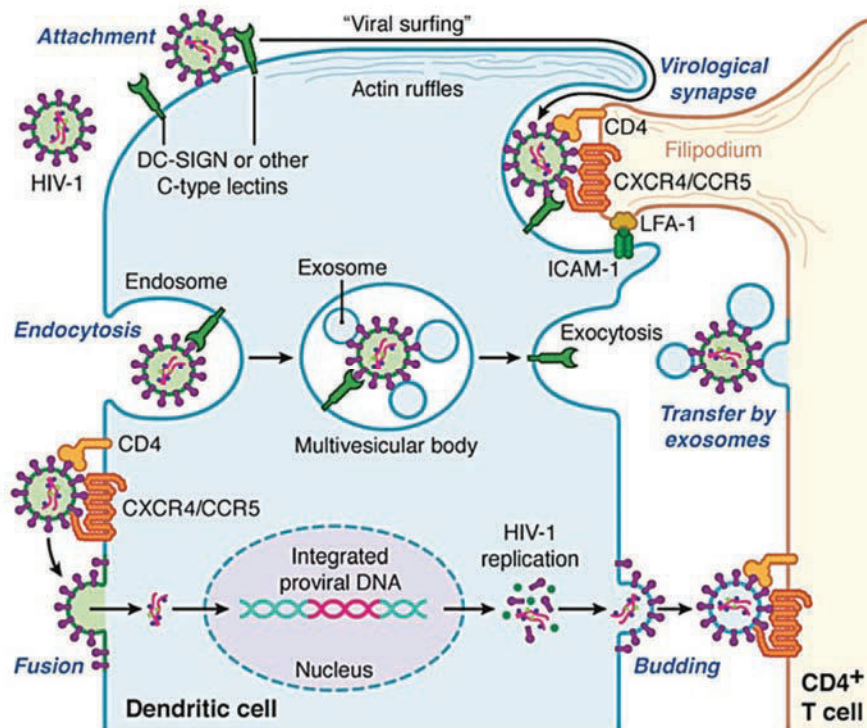


Figure 20. Représentation des 3 mécanismes de transmission employés par le virus dans les cellules dendritiques. Le mécanisme d'attachement consiste à faire glisser le virus à la surface des DCs dans des invaginations où peut se former une synapse virale. Lors de la *trans*-infection (ou endocytose), le virus traverse la DC sans l'infecter et est transmis à une autre cellule cible. Enfin lors de la *cis*-infection, la DC est productivement infectée et libère des virions (316).

5.4. Latence et réservoirs viraux

La persistance du virus dans l'organisme est due à l'intégration du génome viral dans le génome de la cellule infectée qui circule dans un état latent à l'abri des thérapies antirétrovirales et du système immunitaire. Le virus peut ainsi créer des réservoirs viraux contenant des provirus stables et capables de répliquer (317). Il existe deux types de réservoirs viraux : le réservoir tissulaire (incluant les tissus lymphoïdes (318), le tractus gastro-intestinal (319), le système nerveux central (320) et les organes génitaux (321, 322)) et le réservoir cellulaire (incluant principalement les LT CD4+ (284) et les macrophages (323)). Les cellules infectées qui parviennent à survivre à l'infection contiennent le génome viral intégré dans leur génome et peuvent retourner à un état de latence. L'expression virale est considérablement réduite dans ces cellules latentes, seule une faible production de protéine virale Pr55^{Gag} persiste (324). Cet état latent permet au virus de passer inaperçu face à la réponse immunitaire. Des modifications de l'environnement transcriptionnel sont à l'origine de cet état de latence. En effet, la séquestration des facteurs de transcription cellulaires NF-κB et NFAT (impliqués dans l'activation du promoteur viral LTR) (178, 325) et des complexes CDK9 et cycline T1 (impliqués dans l'étape de transcription de l'ARN viral avec la protéine virale Tat) (326, 327) dans le cytoplasme des cellules LT CD4+ quiescentes restreint la production de nouvelles protéines virales. Enfin, des modifications au niveau de la chromatine des nucléosomes, ainsi que des méthylations du promoteur viral renforcent l'état latent du virus (328).

6. Enveloppe du VIH

Le complexe trimérique de l'enveloppe joue un rôle clé dans l'attachement et l'entrée du virus dans la cellule cible en assurant le rapprochement et la fusion des membranes virale et cellulaire permettant la libération du matériel génomique viral dans la cellule.

Contrairement aux autres protéines virales qui sont produites dans le cytoplasme, l'enveloppe est synthétisée au niveau du réticulum endoplasmique rugueux sous forme du précurseur gp160 (329) [FIGURE 21]. C'est lors de son passage par le réticulum

endoplasmique que gp160 s'assemble en trimères et acquiert un grand nombre de O- et N-glycosylations ainsi que des ponts disulfures (330-334). Le précurseur gp160 migre ensuite vers l'appareil de Golgi où il est clivé par la protéase cellulaire furine (335). La forme finale de Env est un trimère d'hétérodimères gp120/gp41 associés de façon non-covalente. Env est la structure du virus la plus exposée au système immunitaire, étant exprimée en surface des virions et des cellules infectées, et elle induit une réponse humorale.

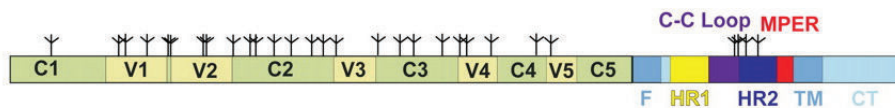


Figure 21. Schéma du précurseur gp160 avec les séquences variables (V1 à V5) et constantes (C1 à C5) de la gp120. Ainsi que les constituants de la gp41 : peptide de fusion (F), les séquences HR1 et 2, boucle disulfure, région MPER, domaine transmembranaire (TM) et la queue intracytoplasmique (CT) (336).

6.1. La protéine de surface (gp120)

La sous-unité de surface gp120 est constituée de cinq régions variables (V1 à V5) formant des boucles intercalées de cinq régions conservées (C1 à C5) (337). Plusieurs résidus Cys disséminés le long de gp120 et gp41 forment des ponts disulfures qui sont à l'origine de la structure tertiaire de Env (330). Les régions V1 à V4 sont exposées en surface de la protéine faisant face à l'environnement extracellulaire et masquent les régions constantes [FIGURE 22]. Les régions V1 et V2 sont de loin les plus variables tant par leur longueur que par le nombre de glycosylations (338, 339). La moitié de la masse moléculaire de gp120 est composée de glycosylations, qui confèrent au virus plusieurs avantages : elles permettent de cacher les structures virales face au système immunitaire (332), contribuent au repliement de l'enveloppe (333) et facilitent l'attachement aux cellules cibles (340). La longueur des régions V1 et V2 et le nombre de sites potentiels de glycosylations augmente durant la phase asymptomatique de l'infection, puis diminue en phase tardive. Ces variations suggèrent que ces modifications favorisent l'échappement du virus face au système immunitaire de l'hôte, quand elles ne sont pas nécessaires peu après la

transmission lorsque la réponse immune n'est pas encore instaurée, ni en phase tardive, alors que le système immunitaire est trop dégradé (253).

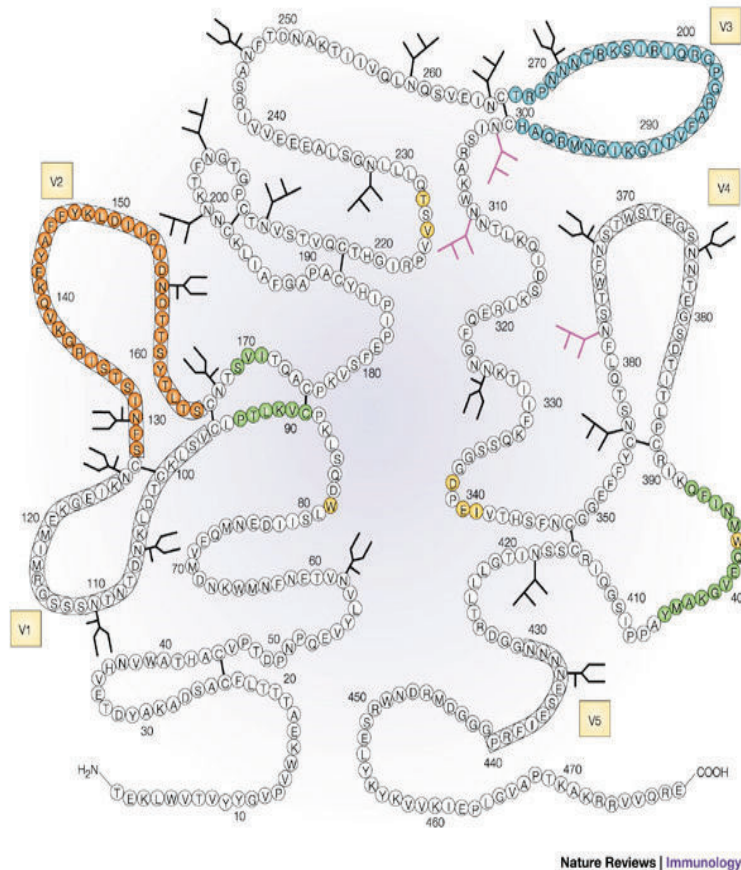


Figure 22. Illustration de la structure de la glycoprotéine gp120. Mise en évidence des boucles variables V1 à V5, ainsi que des épitopes induisant des anticorps neutralisants (séquences colorées) (341).

La liaison au récepteur CD4 se fait au niveau de divers segments dans les régions constantes C3 et C4 de gp120 (342, 343), indépendamment des régions variables (344). L'interaction de la boucle V3 avec un co-récepteur est indispensable pour induire l'entrée du virus dans la cellule cible (345). De plus, la boucle V3 joue un rôle crucial dans le tropisme du virus puisqu'elle détermine si les virus utilisent le co-récepteur CCR5 ou le co-récepteur CXCR4. Le changement de tropisme R5 vers X4 se produit lorsque la charge nette positive de la boucle V3 augmente ce qui permet l'interaction avec les charges négatives à la surface du co-récepteur CXCR4 (346).

6.2. La protéine transmembranaire (gp41)

La sous-unité transmembranaire gp41 a comme première fonction la fusion des membranes virale et cellulaire pour permettre l'introduction du matériel génomique viral dans la cellule cible. De plus, elle assure l'ancrage du complexe Env à la membrane. La protéine gp41 est organisée en trois domaines : le domaine extracellulaire (ou ectodomaine) dans la partie N-terminale suivi du domaine transmembranaire et le domaine intracellulaire (ou queue intracytoplasmique) en C-terminal (334) [FIGURE 23].

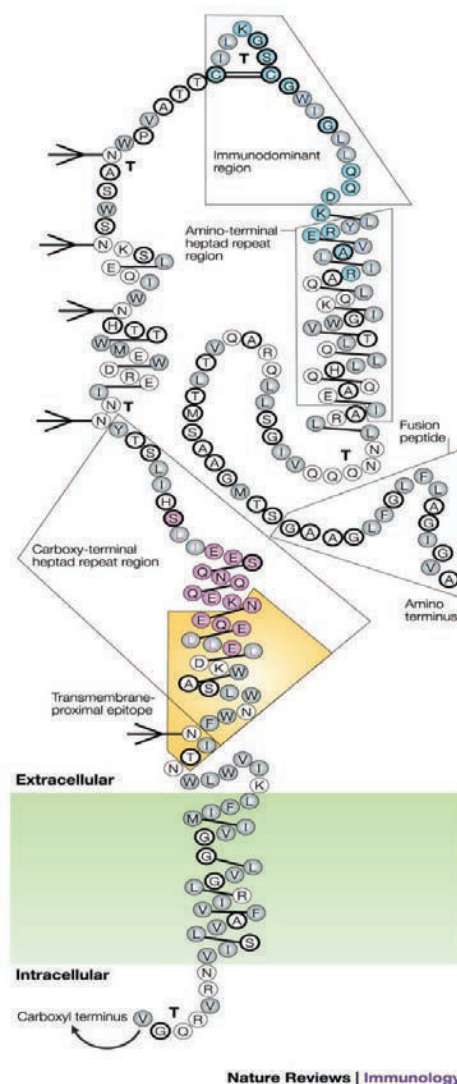


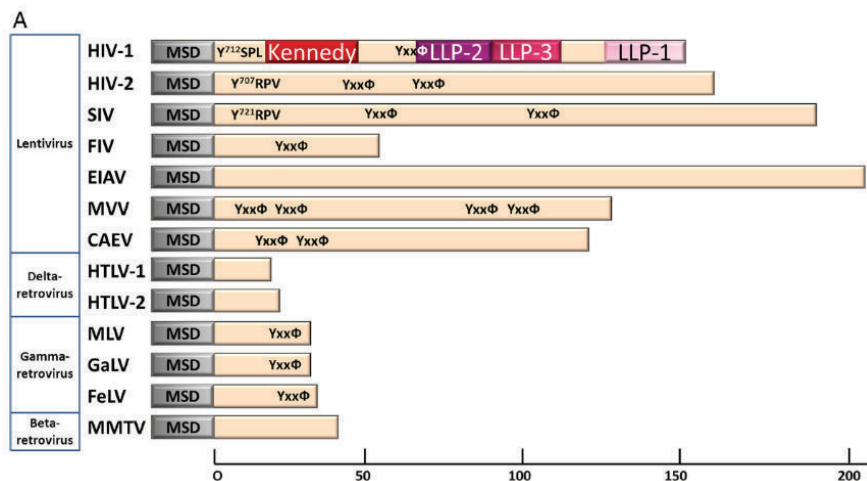
Figure 23. Représentation de la glycoprotéine transmembranaire gp41. Mise en évidence de la région MPER en orange, tandis que les sites glycosylés sont représentés par des arbres (341).

L'**ectodomaine** est la structure déterminante de l'étape de fusion car il contient le peptide de fusion (143, 144), ainsi que les domaines HR1 et HR2 (146) qui forment le 6-HB conduisant à la fusion (**section 3.1**). Ces structures sont conservées entre les différents sous-types et sont la cible du seul inhibiteur de fusion actuellement disponible, l'Enfuvirtide. La région MPER (« membrane-proximal external region ») est située juste avant le domaine transmembranaire et est constituée d'environ 24 acides aminés. Cette région riche en résidus tryptophane est impliquée dans le processus de fusion, bien que les mécanismes ne soient pas connus. Elle est particulièrement conservée, ce qui explique qu'elle est la cible d'anticorps neutralisants (347). Le **domaine transmembranaire** est constitué d'environ 25 acides aminés et sert d'ancrage au complexe Env. La **queue intracytoplasmique de gp41** (gp41CT) est détaillée dans la section suivante.

6.3. La queue intracytoplasmique de gp41 (gp41CT)

6.3.1. Structure

La queue intracytoplasmique de l'enveloppe (gp41CT) située en aval du domaine transmembranaire est constituée de diverses structures présentant des propriétés biophysiques distinctes. gp41CT présente une longueur d'environ 150 acides aminés, elle est particulièrement longue par rapport aux gp41CT d'autres rétrovirus [**FIGURE 24A**] (348). Malgré des différences de séquence [**FIGURE 24B**], la structure de gp41CT est conservée.



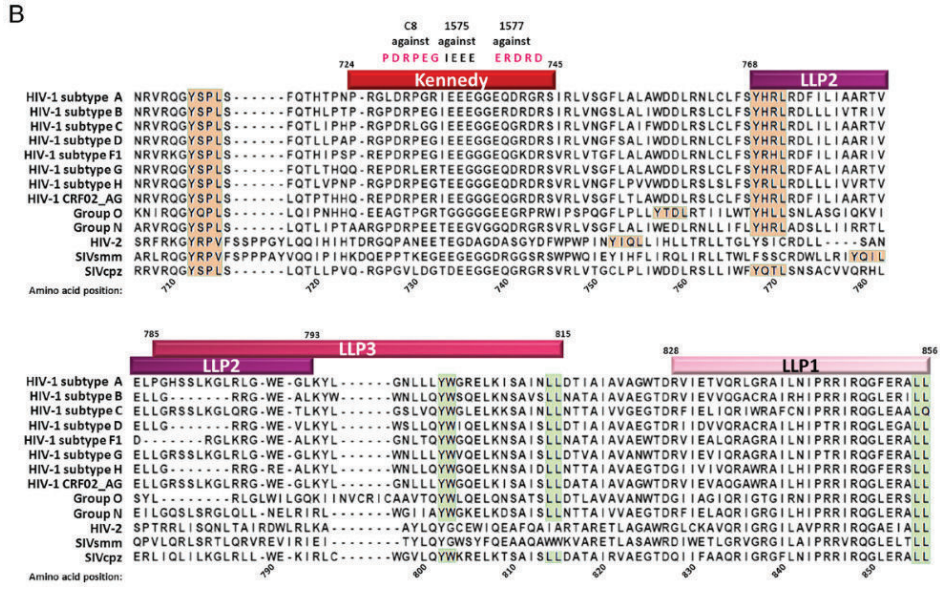


Figure 24. Comparaison de la longueur de la queue intracytoplasmique de divers rétrovirus et alignement de séquences de divers sous-types de VIH, groupes ou types. (A) Comparaison de la longueur de gp41CT et mise en évidence des motifs conservés YxxΦ. HIV, virus de l’immunodéficience humaine; SIV, virus de l’immunodéficience simienne; FIV, virus de l’immunodéficience féline; EIAV, virus de l’anémie infectieuse équine; MVV, virus maedi-visna; CAEV, virus de l’arthrite-encéphalite caprine; HTLV, virus T-lymphotropique humain; MMTV, virus de la tumeur mammaire de la souris; MLV, virus de la leucémie murine; GALV, virus de la leucémie du singe gibbon. (B) Alignement de séquences de gp41CT sous-types A, B, C, D, F1, G, H, CRF02_AG, groupes O et N, type VIH-2 et des virus apparentés SIVmm et SIVcpz. Mise en évidence des motifs conservés (349).

La séquence Kennedy qui suit le domaine transmembranaire est hydrophile et n’adopte pas de structure fixe. Trois structures conservées amphipatiques en forme d’hélices α suivent la séquence de Kennedy, il s’agit des « lentivirus lytic peptides » ou LLP. LLP-2 (AA 768-793) est situé en aval de la séquence Kennedy, LLP-1 (AA 828-856) est à l’extrémité C-terminale de gp41CT (350), tandis que LLP-3 (AA 785-815) est positionné entre LLP-2 et LLP-1, chevauchant partiellement LLP-2 (351). Par ailleurs, LLP-3 est un domaine en fermeture éclair à leucine (« leucine zipper »). Le positionnement des hélices α LLP-1 et 2 présente dans la cellule n’est pas connu, mais leur caractère amphipatique suggère qu’ils longent la membrane phospholipidique, avec la face hydrophobe positionnée sur la face interne de la membrane phospholipidique, alors que leur face hydrophile est orientée vers le cytoplasme (352, 353) [FIGURE 25]. Les premières

propriétés attribuées aux LLPs concernaient la capacité des hélices α à lyser des cellules, d'où le terme de peptides lytiques (354, 355).

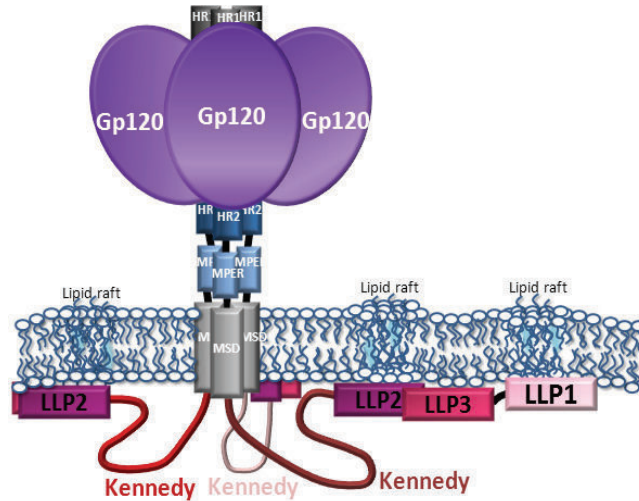


Figure 25. Représentation schématique de la structure du complexe de l'enveloppe avec la mise en avant des divers domaines de la gp41CT (349).

6.3.2. Topologie

Des anticorps dirigés contre des motifs de la séquence Kennedy ont été isolés chez des patients infectés par le VIH suggérant que cette structure pourrait se trouver à l'extérieur du virus (356-358). Dans ce cas, une partie de gp41CT se trouverait à l'extérieur du virion. Dès lors, deux modèles de structure ont été proposés. Le modèle traditionnel soutient que gp41 traverse la membrane une seule fois et que le domaine transmembranaire est une hélice α , tandis que le modèle alternatif, plus controversé, suggère que gp41CT traverse la membrane trois fois par l'intermédiaire 3 feuillets β (356, 359). Le modèle alternatif est difficilement compatible avec le fait que l'un des motifs principaux de signalisation intracellulaire, le motif $Y_{712}SPL$, se trouverait alors à l'extérieur de la cellule. De plus, une étude sur l'émergence de résistances à l'amphotéricine B, un inhibiteur de radeaux lipidiques interférant avec la synthèse d'acides gras, a montré que les variants possédaient un site de clivage par la protéase virale aboutissant à des gp41CT tronquées (360). De tels variants ne peuvent émerger que si le site de clivage dans gp41CT

et la protéase se trouvent tous deux à l'intérieur du virion ou de la cellule infectée. Finalement, un autre modèle a été proposé, selon lequel l'épitope de Kennedy se trouverait à l'intérieur de la cellule cible et à l'extérieur du virion (359, 361). Ces résultats ont néanmoins été contredits par d'autres groupes, qui attribuent la liaison des anticorps à l'enveloppe relarguée dans le plasma ou dans le surnageant de cultures infectées (362, 363). D'autres travaux, basés sur des expériences de biochimie et de transfection, suggèrent également que d'autres parties de gp41CT, et notamment LLP-2, puisse traverser transitoirement la membrane au moment de la fusion et stabiliser le 6-HB (364). Actuellement, la topologie de gp41CT n'est pas encore complètement résolue.

6.3.3. Cellules permissives et cellules non permissives

Certaines études soutiennent que la gp41CT n'est pas indispensable à l'incorporation de l'enveloppe (365, 366), alors que paradoxalement d'autres travaux mettent en avant l'implication de gp41CT dans le processus d'incorporation (367, 368). Cette dichotomie provient du fait que les observations varient en fonction du type cellulaire utilisé pour les expériences. Ainsi certaines lignées cellulaires (HeLa, COS, HEK, M8166, MT4) permettent la synthèse de particules virale infectieuses malgré l'absence de gp41CT (on parle alors de lignées permissives), et étayent ainsi le modèle passif de l'incorporation. Tandis que certaines lignées (H9, CEM, Jurkat) ne permettent pas l'incorporation de l'enveloppe sans la présence de la gp41CT et résultent en des particules non-infectieuses (on parle alors de lignées non-permissives) (366). Au niveau des cellules primaires tel que les LT CD4+ et les macrophages dérivés des monocytes, cibles primaires du virus *in vivo*, l'absence de gp41CT abolit l'incorporation de l'enveloppe et empêche la réplication virale (367, 368). Les phénotypes variables en fonction des lignées cellulaires renforcent l'hypothèse selon laquelle un facteur cellulaire interviendrait dans le processus d'incorporation. Une autre explication serait que dans les lignées permissives, le niveau élevé d'expression de Gag permet de contourner l'absence de gp41CT. Dans ce cas, Env est incorporée passivement dans les virions bourgeonnants (369) (370).

6.3.4. Fonctions

6.3.4.1. Incorporation de Env dans le virion

Pour qu'une particule virale soit infectieuse, il faut qu'elle contienne tous les composants viraux, et notamment, l'enveloppe virale. A l'heure actuelle, il existe 4 modèles d'incorporation de l'enveloppe dans le virion bourgeonnant. Le modèle passif postule que l'enveloppe est incorporée de façon aléatoire sans l'intervention d'un mécanisme cellulaire. Ce modèle est soutenu par le fait que des enveloppes d'autres virus peuvent être incorporées dans les virions à la place de l'enveloppe VIH formant ainsi des pseudovirus (369, 371-374). Par ailleurs, dans certaines cellules, des mutants délétés de gp41CT sont parfaitement incorporés dans les virions et n'affectent pas l'infection (334, 375). Un deuxième modèle postule qu'il y a une interaction directe entre Pr55^{Gag} et Env qui permet le recrutement de l'enveloppe à la membrane pour l'incorporation. Cette hypothèse a été corroboré par deux études biochimiques utilisant des protéines de fusion, de la coimmunoprécipitation ou des peptides *in vitro* (58, 61, 65). Le troisième modèle propose que Env et Pr55^{Gag} se regroupent au niveau d'une plate-forme d'assemblage (par ex. les radeaux lipidiques) facilitant l'incorporation de Env (376). En effet, il a été montré que les deux protéines virales Pr55^{Gag} et Env peuvent s'associer à des régions résistantes aux détergents tels que radeaux lipidiques (377). En l'absence de telles structures, on observe une diminution de la production virale (378). Enfin le quatrième modèle propose que l'interaction entre Env et Pr55^{Gag} n'est pas directe et nécessite l'assistance d'une protéine intermédiaire (379-385) [FIGURE 26]. L'interaction Gag-TIP47-gp41CT dans le processus d'incorporation de l'enveloppe a été suggérée et servirait donc d'intermédiaire entre Gag et Env (385). Toutefois ces résultats ont été infirmés par une autre étude qui observe seulement l'interaction entre Gag et TIP47, mais pas d'influence sur l'incorporation (386).

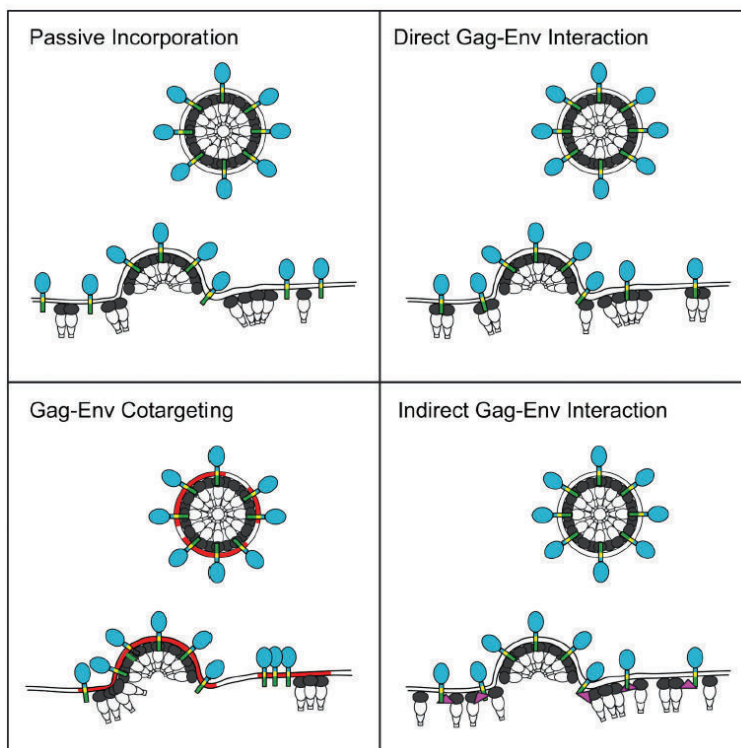


Figure 26. Illustration des modèles d'incorporation de l'enveloppe (334).

Des expériences de production de pseudotypes avec des Env hétérologues (HIV et Ebola) ont montré que les complexes d'enveloppe interagissent aussi entre eux spécifiquement, ne permettant pas d'introduire des enveloppes VIH avec des enveloppes d'autres virus (372, 373). Or ceci va à l'encontre d'un modèle d'assemblage « passif ». Les modèles impliquant une interaction entre Env et Pr55^{Gag} sont ainsi les plus plausibles. En effet, il a été montré que des délétions au niveau du LLP-2 et LLP-3 inhibent l'incorporation de l'enveloppe, cet effet peut être contré par des mutations (V34I) au niveau de la région N-terminale de Pr55^{Gag} correspondant à la matrice (57). A l'inverse, des substitutions dans la matrice diminuent l'incorporation de l'enveloppe (55, 59), et des délétions dans gp41CT permettent de lever cette inhibition (60). Des expériences de mutagenèse dirigée indiquent que le domaine LLP-2 de gp41CT ainsi que les acides aminés L12 et L39 de MA joueraient un rôle dans le processus d'incorporation de l'enveloppe (59, 63). De plus, les gènes *env* et *gag* semblent co-évoluer lors de l'infection par le VIH soulignant encore d'avantage leur interdépendance (387). Enfin, l'étude du bourgeonnement viral dans des

cellules polarisées, telles que les cellules épithéliales, a montré l'accumulation d'Env et de Gag à la phase basolatérale des cellules suivi du bourgeonnement. En absence de Env, Pr55^{Gag} ne polarise pas et reste diffus dans le cytoplasme cellulaire, soulignant encore une fois l'interaction entre Env et Pr55^{Gag} (56, 388).

Bien que l'interaction entre Env et Pr55^{Gag} soit acceptée, ces observations ne permettent pas de déterminer avec certitude s'il s'agit d'une interaction directe ou indirecte.

6.3.4.2. Infectivité

Des mutants tronqués de gp41CT ou tronqués de LLP-1 ou comportant des mutations ponctuelles perdent en capacité infectieuse et ceci a été attribué à des modifications de la structure hélicoïdale de LLP-2 et/ou l'ectodomaine de l'enveloppe (200, 367, 389, 390). Des études biochimiques ont suggéré que l'infectivité dépendait de facteurs tels que l'interaction entre LLP-1 et LLP-2 avec la membrane cellulaire (390-392) ou avec des régions du 6-HB (ectodomaine de gp41) (364, 393). Récemment, il a été montré que des mutants délétés de gp41CT ne perdaient pas leur pouvoir infectieux en tant que virions libres, mais que la transmission de cellule à cellule était fortement diminuée (370).

6.3.4.3. Contrôle de l'infectivité

Les virions immatures sont très rigides et peu infectieux, les étapes d'entrée et de fusion sont fortement réduites (199-201). Le processus de maturation de la particule virale induit par clivage des précurseurs Pr55^{Gag} par la protéase virale confère de la souplesse au virion en libérant MA de CA. La désolidarisation de MA et de CA permet également le mouvement latéral et la coalescence des complexes d'enveloppe à la surface des virions (67, 68, 199, 390, 394). En effet, la fusion du virion avec une nouvelle cellule cible repose sur plusieurs Env engageant les récepteurs et co-récepteurs viraux. Le faible nombre de molécules d'Env exprimées à la surface des virions (83-85) est compensé par la concentration des trimères Env en un foyer (68). Lorsque gp41CT est délétée, le virion conserve sa mobilité latérale, mais n'est plus capable de se rassembler en foyers, ce qui

impacte fortement sa capacité à infecter de nouvelles cellules (67, 68). Des expériences de mutagenèse suggèrent que l'acide aminé K32 de la matrice interagirait avec la gp41CT et influencerait l'infectivité du virus (55). De même, des mutants délétés de gp41CT, de la portion LLP-1 ou encore comportant des mutations de cette région, présentent une capacité de fusion fortement diminuée, aussi bien pour les virions matures (367) qu'immatures (199, 200). Des modifications altérant la capacité de multimérisation de l'enveloppe ont été proposées (391, 394). Ce processus de contrôle de l'infectivité de l'enveloppe par la maturation du virion retiendrait le virus afin de ne permettre qu'aux virus matures, et donc capables d'initier un nouveau cycle de réplication productif, d'infecter une nouvelle cellule.

6.3.4.4. Endocytose et trafic intracellulaire

Suite au clivage des précurseurs trimériques gp160 dans l'appareil de Golgi, les complexes gp120/gp41 migrent vers la membrane phospholipidique de la cellule infectée et sont exprimés en surface. Cette exposition au milieu extracellulaire n'est toutefois que transitoire puisque les enveloppes sont rapidement internalisées grâce à l'interaction de gp41CT avec les protéines du complexe adaptateur de clathrine (AP) et avec d'autres facteurs cellulaires régulant leur trafic à travers le réseau trans-golgien (379, 395, 396). Les motifs comprenant une tyrosine du type YxxL (où x correspond à n'importe quel résidu) interagissent avec les complexes associés aux clathrines 1 à 4 régulant ainsi l'endocytose de protéines de surface. La queue intracytoplasmique de l'enveloppe possède un tel motif juste en aval du domaine transmembranaire. Il s'agit du motif Y₇₁₂SPL qui peut interagir avec les AP-1 à AP-3 (379, 380, 397, 398). Ce motif est extrêmement conservé à travers les groupes et sous-types de VIH soulignant l'importance de ce motif (349). L'interaction du motif Y₇₁₂SPL avec AP-1 permet de réguler le transport de l'enveloppe à travers le réseau trans-golgien puis de la diriger vers la face basolatérale des cellules polarisées (380, 388). D'autre part, le motif Y₇₁₂SPL induit l'internalisation de l'enveloppe en interagissant avec la chaîne μ de AP-2 suivi du transport de l'enveloppe vers un compartiment endosomal ou lysosomal (380, 397). Bien que l'enveloppe ait tendance à être rapidement endocytée après l'expression en surface, l'accumulation de Gag peut bloquer l'endocytose et inclure

ces complexes d'enveloppe dans le nouveau virion en cours de bourgeonnement (382, 395). De plus, le précurseur Pr55^{Gag} peut lui aussi lier les AP, et cette liaison implique les résidus Y₁₃₂ et V₁₃₅ situés à la jonction entre la matrice et la capsid. Par ailleurs, l'interaction de Pr55^{Gag} avec AP-1 et AP-3 contribue à la production virale grâce au transport de Pr55^{Gag} jusqu'aux sites d'assemblages (383, 399, 400). Il est donc possible que les protéines AP jouent le rôle d'intermédiaire dans l'interaction Env/Pr55^{Gag}.

Un deuxième motif d'endocytose a été décrit : il s'agit du motif dileucine LL₈₅₆ à l'extrémité C-terminale de gp41CT. Ce motif LL semble jouer un rôle dans l'internalisation de l'enveloppe ainsi que dans le trafic cellulaire grâce à une interaction avec AP-2 (401, 402). L'absence de ce motif LL engendre une légère augmentation d'enveloppe à la surface de la cellule infectées, par contre lors de l'abolition des motifs Y₇₁₂SPL et LL₈₅₆, on observe un effet synergétique qui résulte en une considérable diminution d'endocytose (401). Ce motif est assez conservé, sauf dans les sous-types A et C où LL est souvent remplacé par LQ. gp41CT est parsemé d'autres motifs dileucine plus ou moins conservés. La mutagenèse de divers motifs dileucine individuels n'a pas montré d'implication dans l'expression de l'enveloppe, néanmoins l'accumulation des mutations de dileucine réduit la fusogénicité ainsi que l'infectivité du virus (403).

Le motif diaromatique Y₈₀₂W₈₀₃ situé dans LLP-3 est particulièrement conservé dans les différents sous-types de VIH (349). Il joue un rôle clé dans le transport rétrograde de l'enveloppe depuis l'endosome tardif vers le réseau trans-golgien en interagissant avec la protéine cellulaire TIP47 (385, 404). Cette interaction permet donc de réguler la distribution intracellulaire de l'enveloppe (384).

6.3.4.5. Activation cellulaire

Outre son rôle dans la régulation du trafic cellulaire de Env, gp41CT contribue également à l'activation cellulaire, par au moins deux mécanismes. Le premier mécanisme est indirect : gp41CT intercepte des protéines cellulaires qui pourraient interférer avec la transcription. Ainsi, gp41CT interagit avec la protéine Luman, un facteur de transcription de la famille CREB/ATF impliqué dans les réponses au stress. La protéine Luman interfère avec la transcription induite par Tat. En interagissant avec la protéine Luman dans le

réticulum endoplasmique, gp41CT la déstabilise et l'oriente vers les voies de dégradation (405-408). Cette interaction a lieu au niveau des AA 751-768 de gp41CT (407). De façon similaire, gp41CT interfère avec RhoA en interagissant avec la protéine p155-RhoGEF qui l'active. Etant donné que RhoA réprime l'expression de gènes, lorsque la gp41CT interagit avec p155-RhoGEF, la gp41CT interfère avec l'activation de RhoA et favorise la transcription virale (409, 410). La leucine L799 dans LLP-3 serait responsable de cette régulation (409, 410).

Le deuxième mécanisme est direct : Une étude récente a montré que gp41CT active la voie NF- κ B par l'intermédiaire du motif tyrosine Y₇₆₆HRL₇₆₉ situé à l'extrémité 5' de LLP-2 (411). Ce deuxième motif YxxL n'est pas impliqué dans le trafic intracellulaire de l'enveloppe, mais participerait à l'interaction de gp41CT avec la protéine cellulaire TAK1 (« TGF- β -activated kinase 1 ») qui induit la phosphorylation de la sous-unité I κ B. L'interaction gp41CT-TAK1 aboutit à la dégradation de la sous-unité I κ B au niveau du protéasome et permet la translocation nucléaire du facteur de transcription NF- κ B. L'attachement des facteurs NF- κ B sur les deux sites de liaison présents sur le promoteur viral LTR induit l'activation de la transcription virale, augmentant ainsi la réplication virale (412, 413). Alors que la majorité des sous-types de VIH arborent deux sites de liaison pour NF- κ B au niveau de leur promoteur, le sous-type E n'en présente qu'un seul alors que les virus de sous-type C possèdent trois sites de liaison (36). Il est intéressant de remarquer que l'activation induite par l'interaction gp41CT-TAK1 est plus prononcée dans les LT CD4+ moyennement activés que dans les cellules fortement activées (411). Ainsi, gp41CT pourrait participer à activer les lymphocytes T infectés pour promouvoir la réplication virale.

6.3.4.6. Interaction avec des protéines virales et cellulaires

Outre l'interaction directe ou indirecte entre gp41CT et Gag décrite dans le paragraphe précédent (**section 6.3.4.1**), plusieurs observations mènent à penser que la gp41CT pourrait aussi interagir avec la protéine virale Nef (369, 414). Il a été proposé que l'interaction Nef-gp41CT puisse favoriser l'incorporation de l'enveloppe dans les virions (414).

La gp41CT interagit également avec plusieurs protéines cellulaires, bien que ces interactions et leur fonction restent peu connues. LLP-1 interagit avec la protéine calmoduline, un intermédiaire de l'induction de l'apoptose via Fas, participant par là à la destruction des LT CD4+ (415-417). La protéine calmoduline joue un rôle crucial dans les changements conformationnels du groupement myristoyl en 5' de Pr55^{Gag} permettant l'ancrage à la membrane cellulaire. Il est donc possible que l'apoptose induite par l'interaction Env/calmoduline n'assure par une fonction propre dans le cycle de réplication virale mais soit un effet secondaire du fait que les deux protéines se rencontrent à la membrane au moment de l'assemblage. L'endocytose de Env dès son arrivée à la membrane pourrait donc constituer un moyen de limiter cette interaction et de préserver la cellule jusqu'à ce que les différents composants viraux s'assemblent à la membrane et que les particules virales puissent s'assembler et bourgeonner.

La gp41CT peut également interagir avec la protéine α -catenine au niveau de la fermeture éclair à leucine LLP-3 (418). Or cette protéine est impliquée dans l'adhésion des cellules, suggérant que l'interaction gp41CT- α -catenine puisse être importante pour la transmission du virus (418, 419).

L'interaction de gp41CT avec le complexe prohibitines 1 et 2 semble aussi importante pour la transmission virale (420). En effet, l'élimination des régions de LLP3 impliquées dans la liaison au complexe prohibitines induit le même phénotype que la délétion de gp41CT, à savoir propagation virale dans des lignées permissives mais inhibition de la propagation virale dans les lignées non-permissives (420). Ce complexe pourrait ainsi être déterminant pour la réplication virale dans les cellules non-permissives, toutefois le mécanisme sous-jacent reste inconnu.

Une étude a montré que la gp41CT pouvait aussi interagir avec la protéine PRA1 (prenylated Rab acceptor 1) (421), néanmoins les niveaux d'incorporation, infectivité ou production virale ne sont aucunement influencés par cette interaction (422).

En conclusion, bien que le rôle de gp41CT ait longtemps été considéré comme secondaire, limité à l'incorporation de Env dans le virion, ce domaine semble également contribuer à moduler l'environnement cellulaire pour favoriser sa réplication.

Objectifs du travail de thèse

Malgré son rôle essentiel dans le cycle de réplication virale, le domaine intracytoplasmique de l'enveloppe a été beaucoup moins étudié que l'ectodomaine. D'importants polymorphismes spécifiques aux divers sous-types de VIH peuvent être observés et notamment au niveau de l'enveloppe virale. Un grand intérêt a été porté à la sous-unité de surface (gp120) ainsi qu'à l'ectodomaine de gp41 (gp41EC), mais cette variabilité n'a pas encore été investiguée dans le cadre de la queue intracytoplasmique de l'enveloppe (gp41CT). Ce projet de thèse vise à déterminer l'impact de la gp41CT sur l'infectivité et la réplication virale en tenant compte des polymorphismes inhérents au sous-type viral.

La majorité des données acquises jusqu'à présent concernant la gp41CT relèvent d'observations biochimiques ou de mutagenèse ou encore des délétions de la gp41CT. Or il a été montré que le phénotype induit par ces mutations ou délétions varie en fonction du type cellulaire utilisé dans les expériences. Certaines cellules, dites permissives, permettent la réplication virale même en absence de gp41CT (ex. les cellules HEK, HeLa, M8166), alors que les cellules dites non-permissives ne la permettent pas (ex. les cellules primaires). De plus, les polymorphismes n'ont jamais été pris en compte puisque les expériences ont été réalisées avec des souches de référence de sous-type B (ex. NL4.3).

Nous nous sommes concentrés sur les sous-types C, le plus répandu dans le monde, et le sous-type B, le plus étudié. Afin d'investiguer l'impact des polymorphismes dans gp41CT, nous avons utilisé des enveloppes VIH primaires de sous-type C et des enveloppes de sous-type B issues de patients infectés. Ces enveloppes primaires ont été introduites dans un clone NL4-3 délété de *env* par recombinaison homologue. Nous avons développé deux vecteurs NL4.3 permettant de générer des virus recombinants exprimant soit l'enveloppe entière (gp160) issue des quasi-espèces de patients soit l'ectodomaine de l'enveloppe (gp140) issu des mêmes quasi-espèces de patients avec gp41CT de NL4.3. Ces paires de virus recombinants ont ensuite été utilisées pour infecter des lymphocytes T CD4+ et des macrophages dérivés de monocytes (MDMs), qui sont des cibles privilégiées

du VIH. Les lymphocytes T CD4+ ont été infectés soit par du virus libre, soit par l'intermédiaire de cellules dendritiques présentant le virus.

Ce modèle permet d'observer l'impact des polymorphismes des sous-types B et C dans les deux types cellulaires sans introduire de mutation ou de délétion, mais également de préserver la diversité des quasi-espèces de patients.

RESULTATS

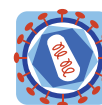
1. The frantic play of the concealed HIV cytoplasmic tail

Santos da Silva E, Mulinge M, Perez Bercoff D.

Publié dans *Retrovirology*, 2013

2.1. Résumé

Tous les rétrovirus possèdent une enveloppe virale qui leur est indispensable pour l'entrée dans une cellule cible. L'ancrage de l'enveloppe est assuré par la sous-unité transmembranaire de la glycoprotéine d'enveloppe. La partie intracytoplasmique de l'enveloppe du VIH (gp41CT) est bien plus longue (~150 acides aminés) que celle d'autres rétrovirus (~40 acides aminés). Pendant longtemps, cette région de l'enveloppe fut l'objet de très peu d'études, en effet des résultats contradictoires compliquaient l'interprétation et des virus tronqués de gp41CT semblaient tout aussi compétents que les virus entiers. La gp41CT était principalement connue pour ses propriétés antigéniques et cytolytiques. La découverte que gp41CT pouvait aussi intervenir dans le processus d'assemblage et d'incorporation de l'enveloppe par l'intermédiaire de l'interaction directe ou indirecte avec le précurseur Pr55^{Gag} a permis d'attirer l'attention sur d'autres possibles propriétés ou interactions importantes de gp41CT. Cette revue relate les connaissances actuelles sur cette région de l'enveloppe : sa structure, sa topologie qui reste controversée, son rôle dans la régulation du trafic intracellulaire de l'enveloppe, mais aussi son implication dans l'activation cellulaire et enfin, les interactions avec des protéines cellulaires et virales. Cette revue porte une attention particulière aux polymorphismes présents à certains sites régissant les activités de gp41CT décrites ci-dessus.



REVIEW

Open Access

The frantic play of the concealed HIV envelope cytoplasmic tail

Eveline Santos da Silva, Martin Mulinge and Danielle Perez Bercoff*

Abstract

Lentiviruses have unusually long envelope (Env) cytoplasmic tails, longer than those of other retroviruses. Whereas the Env ectodomain has received much attention, the gp41 cytoplasmic tail (gp41-CT) is one of the least studied parts of the virus. It displays relatively high conservation compared to the rest of Env. It has been long established that the gp41-CT interacts with the Gag precursor protein to ensure Env incorporation into the virion. The gp41-CT contains distinct motifs and domains that mediate both intensive Env intracellular trafficking and interactions with numerous cellular and viral proteins, optimizing viral infectivity. Although they are not fully understood, a multiplicity of interactions between the gp41-CT and cellular factors have been described over the last decade; these interactions illustrate how Env expression and incorporation into virions is a finely tuned process that has evolved to best exploit the host system with minimized genetic information. This review addresses the structure and topology of the gp41-CT of lentiviruses (mainly HIV and SIV), their domains and believed functions. It also considers the cellular and viral proteins that have been described to interact with the gp41-CT, with a particular focus on subtype-related polymorphisms.

Keywords: HIV-1, Lentiviruses, Envelope gp41 cytoplasmic tail, Envelope trafficking, Viral assembly, Envelope intracellular interacting factors

Review

Background: Env and the entry process

The main target cells for the Human Immunodeficiency Virus type 1 (HIV) and Simian Immunodeficiency Virus (SIV) are CD4-expressing cells, namely CD4⁺ T lymphocytes, dendritic cells (DCs) and macrophages. The first step of the viral cycle, involving anchoring to the target cell membrane and entry, is mediated by the envelope glycoprotein (Env). Env is composed of two non-covalently linked subunits, SU (surface glycoprotein or gp120) and TM (transmembrane glycoprotein or gp41), displayed as heterotrimeric spikes on the surface of virions and infected cells [1-6]. The two Env subunits are the products of the proteolytic cleavage of a highly glycosylated gp160 precursor protein by the cellular protease Furin in the Golgi apparatus. gp120 is responsible for binding to the CD4 receptor and the coreceptor expressed at the cell surface of target cells. gp120 is composed of several variable and constant regions; it comprises a heavily glycosylated outer

domain and an inner domain, which are linked by a structure called the bridging sheet. Mature Env thus forms a cage-like structure with an interior void; this cage-like structure is believed to restrict antibody access [7]. gp41 is non-covalently linked to gp120 and the N-terminal ectodomain of gp41 contains a hydrophobic, glycine-rich fusion peptide that mediates fusion of the viral and target cell membranes. Binding of gp120 to the CD4 receptor induces conformational changes that expose the third hypervariable region (V3 loop) of gp120, which in turn binds one of the co-receptors, CCR5 or CXCR4 [8-12]. Binding to the co-receptor triggers further conformational changes within gp41, which adopts the so-called "fusion-active" state required for bringing the viral and cellular membranes into close contact and allowing fusion. These conformational changes involve two leucine zipper-like motifs, the heptad repeat 1 (HR1) and heptad repeat 2 (HR2): HR2 folds in an anti-parallel fashion onto the pre-formed trimer of HR1 helices, allowing HR1 and HR2 to assemble into a highly stable antiparallel six-helix bundle structure, which juxtaposes the viral and cellular membranes, and allows fusion of the viral and cellular membranes [2,3,5,6,13].

* Correspondence: danielle.perezbercoff@crp-sante.lu
Laboratory of Retrovirology, CRP-Santé, 184 Val Fleuri, Luxembourg L-1526, USA

Most of the Env precursor is retained in the endoplasmic reticulum (ER) or the cis-Golgi compartments and only a small proportion progresses through the trans-Golgi network (TGN) and reaches the cell membrane [14-17]. As it is synthesized and oligomerizes in the rough ER (RER), Env is extensively N- and O-glycosylated, sulfated and palmitoylated concomitantly with its translation and transit through the TGN [1,18-24] reviewed in [25]. Glycosylation of gp120 accounts for nearly half of its molecular mass. This substantial glycosylation is believed to ensure correct protein folding and to favor immune escape by providing a glycan shield against neutralizing antibodies. As the disease progresses, viral strains lose potential N-glycosylation sites (PNGs) and display notably reduced glycosylation; these strains are consequently less well shielded from neutralizing antibodies [26,27]. Nonetheless, such viral strains appear late during infection, concomitantly with the decline in CD4⁺ T cell counts, such that the immune system is unable to trigger an efficient B-cell response; consequently, evading the immune system is no longer necessary [28].

Env is the most variable region of the HIV genome, and inter-subtype diversity is typically up to 25-35%. This variability is not evenly distributed throughout the *env* gene: the gp120 ectodomain is the most variable, the gp41 ectodomain is the least variable, and the gp41 cytoplasmic tail (gp41-CT) lies somewhere in between, with an intermediate level of sequence diversity and phylogenetic evolution [29]. Experimental data obtained from peptides and by sequence analysis and modeling indicate that the structural and physicochemical properties of the gp41-CT are surprisingly highly conserved despite the sequence variations. Presumably, this reflects constraints on its evolution imposed by crucial functional roles. However, these roles have mostly been explored using the NL4-3 reference strain or synthetic peptides.

Aims of the review

The ectodomain of Env has been the subject of much interest and substantial investigations. The structural and functional characterization of the HIV and SIV gp41-CTs has received far less attention, and indeed, there are numerous contradictory results. Early studies of the gp41-CT mainly addressed the immunogenic properties of the Kennedy epitope, or the viroporin properties of the α -helical lentiviral lytic peptides LLP-1, LLP-2 and LLP-3 and their role in viral incorporation and infectivity. The discovery that the gp41-CT interacts with the p55^{Gag} precursor during viral assembly and ensures Env incorporation into virions has led to a reconsideration of the functional importance of this domain. It was, nevertheless, only very recently that its role in modulating Env expression and viral infectivity through a plethora of interactions with cellular partners started to

be appreciated. Three recent reviews are relevant: they address the role of the HIV-1 gp41-CT in Env trafficking, incorporation and viral assembly [25], the biophysical properties and topology of the gp41-CT [30] and the interactions of the gp41-CT with cellular factors [31]. Parallel spectacular advances in cell and virion imagery, and very recent studies on the biophysical characteristics of mature and immature virions based on Atomic Force Microscopy (AFM) [32] and STED microscopy [33] provide new insights into the contribution of the gp41-CT to viral fusion efficiency. Here, we review current knowledge on the gp41-CT and its believed structure and function as a regulator of viral infectivity. In particular, we focus on variability and subtype-related specificities, as most studies on the gp41-CT are based on the NL4-3 reference and mutants thereof, in sharp contrast to studies on the Env ectodomain.

Structural determinants and topology of the gp41-CT

Structure of the gp41-CT

The gp41-CT of lentiviruses is unusually long (~150 amino acids) and substantially longer than the tails of other retroviruses (< 50 AA) [34] (Figure 1A). Although amino acid (AA) sequence identity is not conserved, both secondary structures and various endocytic and trafficking motifs are highly conserved across HIV-1 clades and even among lentiviruses in general [29,30] (Figure 1B). Immediately downstream from the membrane-spanning domain, gp41-CT has a loop containing the highly immunogenic Kennedy epitope (KE) which is not believed to adopt any particular conformation. The KE in fact harbors three consecutive epitopes (P₇₂₈DRPEG₇₃₂, I₇₃₃EEE₇₃₆ and E₇₃₉RDRD₇₄₃) [35]. Antibodies against the KE (SR1) display neutralizing activity against the infectivity of free virus and impair cell-to-cell spread by inhibiting Env-mediated fusion [36-38]. ERDRD is a conformational epitope and elicits production of neutralizing antibodies; the linear IEEE epitope seems to deflect the humoral response to favor viral immune escape from a neutralizing response against ERDRD [35].

The KE is followed by three α -helical motifs named lentiviral lytic peptides (LLP)-2 (AA 768 to 793 of NL4-3), LLP-3 (AA 785 to 807) and LLP-1 (AA 828 to 856) [39-41] (Figure 1B). LLP-3 partly overlaps LLP-2, and has been less studied than the other two LLP domains. Biochemical analyses with LLP peptides indicate that although primary amino acid identity is not conserved across clades (with the exception of the more conserved LLP-1), these domains adopt an α -helical structure in a membrane-mimetic environments and random coil conformations in an aqueous environment [29,30]. Early studies focused on determining the role and biochemical properties of these domains; they were based on synthetic peptides and three-dimensional computer modeling [39,40,42] or point mutations and truncation mutants of the gp41-CT, analyzing Env incorporation into virions [43-46] (detailed below),

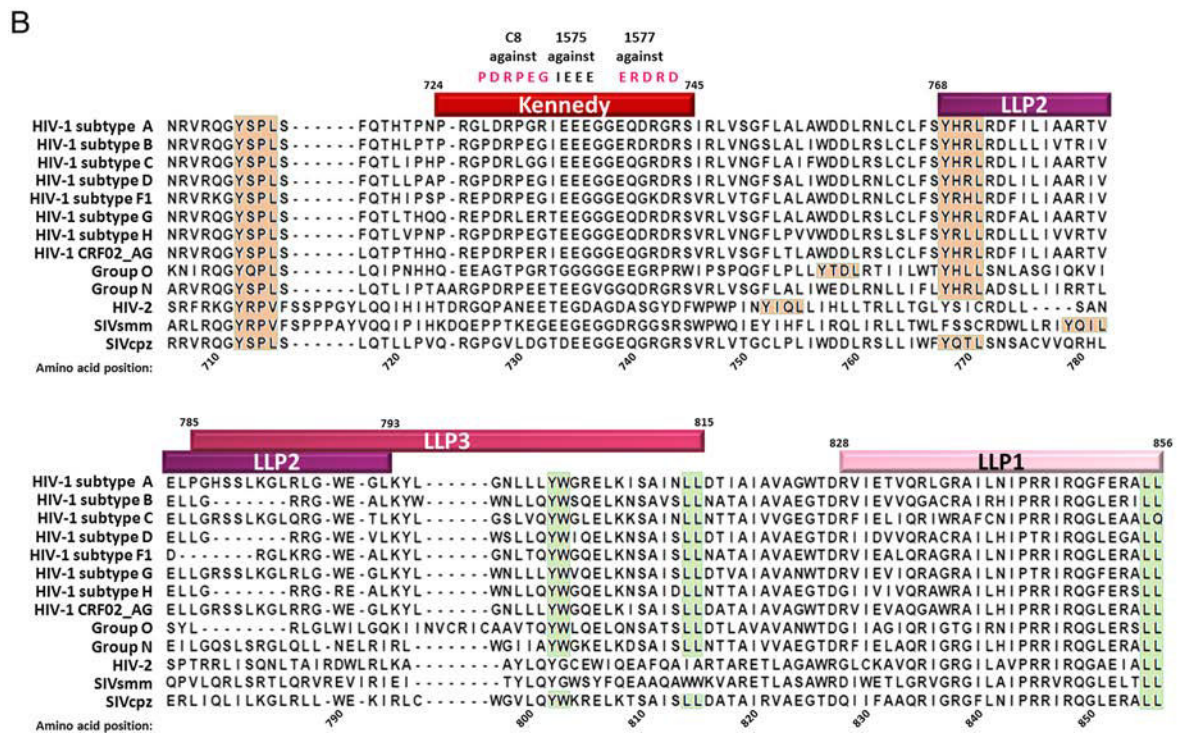
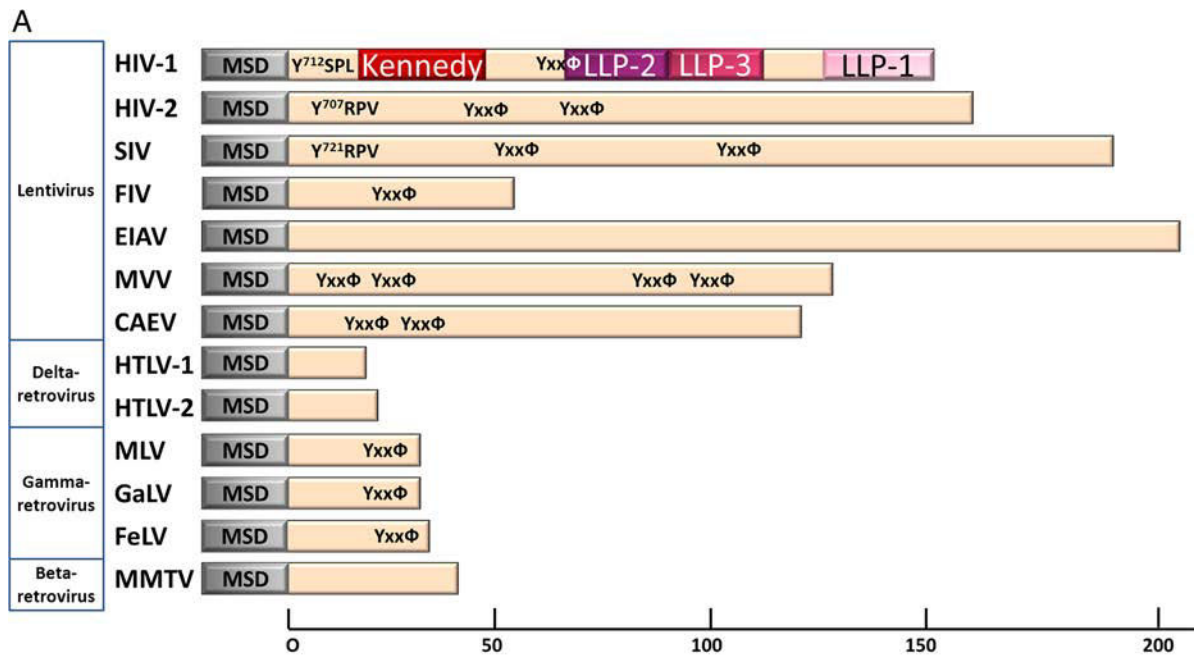


Figure 1 Lengths of the cytoplasmic tails of various retroviruses and sequence alignment of different HIV-1 subtypes, clades and types. **(A)** Comparison of the lengths of the gp41-CT of Human Immunodeficiency Virus type 1 (HIV-1), HIV type 2 (HIV-2), Simian Immunodeficiency Virus (SIV), Feline Immunodeficiency Virus (FIV), Equine Infectious Anemia Virus (EIAV), Maedi-Visna Virus (MVV), Caprine Arthritis Encephalitis Virus (CAEV), Human T-lymphotropic Virus type 1 (HTLV-1), Human T-lymphotropic *Vtype* 2 (HTLV-2), Mouse Mammary Tumor Virus (MMTV), Murine Leukemia Virus (MLV) and Gibbon Ape Leukemia Virus (GaLV); conserved YxxΦ motifs (where Φ represents a hydrophobic residue) are highlighted. **(B)** Sequence alignment of the gp41-CT from various lentiviral (HIV and SIV) types and subtypes: HIV-1 subtypes A (92UG037), B (HXB2), C (ETH2220), D (SE365), F1 (93BR020), G (175), H (90CF056), CRF02_AG (93TH065), clade O (ANT70) and clade N (YBF106), HIV-2 (UC2), SIVcpz (Ptt_04CAM155) and SIVsmm (H635). AA positions are aligned against the NL-3 reference used in most studies of the gp41-CT. Lentiviral lytic peptide (LLP) domains, Kennedy epitopes and antibodies directed against them are shown. Conserved YxxΦ motifs are highlighted in orange; conserved dileucine motifs, and motifs involved in cell trafficking are highlighted in green.

viral infectivity [47] and syncytia formation [46]. In the peptide form, the LLP domains interact with the plasma membrane [41,48,49] and thereby decrease bilayer stability [41,50] or alter the ionic permeability of membranes [48,50,51]. Intracellular localization studies suggested that the LLP helices may lie adjacent to and line the plasma membrane [49] (Figure 2A). These observations have led to the idea that the LLP domains are involved in fusogenicity, immunogenicity, and in conformational regulation of Env accompanying viral maturation and infectivity (see below, section 'Viral stiffness, viral maturation and infectivity').

Functional domains of the gp41-CT

Mutational analyses and work with truncation mutants of specific LLP-1 or LLP2 domains indicate varying Env expression and processing phenotypes, depending on the extent of the truncation and on the cell type. Despite subtle differences in the extent of the truncations studied, it appears that deleting the C-terminal domain of LLP-1 diminishes virion infectivity and cell-to-cell spread, due to decreased Env incorporation into virions [43-46,52] and to diminished Env expression and stability of the gp41/gp120 heterodimeric complex both at the cell membrane and in virions [44,53]. Env protein synthesis and processing in transfected HeLa or HEK293 cells is however unaffected by truncations of the C-terminal domain of LLP-1 [43,46,53]. Larger truncations, i.e. truncation of the full gp41-CT, or the region downstream from LLP-3 (i.e. deleting upstream from LLP-1), or even LLP-1 deletions or point mutations in this region dictate Env fusion capacity of mature [46] and immature virions [47,54]. Some authors attributed this phenotype to failed Env multimerization, a prerequisite for viral infectivity [55,56]; others proposed that truncation of LLP-1 induces modifications of the helical structure of LLP-2 and/or alterations in the structure of the Env

ectodomain, suggesting an inside-out regulation of viral infectivity and fusion capacity [46,47,54,57,58]. Biochemical studies involving the use of synthetic peptides found that viral infectivity is dependent on interactions of LLP-1 and LLP-2 with the cell membrane [49,56,58] and/or with regions of the gp41 ectodomain, and particularly the HR1-HR2 6-helix bundle [29,59,60]. For SIVmac239, truncations of the gp41-CT affected the fusion capacity of Env in HeLa-CD4 cells by changing the conformation of the Env gp41 ectodomain [61] or the amount of gp120 on the surface of the virion due to an unstable gp120-gp41 association [62]. Indirect support for gp41-CT affecting the conformation of the gp41 ectodomain has been provided by studies showing that truncations of the gp41-CT affect the conformation of gp120 [57] and of the gp41 ectodomain [63]; also, such truncations interfere with the accessibility of these domains to antibodies targeting various epitopes including the CD4 or co-receptor binding sites [57] or the Membrane Proximal External Region (MPER) (neutralizing antibodies such as 2F5 and 4E10) [63]. In addition, a L49D mutation in the matrix protein (MA) of the p55^{Gag} precursor, which interacts with the gp41-CT (see below, section 'Viral assembly, Env incorporation into virions and viral infectivity'), is associated with decreased amounts of gp120 at the surface of the virion without affecting gp41 incorporation [64]. This suggests that modifications to the MA/gp41-CT interaction may affect the level of gp120 incorporation [64]. This defect could be reversed by truncating the gp41-CT or disrupting the Y₇₁₂SPL endocytic motif (i.e. impairing Env down-regulation, see section 'Env trafficking and sorting through the TGN'). This implies that altering the interaction between p55^{Gag} and the gp41-CT affects the conformation of gp41 and thereby the incorporation of gp120 into virions [64]. Although these reports are descriptive and the proposed mechanisms are divergent, these studies agree that

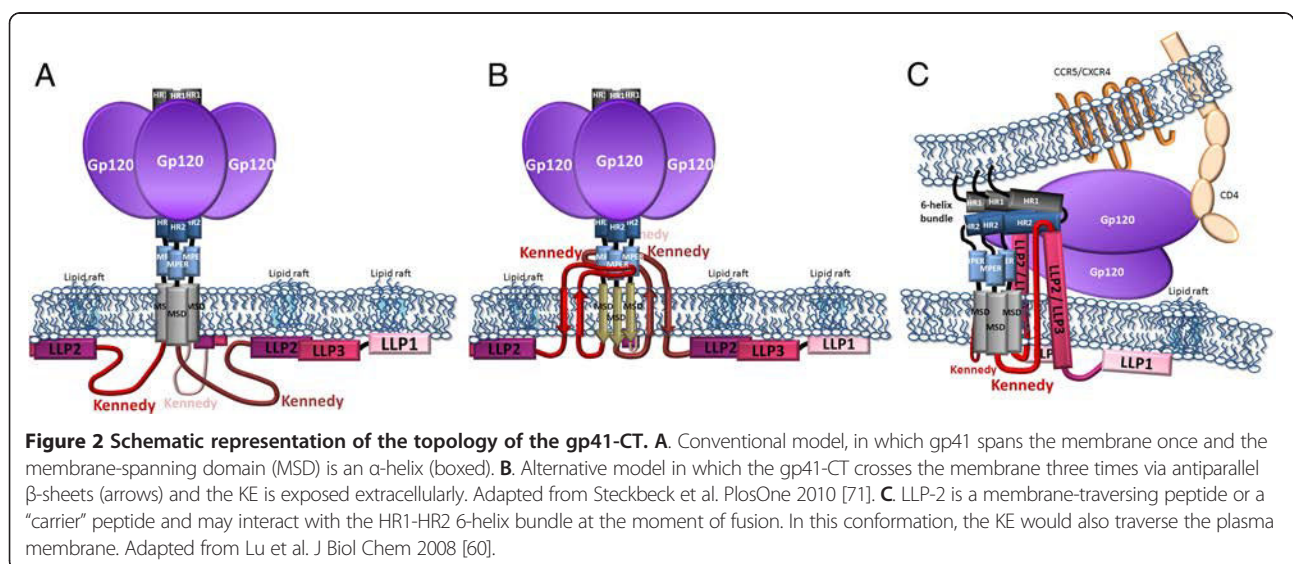


Figure 2 Schematic representation of the topology of the gp41-CT. A. Conventional model, in which gp41 spans the membrane once and the membrane-spanning domain (MSD) is an α -helix (boxed). **B.** Alternative model in which the gp41-CT crosses the membrane three times via antiparallel β -sheets (arrows) and the KE is exposed extracellularly. Adapted from Steckbeck et al. PlosOne 2010 [71]. **C.** LLP-2 is a membrane-traversing peptide or a "carrier" peptide and may interact with the HR1-HR2 6-helix bundle at the moment of fusion. In this conformation, the KE would also traverse the plasma membrane. Adapted from Lu et al. J Biol Chem 2008 [60].

the gp41-CT may modulate the conformation of the Env ectodomain [46,47,54,57,58] and the stability of the 6-helix bundle [29,59,60] thereby affecting fusion capacity and sensitivity to neutralization [57,63].

The dileucine motifs that span the gp41-CT LLP helices play a role in viral replication, as has been demonstrated through a cumulative mutagenesis approach [65]. Bhakta et al. reported a cumulative effect on the infectivity of free virions and on viral propagation that did not correlate with modifications in viral incorporation or expression at the cell membrane [65]. Importantly, disruption of individual dileucine motifs had only a moderate impact, whereas the cumulative effect was substantial, suggesting a degree of flexibility, and tolerance of mutations of single dileucine motifs [65]. This study reconciles early and more recent reports on the role of the gp41-CT, as it suggests that impaired replicative capacity and viral spread may result from disrupting the hydrophobic properties of the LLP-2 α -helix: this disruption may impair interactions with the cell membrane and/or membrane translocation and interaction with the HR1-HR2 6-helix bundle [65]. Another possible explanation is that altering the biochemical amphipathic properties of the gp41-CT, or simply disrupting the LL dileucine domains, interferes with Env/Env interactions and coalescence [33,55], thereby affecting infectivity. This issue requires further investigation. Intriguingly, a highly conserved seven AA insertion (NSLKGLR) between R₇₈₇ and R₇₈₈ (LLP-2 domain) is found in many subtype C strains where the dileucine motifs spanning the gp41-CT are disrupted (ESS, MM and DPB, unpublished observation). The two supplementary α -helical turns result in three arginine residues being aligned side-by-side on the hydrophilic face of the α -helix. Whether adding these two α -helical turns to LLP-2 modifies its biophysical properties remains to be established.

The gp41-CT also harbors many functional determinants, including internalization motifs, for example: two Yxx Φ motifs (Φ representing a hydrophobic AA, generally L in HIV and V in SIV), one immediately downstream from the transmembrane domain (membrane-proximal Yxx Φ) and one within LLP-2; a Y₈₀₂W₈₀₃ diaromatic motif within LLP-3; two inhibitory sequences named IS1 (AA 750 to 763 of HXB2) and IS2 (AA 764 to 785); and the various dileucine (LL) motifs spanning the gp41-CT (Figure 1B). The role of these motifs in Env trafficking and protein-protein interactions is considered in more detail below (section 'Interactions of the gp41-CT with cellular factors and intracellular regulators').

Topology of the gp41-CT

gp41 is generally thought to be a type I membrane-spanning protein, with one α -helical membrane spanning

domain (MSD) (AA 671–705) [66]. However, alternative models have been proposed, in which the gp41-CT lies partly outside the virus or the infected cell, crossing the membrane three times [38,60,67–71] (Figure 2B and C).

The Kennedy Epitope

Serum from HIV-infected individuals contains antibodies against epitopes within the KE. This has led to the hypothesis that this region is exposed extrvirally and extracellularly, consistent with gp41 spanning the membrane three times rather than once [38,67–70]. In this model, the membrane-spanning domains form three antiparallel β -sheets [30,71] (Figure 2B). In support of this model, IgG against the gp41-CT-encoded epitopes P₇₂₈DRPEG₇₃₂, I₇₃₃EEE₇₃₆ and E₇₃₉RDRD₇₄₃ bound and neutralized free virions, and protease digestion inhibited antibody binding [69]. Other studies showed that neutralization by these antibodies extended to cell-to-cell fusion [38]. However, this model places the Y₇₁₂SPL internalization motif outside the cell such that it would not allow and ensure an interaction of Env with cellular partners (see section 'Env trafficking and sorting through the TGN'). Moreover, experiments performed with amphotericin B methyl ester (AME), a cholesterol-binding compound that interferes with virion-cell membrane fusion, showed that AME-resistant HIV variants contained mutations within the gp41-CT that create a cleavage site for the viral protease within the Y₇₁₂SPL motif [72]. By cleaving the gp41-CT, HIV mimics the shorter gp41-CT of other retroviruses to restore Env fusogenicity [72,73]. (In the case of SIV, AME-resistant SIV gp41-CT displayed a premature STOP codon [72], and the topology of the gp41-CT does not affect its capacity to arise). Cleavage of the gp41-CT by the viral protease is not compatible with this epitope lying outside the virion. A more dynamic view of gp41-CT topology has recently been proposed to reconcile these observations [71] (reviewed in [30]). This model posits that the KE can adopt various distinct topologies: inside the virion, the KE would lie intravirally (Figure 2A), whereas in transfected/infected cells, the KE would lie outside the cell (Figure 2B) [71]. In support of this model, the authors present evidence that the gp41-CT of all HIV clades and of SIV share a highly conserved enrichment in arginine over lysine [29,40]. Arginine-rich peptides may be located within lipid environments and may readily cross biological membranes due to their guanidium group; they can also carry soluble proteins across membranes [74–77]. This dynamic model is compatible with the viral protease cleaving the gp41-CT in the virion under AME selective pressure. Note, however, that the authors compared the location of the KE in virions (i.e. in entities containing MA) with that in cells transfected with a construct encoding Env only rather

than in cells transfected with a full HIV coding sequence. The interaction between the gp41-CT and p55^{Gag} (see section 'Interaction of the gp41-CT with Gag') is not taken into account in this model and it may not be excluded that the absence of Gag in the experimental setup may bias the topology of the gp41-CT, including that of the KE.

The dynamic topology model still predicts that the Y₇₁₂SPL internalization motif is outside the cell: this is not consistent with the regulation of Env expression at the plasma membrane of infected cells through adaptor protein (AP)-mediated internalization (see section 'Env trafficking and sorting through the TGN'). The authors [71] do not address this point. This dynamic model has been opposed on the grounds that the model for a dynamic intra/extra viral localization of the KE is based solely on indirect observations, and in particular labeling with antibodies raised against this epitope [78]. In a very recent study based on the SIV gp41, it was convincingly shown that the reported surface labeling was due to the release of Env from expressing cells into the supernatant; shed Env subsequently attaches to the surface of cells in the culture [78]. The authors report that transferring supernatant from Env-transfected cells to mock-transfected cells was sufficient for labeling of the mock-transfected cells. Furthermore, the PNG in the KE was never glycosylated, and both serum and monoclonal antibodies against the KE failed to neutralize even highly neutralization-sensitive strains [78].

LLP-2

Biochemical studies led to the suggestion that LLP-2 may also be a traversing peptide, and that it interacts with the extracellular 6-helix bundle at the moment of fusion [60] (Figure 2C). LLP-2 exposure may be induced by CD4 and CCR5 binding, and be highly transient, as implied by the observations that antibody binding is observed at 31.5°C but not at 37°C and that LLP-2 peptides bind peptides mimicking the 6-helix bundle in biochemical experiments [60]. The mechanisms underlying this crossing of the membrane are unclear; the exact extent of membrane crossing by LLP-2 has not been investigated, so this remains speculative. As this study was based on a biochemical approach using peptides and antibody binding, the possibility of a biased interpretation due to labeling of shed Env subsequently bound to the cell surface, as described by Postler *et al.* for KE epitopes [79], cannot be excluded. Furthermore, the absence of other viral proteins from the Env-transfected cell system pleads for caution in interpreting the results, as indicated by the authors themselves [60]. Furthermore, Steckbeck *et al.* described the introduction of known epitopes into LLP-2, and failed to detect any extracellular or extraviral labeling, whereas similar epitopes inserted within KE epitopes were successfully detected [71].

These diverse results are such that caution is still required for any conclusions concerning a cell-versus-virion conformation of Env. It remains unclear whether there are such transient rearrangements and biochemical adjustments of Env in response to modifications in the membrane lipid or protein environment during viral assembly or at the moment of fusion; the corresponding observations may be experimental artifacts that do not reflect to biological realities. No images are available of the conformation and distribution of Env gp41-CT in the immature forming virion or in the mature virion. If the gp41-CT does adopt different topologies and if both the KE and LLP-2 are indeed membrane-traversing peptides, it would be interesting to investigate whether both can be found extracellularly at the same time, or whether such a conformation would be too highly unfavorable.

Open questions

A series of questions remain unanswered. For example, why is there such strong constraint on the structure of the LLP peptides but not on their sequences? How does this structure favor infectivity, motility or clustering of the Env in the virion? Does the conformation of the gp41-CT change upon virion maturation p55^{Gag} cleavage by the viral protease? Does the gp41-CT really adopt different topologies? If so, what triggers the switch from an intraviral/intracellular to extraviral/extracellular localization of KE or of LLP-2, and how are the KE and LLP-2 retained intravirally or "freed"? And what would the function of transiently exposing the highly immunogenic KE be? Alternatively, if the immune response elicited by the KE is due to Env shedding rather than to extracellular exposure, could this be a viral strategy to evade the immune system, by providing immunodominant domains that are subsequently not accessible to antibodies as they are located inside the virion and infected cells?

Viral assembly, Env incorporation into virions and viral infectivity

Env is produced in the RER and the Golgi network, whereas the p55^{Gag} and p160^{GagPol} protein precursors and the accessory proteins are synthesized by cytosolic ribosomes. Viral assembly thus requires viral components synthesized in distinct cellular compartments and the genomic RNA to come together and to interact at the budding interface. Viral assembly is a highly regulated phenomenon, dictated by intracellular trafficking.

HIV assembly and budding occurs at the plasma membrane (PM) [80-84] in detergent-resistant-membrane lipid raft-enriched domains [73,85-87] (Figure 3). The p55^{Gag} precursor is a polyprotein, including the structural proteins of HIV, namely the matrix protein (MA) lining the cell-derived viral envelope, the capsid protein (CA), constituting the viral cone-shaped capsid containing the viral

RNA genome, the nucleocapsid (NC, p7), associated with the viral RNA genome, and p6, regulating the viral protease, as well as two spacer peptides, SP1 and SP2. MA drives and regulates p55^{Gag} targeting to the PM by preferentially interacting with phosphatidylinositol 4,5 bisphosphate [PI(4,5)P2] [88-90]. Once it has reached the PM, the p55^{Gag} precursor is anchored by its N-terminal myristoylated moiety [91,92] and stabilized by basic residues 84–88 [89,90,92]. The myristoylated moiety is cotranslationally coupled to the p55^{Gag} MA domain, and its exposure is regulated by binding to calmodulin: binding to calmodulin induces conformational changes within MA [93,94] which lead to p55^{Gag} binding to lipid rafts [95-97], where it oligomerises [73,86,87,98], through the C-terminal domain of CA and the α -helical spacer peptide SP1,

leading to the formation of the immature lattice [99,100]. In solution, MA within the p55^{Gag} precursor assembles in trimers [101]. In cholesterol-rich membranes composed of phosphatidylcholine, cholesterol and [PI(4,5)P2], myristoylated MA assembles in hexamers of trimers interconnecting the radially oriented immature p55^{Gag} lattice [99,102,103].

Four models of viral assembly and budding have been described exhaustively in three recent reviews [25,104,105] and will therefore not be addressed in detail again here. Briefly, the “Passive incorporation model” posits that HIV assembly occurs by random co-localization of p55^{Gag} and Env at the cell membrane; it is supported by the observations that a number of cellular membrane proteins are incorporated into virions without stringent restriction, that

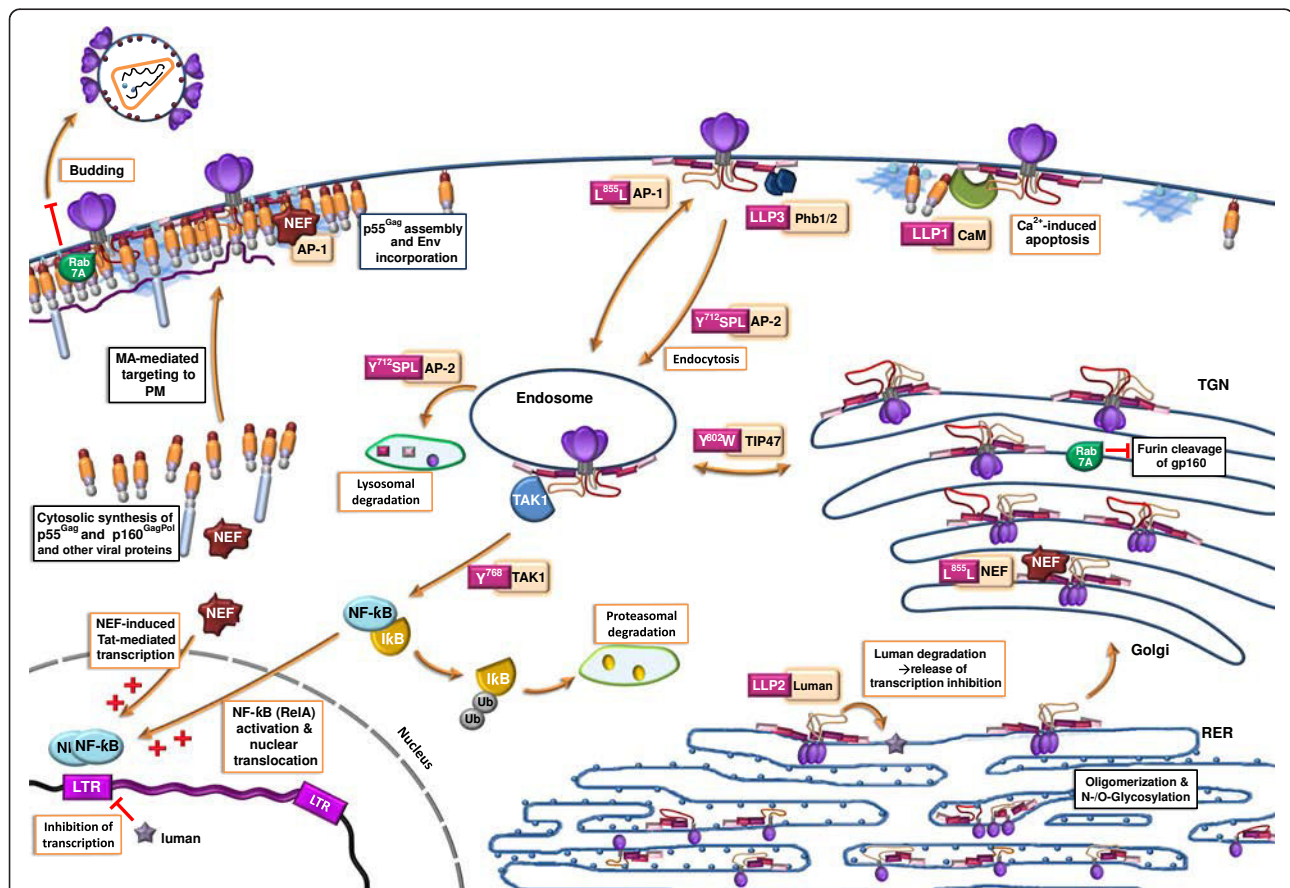


Figure 3 Schematic representation of the interacting partners of the gp41-CT in the cell. The Env precursor gp160 is synthesized in the rough endothelial reticulum (RER), where it is glycosylated and oligomerizes into trimers. Furin cleavage in the Golgi apparatus enables the formation of the gp120-gp41 structure. Once it reaches the plasma membrane (PM), Env is subject to endocytosis, via the Y₇₁₂SPL and L₈₅₅L₈₅₆ motifs interacting with AP-1 and AP-2. Cycling through the trans Golgi network and between endosomes is ensured through interactions with TIP47, via the Y₈₀₂W₈₀₃ diaromatic domain, and with AP-1 via Y₇₁₂SPL. Env is targeted to the PM through Y₇₁₂SPL interaction with AP-1 and AP-3. The p55^{Gag} precursor is synthesized in the cytoplasm and accumulates at the PM in lipid raft-rich domains. Interaction of the gp41-CT (through LLP-2) with the Matrix protein (MA) ensures Env incorporation into the budding virion. gp41-CT interacts with Nef through L₈₅₅L₈₅₆. NF- κ B activation through phosphorylation of I κ B is mediated by interaction of the Y₇₆₈ with TAK-1. Interference with luman-mediated inhibition of transcription is mediated by LLP-2. Abbreviations: RER: rough endoplasmic reticulum; TGN: trans Golgi network; CaM: calmodulin; LLP: lentiviral lytic peptide; TAK: TFG- β -activating factor; AP: adaptor protein; TIP47: Tail-interacting protein 47 kDa; Ub: ubiquitin; Phb: Prohibitin.

HIV-1 can be pseudotyped by a variety of retroviral and non-retroviral envelopes (*e.g.* alphaviruses, ortho/paramyxoviruses, flaviviruses, etc.) [106-109], and by the fact that gp41-CT truncations do not affect Env incorporation in some cell types (*e.g.* the HeLa cell line) (reviewed in [25,104]). The three other models, in contrast, propose that p55^{Gag} and Env are co-targeted to assembly platforms in a highly regulated fashion, either through direct p55^{Gag}/Env interaction, or through an indirect interaction mediated by lipid rafts, or by other cellular factors (for details, see [25]). Indeed, several lines of evidence suggest that p55^{Gag} and the gp41-CT interact [52,110-118]. However, whether the MA/gp41-CT interaction is direct or involves other partners remains controversial and requires further investigation: coimmunoprecipitation studies and cell-free protein-protein interaction studies suggest a direct interaction between gp41-CT and p55^{Gag} [112,113,116], but further interactions involving cellular or other viral proteins, such as adaptins AP-1 through AP-4 or TIP47, lipid rafts themselves and other lipid raft components have also been described. One possible explanation for this diversity of reports is that the interacting partners may have stabilizing or facilitating effects, that are not required at all moments, or that are inessential in some cell types. The observation that gp41-CT truncation alters the localization and affects polarization of the viral capsid at the viral synapse (VS) in some cell types (see section 'Phenotypes of gp41-CT truncations') supports this view [119,120].

Interaction of the gp41-CT with Gag

In the absence of Env, Gag and viral-like particles (VLPs) bud in a non-polarized manner from the epithelial cell membrane suggesting the existence of a p55^{Gag}/Env interaction. Env drives p55^{Gag} accumulation and virion budding from the basolateral surface of polarized cells (MDCK cells in early reports, then extended to all polarized cells) [111,121,122]. This polarized release was shown both to be mediated by the Y₇₁₂SPL motif of the gp41-CT and to require interaction between Env and MA [111,121,122]. Likewise, truncation of the gp41-CT of a SIV strain resulted in redistribution of cell surface envelope molecules from localized patches to a diffuse pattern that covered the entire plasma membrane [123]. Conversely, Env glycoproteins produced alone are randomly scattered across the cell plasma membrane, whereas in the presence of p55^{Gag} they are recruited to budding sites, suggesting active recruitment to these sites [109].

Further evidence that a Gag/Env interaction is required for the process of viral assembly arises from the observation that although truncation of the entire gp41-CT generally has little or no effect on Env incorporation, and even increases Env expression at the cell membrane, deletions downstream of the KE (ablating the LLP domains) or small deletions within LLP-2, severely hamper Env incorporation

[43-46,52]. This defect can be reversed by mutations (V34I) in MA [52]. Conversely, it was reported that mutations in the p55^{Gag} precursor MA protein result in decreased plasma membrane expression of Env [110], and in lower Env stability and virion incorporation [110,119,124,125]. The discovery that mutations in MA interfere with Env incorporation in 'non-permissive' cells and that pseudotyping with shorter Envs (MLV or a truncated HIV-2) or truncating the HIV-1 gp41-CT reversed this impairment confirm that the gp41-CT interacts with the p55^{Gag} precursor [52,114,115,118]. Importantly, these findings have led to the idea that the gp41-CT plays a role in assembly during the late stages of the viral cycle, rather than in Env fusogenicity and in entry during the early stages of the replication cycle [52,114,118].

The MA/Env interaction has variously been mapped to L12 and L30 within MA [52,114,118], to K32 [110], and to the MA-CA junction [114-116,118]. The defect in Env incorporation into virions containing the L12E mutation in MA can be reversed by truncations of the gp41-CT depriving it of the LLP domains [114,115,118], and MA AA L30 has been shown to interact with the C-terminus of LLP-2 [52]. Other studies based on progressive HIV-1 or SIV gp41-CT truncation mutants (67 AA, or a central peptide of 93 AA) confirmed that this interaction mapped to LLP-2 [52,112,113,126]. Another group however repeatedly mapped the interaction to the 28 C-terminal AA (*i.e.* to LLP-1) [47,54,116]. Despite the diversity of truncations that have been studied, the observation that removal of 59 to 90 AA from the gp41-CT strongly impairs Env incorporation into virions whereas removing 93 AA from the C-terminus does not affect Env incorporation [115,118] suggests that LLP-2 does not adopt an appropriate conformation in the absence of LLP-1, and fails to accommodate the p55^{Gag} precursor. Lastly, Env and MA sequences from patient-derived viral strains co-evolved over time [127], providing further *ex vivo* support for a gp41-CT/p55^{Gag} interaction.

The gp41-CT/p55^{Gag} interaction seems to tolerate a degree of flexibility, as MA mutations that abolish incorporation of HIV-1 Env do not interfere with the incorporation of heterologous retroviral Envs with short CTs [115,118] and as HIV can be pseudotyped with a variety of retroviral and non-retroviral Env glycoproteins [106-109]. This contrasts with VSV cores, which cannot be pseudotyped by HIV gp160 [128]. The Env/Env interaction mediated by the gp41-CT [33], in contrast, is highly specific as recent studies mixing Env glycoproteins from lentiviruses (HIV or SIV) and from two retroviruses (RSV and MLV) to produce pseudovirions show that Env clustering at the plasma membrane is dependent on the Env gp41-CT and is limited to autologous Envs [106,109]; also in another study, the formation of hybrid pseudovirions was not tolerated, although in this case, the non-retroviral Ebola Env glycoprotein and HIV were mixed [108].

Viral stiffness, viral maturation and infectivity

It is currently believed that the p55^{Gag} precursor orchestrates viral assembly through interactions with all viral components to be incorporated, and that the gp41-CT/p55^{Gag} interaction is involved in controlling viral infectivity in a maturation-dependent fashion. The efficiency of entry and fusion is lower for immature than mature HIV-1 particles [47,54,129]. Similar results have been reported for the SIV Env-CT [117]. This inefficiency can be overcome by truncation of the gp41-CT or by LLP-1 truncations [47,54,114].

It has been proposed that the plasticity of the lentiviral particle controls its infectivity and fusion capacity [32,130]. Immature virions are 14 times stiffer than mature particles [32]. The structural rearrangements associated with p55^{Gag} maturation by the viral protease transform the stiff immature viral particle into a mature particle with a thin protein shell and conical core; its ability to enter cells depends on its softness [32,130]. p55^{Gag} maturation by the viral protease into its distinct components is a regulated and sequential process with particular kinetics [131]: the first cleavage is that of the SP1-NC, and it induces the structural switch of Gag that controls maturation and activates Env [54,99]. STED microscopy has recently provided visual evidence of such regulation: Env spikes at the surface of mature virions coalesce into foci, whereas in the less infectious immature virion, Env spikes remain scattered across the virion surface [33]. Proteolytic maturation of p55^{Gag} allows Env lateral motility and clustering into foci [33,132]. However, although truncation of the gp41-CT also favors Env motility on immature particles, it does not result in Env clustering: this provides evidence that gp41-CT-mediated Env clustering optimizes viral infectivity [33,132], confirming prior reports showing that LLP-1 and LLP-2 mediate Env clustering [55], partly via Arg residues [56]. Pang *et al.* [32] recently used atomic force microscopy to demonstrate a correlation between viral infectivity and virion stiffness. Using different constructs that separate the Env 'fusion-activity' (Env Δ CT construct) from the 'stiffness/softness properties' (GFP-gp41-CT construct), they found that the gp41-CT stiffens immature virions in a concentration-dependent fashion, thereby impairing infectivity of immature (but not mature) particles [32]. Because VSV-G pseudovirions were similarly affected, the authors conclude that particle stiffness directly regulates immature viral entry [32]. Intriguingly, there was no direct interaction between the Env Δ CT and the GFP-gp41-CT constructs, implying that the gp41-CT directly stiffens immature viral particles, and that virion stiffness controls virion fusion capacity [32]. These studies indicate biophysical mechanisms underlying the control of viral infectivity by protease-mediated p55^{Gag} cleavage [32,33,132]. Although both groups related viral infectivity to viral

stiffness, they draw opposite conclusions about the roles of viral stiffness and of the gp41-CT: Chojnacki *et al.* [33] propose that the stiffness of the immature particle interferes with Env motility and clustering through the gp41-CT, whereas Pang *et al.* [32] propose that it is viral stiffness itself that is controlled by the gp41-CT through an as yet undefined mechanism possibly involving the arrangement or stability of the immature Gag shell. In other words, stiffness impairs Env motility and gp41-CT-mediated Env clustering and infectivity [33,132], or, alternatively, stiffness is a consequence of the presence of the gp41-CT [32]. Either way, Env ensures minimal fusion until the virus is mature, suggesting a mechanism whereby unprocessed p55^{Gag} "withholds" Env until full maturation by the viral protease is complete through an inside-out regulation mechanism [54,58,129].

Control of viral infectivity

The p55^{Gag} precursor is trafficked to and accumulates at the plasma membrane where it recruits viral components (including Env, viral genomic RNA, viral accessory proteins). However, interactions of the gp41-CT with cellular trafficking regulators (discussed below, 'Interactions of the gp41-CT with cellular factors and intracellular regulators') down-modulates Env as soon as it reaches the plasma membrane such that it remains scarce until p55^{Gag} oligomerization is complete or, at least, sufficiently dense in the assembly platforms to ensure efficient viral assembly and release [133] (Figure 3). This mechanism for the regulation of the surface abundance of Env protein may protect infected cells from Env-dependent cytopathic effects, from Env-induced Fas-mediated apoptosis and/or from Env-specific immune responses [133-137]. During, or shortly after virus release, the viral protease cleaves the p55^{Gag} and p160^{GagPol} precursors, resulting in structural and morphological rearrangements of the virion that enable Env fusion capacity [47,54,129]. gp41-CT interacts with p55^{Gag} with higher affinity than with mature MA [129], and this loosened interaction probably favors changes in Env conformation after viral maturation [47,54] and Env lateral motility [33]. With the completion of maturation, the virion becomes maximally infectious and can start a new round of infection. The scarcity of Env spikes at the surface of virions appears to be a compromise between efficient fusion capacity, through virion softness, clustering of Env into foci, and immune escape.

This mechanism for controlling infectivity differs from that of other retroviruses, such as Murine Leukemia Virus and Mason-Pfizer monkey virus, where the viral gp41-CT is cleaved by the viral protease in the released virion, resulting in Env becoming fusion competent [138,139].

Open questions

Clearly, p55^{Gag} is the main pillar of HIV assembly, and recruits the constituents of HIV virions, orchestrates their assembly and controls infectivity of viral particles. Nevertheless, many questions concerning Env recruitment and incorporation into virions remain unresolved. Do p55^{Gag} and Env interact directly or is this interaction indirect? Or, alternatively, are there many contact points, both direct and indirect? In this case, are these multiple interactions permanent and simultaneous, or is there a sequential order important for the control of viral assembly and/or infectivity? Which Env determinants are involved? Many cellular proteins have been described to interact with the gp41-CT (detailed below, section 'Interactions of the gp41-CT with cellular factors and intracellular regulators'), but it is not known which bridge the p55^{Gag}/Env interaction and which serve other purposes. It is unclear which are, as a consequence, incorporated in newly synthesized viral particle, and if they are, whether they are included passively or contribute to the initial steps of the following infection round.

Phenotypes of gp41-CT truncations

Despite numerous studies addressing the role of the gp41-CT domain, the roles of particular determinants remain poorly documented. One reason why the gp41-CT remains poorly understood is that the effects of gp41-CT truncations and/or mutations are cell-type dependent. For example, many transformed T-cell lines (*e.g.* the cell lines H9, MT-2, Jurkat, and CEMx174) and primary target cells of HIV (primary CD4+ T cells and macrophages) do not sustain replicative spread of viruses with a truncated gp41-CT; these cells are referred to as 'non-permissive' for gp41-CT truncation. Other cells, in contrast, such as the T cell lines MT-4 and C8166 and several laboratory cell lines (HEK293T, HeLa, and COS) are less affected by full or partial truncations of the gp41-CT and sustain replication of the corresponding viruses (termed 'permissive' cells) [43-45,119,120,140-142]. Truncation of gp41-CT decreases Env incorporation into virions by about 10-fold in 'non-permissive' cell lines and in primary HIV target cells (*i.e.* primary PBMCs and monocyte-derived-macrophages), but has a much smaller effect in 'permissive cells' [119,120]. An illustrative example, albeit extreme, is that truncation mutants have different effects in macrophages [119], perhaps owing to differentiation and culture conditions. Compromised infectivity and replication capacity in 'non-permissive' cells has been linked to altered localization of the viral capsid that failed to accumulate and polarize at the viral synapse [119,120], but not to increased gp120 shedding from virions nor to reduced Env cell-surface expression [110,119,120,140]. HIV and SIV viral particles contain relatively small amounts of Env (~7-14 Env trimers/virion) [143-145], due to both the massive

recycling of neo-synthesized Env and to Env shedding. These findings and comparisons are thus difficult to interpret and their biological relevance is unclear.

It is not clear how gp41-CT truncations may impact Env incorporation into virions without affecting Env expression at the PM or Env trafficking through the ER and Golgi networks, as one could expect Env incorporation into virions to be related to the amount of Env at the PM. One possible explanation is that inherent differences between cell types in the expression or localization of host factors play a role in viral assembly; this would account for the cell-type dependent phenotypes of gp41-CT truncations and mutations. A second possible explanation is that because the gp41-CT activates the NF- κ B canonical pathway [79], gp41-CT truncations interfere with the cell activation status (see section 'Activation of transcription'). Cell types and cell lines differ in their basal activation levels and in their dependence on NF- κ B, and it is therefore conceivable that truncations of the gp41-CT could translate into cell-type-dependent phenotypes [31].

One recent study [141] compared the infectivity of sub-saturating titers of wild-type and of a HIV-1 gp41-CT-truncated mutant in various gp41-CT truncation 'permissive' (MT-4, C8166) and 'non-permissive' (H9, MT-2, Jurkat, CEM-SS) cell lines: the 'permissive' and 'non-permissive' phenotypes of target cells correlated with the level of gene expression, and particularly of p55^{Gag} production and processing to mature capsid (CA), rather than with Env incorporation into the virion [141]. In 'non-permissive' cells, viral assembly was compromised as a consequence of p55^{Gag} failing to accumulate and polarize at the viral synapse (VS), whereas in 'permissive' cell lines, high levels of p55^{Gag} overrode and compensated for the absence of the gp41-CT [141]. This study could reconcile the cell-type dependence of Env incorporation with a dual role for the gp41-CT, being involved in Env incorporation into virions and polarized budding on one hand and in cell activation and regulating viral gene transcription on the other.

The situation is more clear-cut for the SIV gp41-CT. SIV Env truncations have been reported to arise spontaneously when culturing SIV in human cell lines, and then to revert back following culture in simian cells [146-149]. These truncations increased Env surface expression, fusogenicity [61,150] and infectivity *in vitro* [147,151]. These SIV gp41-CT truncation mutants are replication competent both *in vitro* and *in vivo*, but lack full pathogenicity *in vivo*: macaques infected with a LLP-1- and LLP-2-defective SIVmac239 initially had viremia, but the viral load declined with time to below the detection threshold; the infected macaques never progressed to AIDS [152]. A similar phenotype has been reported for HIV-2 [147,153]. Spontaneous truncations of the gp41-CT of SIV cultured in human cell

lines and reversion in rhesus PBMCs may reflect the presence of a restriction factor whose action is inhibited by the gp41-CT in rhesus cells, but which is absent from human PBMCs or ineffective against SIV [79]. Alternatively, spontaneous truncations of the gp41-CT in human PBMCs or T cell lines may improve viral infectivity by enhancing Env expression and thereby incorporation into virions. The truncation of the SIV gp41-CT observed in human cell lines may remove signals mediating Env trafficking such that the higher Env density at the surface of SIV virions is preserved. Further studies to elucidate how these phenomena are related and the real consequences of Env levels in the virion may reveal new leads for the development of strategies to control viral assembly.

Interactions of the gp41-CT with cellular factors and intracellular regulators

A number of cell factors have been described to interact with the gp41-CT: in some cases, the interactions have simply been described without any evidence of significance, and in others, the significance is controversial. Nonetheless, most of these interactions between the gp41-CT and cellular factors contribute to improving viral replication, by promoting viral gene expression, enhancing viral infectivity, or interfering with intrinsic cellular restriction factors or immunity. Innate immunity has developed many (often interferon-induced) pathways to counter gene expression and the late stages of the viral replication cycle, namely assembly and budding, as these are essential steps for viral propagation. It seems likely that such factors are, at least in part, responsible for the cell-type-dependent phenotypes observed.

Figures 3 and 4 summarize cellular and viral proteins interacting with the gp41-CT and their intracellular localization.

Env trafficking and sorting through the trans Golgi network (TGN)

Golgi retrieval signals function to return proteins which have left the Golgi to that compartment; Golgi retention signals, by contrast, are generally found in the transmembrane domain of Golgi and ER resident proteins. The main Golgi retrieval signals are cytoplasmic domain tyrosine-based or dileucine motifs [154,155]. The gp41-CT contains both Golgi retrieval (tyrosine-based and dileucine-based) and Golgi retention motifs [156]. They regulate Env trafficking through the endocytic pathway [157-159].

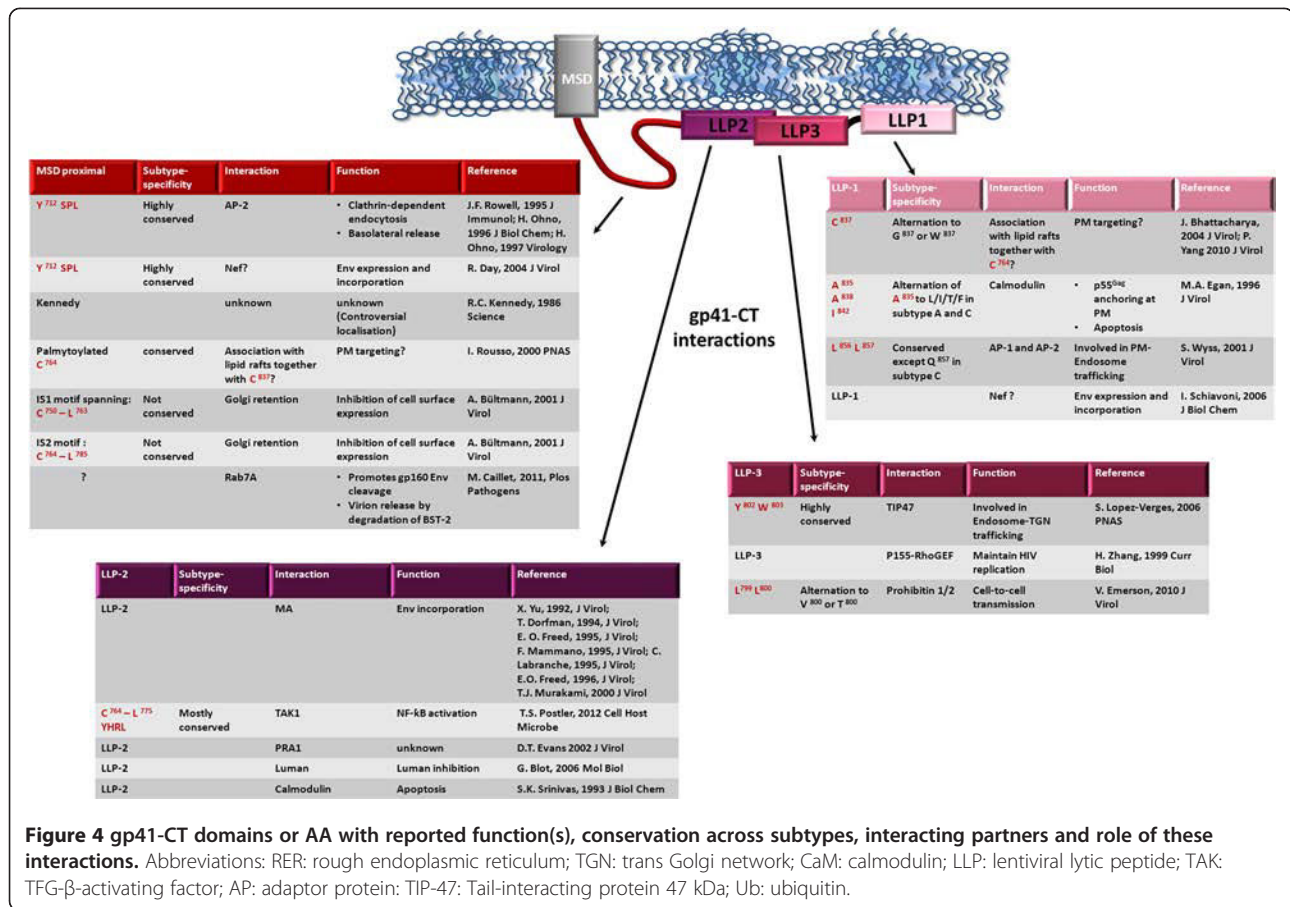
AP-mediated Env endocytosis

As soon as Env comes into contact with the plasma membrane, it is endocytosed. Time course analyses show that 50% of Env, but only 15% of a gp41-CT-truncated Env, is internalized after 60 minutes [157]; and similar time

courses have been reported using a chimeric CD4 molecule fused to the gp41-CT [156]. These observations indicate that the gp41-CT mediates endocytosis. Env is endocytosed via interactions with the clathrin adaptor complex (AP proteins) [157-159]. Clathrin adaptors sort and direct proteins through the endocytic pathway. The highly conserved Y₇₁₂SPL motif of HIV (and Y₇₂₁XXΦ of SIV) immediately downstream from the transmembrane region has been implicated in regulating Env intracellular trafficking by binding clathrin adaptor AP-2 μ (medium) chains [157-159] (Figures 3 and 4). The Y₇₁₂SPL motif can also interact with the AP-1 and AP-3 adaptor complexes through μ1 and μ3A, respectively [159]. Whereas the interaction of the gp41-CT Y₇₁₂SPL motif with AP-2 leads to Env internalization, interaction with AP-1 regulates Env trafficking through the TGN and addresses Env to the basolateral PM in polarized cells [159] (Figures 3 and 4). The AP-2 adaptor complex also mediates p55^{Gag} internalization through Y₁₃₂ and V₁₃₅ at the MA-CA junction [160], and the AP-1 μ [161] and AP-3 β and δ subunits contribute to p55^{Gag} release. They do so by transporting it to intracellular sites of active budding, such as MVBs and the PM [162,163] and/or by facilitating its interaction with other cellular partners such as Tsg101 [161]. The interaction between Env and various proteins of the AP clathrin adaptor complex may therefore be involved in the p55^{Gag}/gp41-CT interaction and determine the fate of Env (targeting to the lysosomal compartment for degradation, or to the basolateral plasma membrane for incorporation into virions).

Y₇₁₂SPL-mediated endocytosis is suppressed in the presence of the HIV-1 p55^{Gag} precursor polyprotein [133,164]. This suggests that p55^{Gag} interferes with the Env/AP-2 interaction and that the amount of Env at the cell surface is regulated by the AP-2 versus p55^{Gag} balance. AP-2 may thus eliminate any Env protein that is not associated with p55^{Gag} and that is therefore not destined for incorporation into virions [133,164]. Env internalization may proceed until sufficient p55^{Gag} has been synthesized and has accumulated at the site of viral assembly, at which point, p55^{Gag} would interact with Env directly or indirectly, thereby trapping Env into the nascent virion [133,165]. In the case of SIV, mutating the Y₇₂₁ in agp41-CT truncated Env resulted in increased Env expression at the plasma membrane, whereas mutations of Y₇₂₁ in a full-length gp41-CT context had little impact on Env expression at the plasma membrane [123,166]. Presumably, other motifs involved in endocytic regulation overcome the single mutation.

Additional distal determinants also regulate Env internalization [164]. These have now been identified as being the most distal C-terminal dileucine motif [167,168] and/or IS1 and IS2 Golgi retention sequences [156]. Recruitment of AP-1 [167] and AP-2 [168] by the most C-terminal dileucine motif ensures correct post-Golgi routing of Env,



and restricts cell surface expression of Env in conjunction with the membrane-proximal Y₇₁₂SPL motif [167,168] (Figures 3 and 4).

Both the Y₇₁₂SPL motif and the most C-terminal dileucine motif are extremely well conserved across HIV-1 subtypes (except subtype C), and also in HIV-2 and SIV strains, evidence of their importance (Figure 1B). Similar signals in cellular proteins or in the glycoproteins of other viruses (*e.g.* alpha herpesviruses) affect endocytosis as well as intracellular trafficking in endosomes and the TGN, and can determine basolateral versus apical sorting (reviewed in [169,170]). It is nevertheless intriguing that subtype C viruses harbor a LQ rather than LL (dileucine) at this position in over 60% of cases. As the requirement for an intact C-terminal dileucine motif for correct interaction with AP-1 and with Nef (see below, section ‘Assembly’) seems to be quite stringent [167], these observations raise three possibilities: (i) other motifs are involved, or (ii) Env trafficking differs between subtypes, and differences in sub-cellular localization are less detrimental to viral assembly and infectivity than previously believed, or (iii) subtype C Env internalization is less efficient and more Env is expressed at the surface of infected cells and possibly on virions of

subtype C than of other subtypes; this could be a cause or consequence of the weaker antibody response or of the lower replicative capacity.

Another tyrosine-based motif, Y₇₆₈HRL, is present in the gp41-CT. Although this motif also interacts with AP-1, AP-2 and AP-3, it is not involved in endocytosis of Env, perhaps because of its localization in the gp41-CT, but may mediate NF-κB gene expression [79] (see below, section ‘Activation of Transcription’). Likewise, one or two other YxxΦ motifs are present in various SIV strains and in HIV-2, but none has been shown to mediate endocytosis [166].

Golgi retention

Most Env is retained in the ER, and from there ubiquitinated and degraded by the proteasome. Only a small proportion actually reaches the Golgi and proceeds to the cell surface. Nevertheless, Env is also retained in the Golgi [156]. The IS1 (AA 750–763) and IS2 (AA 764–785) regions in the gp41-CT reduce Env surface expression by retaining Env in the Golgi, independently of AP-1 and AP-2 [156] (Figure 4). These sequences include LL (or IL motifs, which behave similarly to LL in Golgi retrieval [171]), and result in retention in the Golgi of gp160 and of

chimeric proteins composed of the gp41-CT fused to the CD4 receptor or to immunoglobulin extracellular/transmembrane domains. These sequences are also highly conserved across subtypes [156] (Figure 1B).

TIP47

The leucine-zipper domain, LLP-3, also contains a highly conserved $Y_{802}W_{803}$ diaromatic motif. This motif has been found to be involved in the retrograde transport of Mannose-6-Phosphate Receptors and in lipid droplet biogenesis. The $Y_{802}W_{803}$ motif in Env ensures the retrograde transport of Env from late endosomes to the TGN (Figure 3) and thereby contributes to the regulation of the intracellular distribution of Env, and to Env incorporation into the virion, particularly in macrophages [165,172-175]. Lopez-Vergèz et al. reported that TIP47 is required for the $p55^{Gag}/gp41$ -CT interaction [165]. Disruption of the $p55^{Gag}/TIP47$ interaction through mutations in MA or by TIP47 knock-down causes $p55^{Gag}$ to localize in scattered dots in the vicinity of the PM. These observations led to the suggestion that TIP47 acts as a linker between Env and $p55^{Gag}$, and serves as a bridge for efficient Env incorporation [175]. However, other authors report not being able to reproduce these findings [25].

Rab7A and other RabGTPases

Rab proteins are early and late-endosome associated GTPases that regulate specific steps in intracellular membrane trafficking. They recruit tethering, docking and fusion factors, as well as the actin- and microtubule-based motor proteins that facilitate vesicle traffic [176-180]. In a study aiming to identify cellular trafficking factors involved in HIV assembly and budding, Caillet et al. report that Rab4A, Rab6A, Rab8A, Rab9A, and Rab11A moderately decreased viral infection (by 30-60%) in HeLa cells, probably in relation to their roles as regulators of the exocytic pathway [173,181,182]. Rab7A knock-down resulted in a > 30-fold decrease in viral propagation [182]. Rab7A is involved in regulating exit from the late endosome/MVB by controlling the maturation and fusion of late endosomes and phagosomes with lysosomes, their intracellular motility when loaded with lipids, and their interaction with the cytoskeleton for migration along microtubules [176,178-180,183-185]. Of particular interest is the finding that Rab7A has a dual activity. Rab7A knock-down experiments using siRNA also showed that Rab7A regulated and allowed gp160 processing to gp120 + gp41 and incorporation of mature Env into particles (Figure 3). The authors propose that Rab7A may either ensure correct Env trafficking through furin-containing compartments, or ensure appropriate co-localization of Env and/or of furin; alternatively, Rab7A may modulate Env expression, and in the absence of Rab7A, Env is

expressed to levels that exceed the processing capacity of furin [182].

Rab7A knock-down also interfered with viral release, resulting in the accumulation of gp160 and viral components at the PM, reminiscent of a BST-2/Tetherin phenotype [182]. The authors report that Rab7A is required for sorting BST-2/Tetherin to lysosomes for degradation through molecular mechanisms that remain to be elucidated [182] (Figure 3). Rab7A is also involved in the late maturation of the final autophagic vacuoles [186,187].

Strikingly, the role of Rab7A shares similarities with that of Nef (see section 'Nef' below) in mediating increased Env at the PM [188] and in intercepting the autophagic processing of Gag to increase virion assembly and egress [189,190]; both also affect traffic through the endocytic pathway [106]. It would be interesting to find out whether Nef intersects the Rab7A or Rab5 signaling cascades.

Rab9A is a mediator of late endosome-to-TGN trafficking involved in the replication of many viruses, including Marburg Virus, Filoviruses and Measles. It interacts with TIP47 and PRA1 (prenylated Rab acceptor 1), both cellular interacting partners of gp41-CT [173]. PRA1 has been reported to interact with lentiviral gp41-CT (SIV, HIV-1 and 2, EIAV and FIV) in a 2-hybrid system [191] without modulating viral release, infectivity or Env incorporation [192]. The significance of these interactions remains unclear, but as both Rab9A and PRA-1 are involved in vesicular trafficking, it is possible that they participate in regulating Env trafficking through the Golgi.

Assembly

Detergent-resistant lipid rafts

Detergent-resistant lipid rafts are cellular microdomains enriched in cholesterol and sphingolipids and which contain proteins. Lipid rafts are thought to play an important role in many cellular processes including signal transduction, membrane trafficking, protein oligomerization, cytoskeletal organization, and pathogen entry (reviewed in [193-195]). Lipid rafts are thought to be central to the assembly and infectivity of numerous enveloped viruses, including lentiviruses. The lipid bilayer of HIV virions is highly enriched in cholesterol and sphingomyelin, both of which are major components of lipid rafts [196,197]. Also, $p55^{Gag}$ and Env and have been co-localized with lipid raft-associated proteins [73,85-87]. Env incorporation into lipid rafts optimizes its incorporation into virions and confers viral infectivity [198]. Conversely, excluding HIV from lipid rafts impairs viral infectivity [85,199-203]. The 12 C-terminal amino acids of LLP-1 and portions of LLP-3 [43,44,49,204], and also palmitoylated cysteines C_{764} and C_{837} , and bulky hydrophobic side chains, have been implicated in targeting Env to lipid rafts [198,205] (Figure 4). In contrast to the reports by Bhattacharya [198,206], Chan et al. [207] found

that Cys to Ser and Cys to Ala mutations had no significant effect on Env expression, trafficking, interaction with lipid rafts and incorporation into virions, or on the fusion efficiency or infectivity in single-cycle infections, despite decreased palmytoylation of Env [207].

Other studies argue against any substantial direct targeting of Env to lipid rafts, and suggest, rather, that it is p55^{Gag} that recruits and anchors Env to lipid rafts [85]. Various observations imply that p55^{Gag} drives Env to lipid rafts. First, mutation of residue S4 in MA abolishes the interaction of p55^{Gag} with lipid rafts resulting in reduced Env incorporation into virions, presumably by inhibiting the ability of p55^{Gag} to target or hold Env within raft microdomains [208]. Second, independent studies have reported that in the absence of p55^{Gag}, or in the presence of MA or gp41-CT mutations that abolish Env incorporation into virions, Env was not found associated with detergent-resistant membranes [198,206]. The observation that neither the cysteine residue in LLP-1 nor the presence of bulky AA is conserved between subtypes raises further doubts about a direct interaction between Env and lipid rafts. This polymorphic flexibility is more consistent with the view that Env is recruited to lipid rafts through p55^{Gag}, and/or that other determinants are also involved. Nonetheless, it cannot be excluded that under certain circumstances, particularly in transfection experiments involving strong viral gene overexpression, Env may be passively incorporated into virions; a phenomenon of this type would explain the contrasting results reported.

In the cases of other enveloped viruses, such as Influenza Virus [209] and Respiratory Syncytial Virus [210], the matrix/lipid raft interaction is favored by, or even dependent on, the presence of the respective envelope cytoplasmic tails. Whether lentiviral gp41-CTs also somehow influence the MA/lipid raft interaction has not been described.

Nef

Interplay between the Env gp41-CT and the viral accessory protein Nef has been reported to increase viral infectivity [211,212]. The viral accessory protein Nef is a pleiotropic non-enzymatic, myristoylated, HIV and SIV lentivirus pathogenesis factor. Nef increases viral infectivity through various, not fully elucidated, mechanisms involving immune escape [213-216] (reviewed in [217]), increased viral infectivity and propagation [106,212,218-222], and perturbed immune activation, thereby promoting a favorable environment for viral replication [90,216,219,223-227]). Most of Nef's functions are due to its ability to influence the cellular environment, mainly through protein-protein interactions which interfere with cell-receptor trafficking or are involved in T cell receptor (TCR)-induced signaling [228].

It has been proposed that Nef promotes Env gp160 precursor processing and PM expression of the mature gp120 and gp41 products by abrogating their retention in the cis-Golgi [106,212], and by specifically augmenting Env accumulation at the PM [212]. Enhanced Env at the PM was found to be dependent on the C-terminal dileucine motif of the gp41-CT and on the C-terminal residues of Nef (positions 181-210), and it was suggested that Nef increases Env incorporation into virions [212]. Whether the gp41-CT Golgi retention sequences IS1 and IS2 are also involved has not been investigated.

Others have proposed that Nef may modulate cell signaling thereby altering intracellular trafficking and augmenting Env accumulation in late endosomes/MVBs [106]. Nef-insensitive Env glycoproteins from various enveloped viruses share pH-dependence (Influenza-HA, VSV-G and HCV), and most importantly, lack endocytic traffic motifs, budding in compartments other than MVBs (HCV) or at the cell PM (Influenza-HA, VSV-G). Glycoproteins needing Nef for optimal assembly harbor determinants of intracellular trafficking through the endocytic pathway and assemble in MVBs [106,229]. Furthermore, the role of Nef in Env and p55^{Gag} precursor co-localization could be linked, at least to some extent, to Gag expression levels, and this may explain the cell-dependence and the gp41-CT 'permissive' versus 'non-permissive' phenotypes. The incorporation into pseudovirions of some retroviral and lentiviral Envs is increased in the presence of Nef, but not for those for which Env incorporation was already optimal [106]. Sandrin *et al.* conclude from these observations that Nef expression optimizes co-localization of retroviral Envs with p55^{Gag} in late endosomes/MVBs specifically for those Envs that inherently do not accumulate in the endosomal pathway [106]. This is consistent with the observation that Nef [231-233], the gp41-CT [167,168] and p55^{Gag} [160-163] all interact with adaptins AP-1 through 3, suggesting these intermediates may be involved. It would be interesting to assess whether this observation is relevant to the findings of another study: the absence of the scaffold protein Dlg1 was described to restrict late steps of the HIV cycle due to Gag and Env rerouting and sequestration in a tetraspanin-rich (CD63+ and CD82+) late-endosome and plasma-membrane-related compartment [230].

Nef also interacts directly with the p55^{Gag} and p160^{GagPol} precursors via p6* in the overlap region between p6 and *pol* [234,235], and increases p55^{Gag} and p160^{GagPol} processing and egress via AIP-1/ALIX, a critical intermediate in the formation and proliferation of multivesicular bodies (MVBs) [221,233,234]. It has been suggested that the Nef-induced p55^{Gag} accumulation in late endosomes/MVBs is linked to autophagy. Autophagy is a cell survival and host defense mechanism by which

cells degrade their own cytoplasmic constituents during starvation and microbial invasion; it involves clearing damaged organelles, macromolecular aggregates and intracellular microbes through double-membrane vacuoles, termed autophagosomes, which typically mature into amphisomes or autolysosomes after fusing with MVBs or lysosomes [236-238]. Nef has been suggested to increase virion assembly and egress by intercepting the autophagy process [189,190]; however, the mechanisms of HIV p55^{Gag} incorporation into the autophagosome leading to the release of HIV Gag particles into the extracellular environment remain largely unknown.

It is important to keep in mind, however, that recent work to identify the localization of HIV assembly in different cell types, and particularly in 293T cells and in macrophages, were performed using viral-like particles (VLPs) and VSV-G pseudotypes which target the p55^{Gag} precursor to the PM. From there, it either buds releasing new infectious viral particles or is endocytosed and accumulates in late endosomes and MVBs [80,81,239]. Most of these studies are based on the use of VLP and of pseudovirions comprising a heterologous Env, and on large p55^{Gag} or VLP/pseudovirion input, i.e. high Gag overexpression. The precise fate of p55^{Gag} precursor protein that has not budded from the PM and that has been endocytosed into late endosomes and MVBs is not known; more importantly, it remains to be established how and where Env and Nef interplay with the p55^{Gag} precursor to allow the release of fully infectious virions.

The Nef/gp41 interplay also involves other mechanisms independent of the gp41-CT and of its trafficking domains. Another function of Nef is to promote the biogenesis of viral assembly platforms including rafts and MVBs [221,222]. Nef can accumulate in lipid rafts and thereby contribute to infectivity of progeny virions by increasing the incorporation of lipids into viral membranes [222]. Whether Nef further favors or stabilizes the insufficient interaction of Env with lipid rafts, in conjunction or not with Gag, is still not known. In another study, the N-terminal region of Nef and its myristoylated moiety was shown to decrease the sensitivity of the gp41 ectodomain to neutralization by antibodies targeting the MPER epitopes that are embedded in the virion lipid bilayer (2F5 and 4E10) [240-242] and thereby contribute to viral escape from the humoral immune response [243]. This activity did not affect Env incorporation into virions, and the authors suggest that the susceptibility of neutralization to these epitopes may be modulated by Nef-mediated alteration of the lipid bilayer composition [243].

The consequences of Nef deletions, like those of gp41-CT truncations, are cell-type dependent [244,245]. In 'Nef non-permissive' cells, Nef deletion affects the early stages of viral infection and only slightly

modulates the late stages of the viral replication cycle (assembly, release and maturation) [244,245]. Although there has been no direct pair-wise comparative study of gp41-CT truncations and Nef deletion mutants to our knowledge, it is interesting that there is some overlap between the gp41-CT and Nef 'permissive'/non-permissive' phenotypes (e.g. MT-4 cells are 'permissive' to both, whereas primary CD4 cells and macrophages, and H9, Jurkat and CEMx174 cells are 'non-permissive' to both [119,120,244,245]). The reported effects of gp41-CT truncations and of Nef deletions on viral replication can differ in a given 'non-permissive' cell type (e.g. C1866 cells are 'permissive' to gp41-CT truncations [119,120] but are partially 'non-permissive' to Nef truncations) [244,245], although such comparisons should be interpreted with caution as the results come from different groups with different experimental set-ups. Day *et al.* reported that the incorporation of an Env Y₇₁₂A mutant into virions was impaired in the absence of Nef in MT-4 cells but not in HEK293 cells [211]. This is intriguing because MT-4 cells are 'permissive' to both gp41-CT truncation and Nef deletion. One would have expected the Env Y₇₁₂A ΔNef double mutant not to display impaired Env incorporation when produced in this cell type. One possible explanation is that in gp41-CT 'permissive' cells, Nef plays a "compensatory" role: in the absence of Nef, the gp41-CT 'permissive' cell-line becomes 'non-permissive' to gp41-CT mutations. Alternatively, the correct routing of Env and/or Nef to intracellular trafficking compartments may play a crucial role in viral assembly and Env incorporation. Mutations disrupting the Y₇₁₂SPL endocytic motif and truncation mutants may behave differently.

Calmodulin

Calmodulin is a calcium-induced mediator of FAS-mediated apoptosis. The gp41-CT binds calmodulin [246,247] and induces apoptosis of T cells, thereby accounting for most of the CD4+ T cell decline that characterizes HIV infection [134,248]. Amino acids Ala₈₃₅, Ala₈₃₈ and Ile₈₄₂ of LLP-1 have been identified as being involved in calmodulin binding and inducing Ca²⁺ flux and DNA fragmentation in transfected cells [134,136,137,246,247] (Figure 4). Point mutations of the gp41-CT (A835W, A838W, A838I, and I842R) eliminate co-immunoprecipitation of Env with calmodulin but have no significant effect on viral production or Env expression [135,137]. Therefore, active Env cycling through the endocytic pathway may prevent Env-mediated apoptosis and thereby avoid premature cell-death and ensure viral assembly [133,164]. Because the gp41-CT/calmodulin interaction is deleterious to the virus by itself, it is likely that this interaction is "collateral damage" from other actions of calmodulin in viral assembly. These other actions include ensuring the conformational changes of MA so as to expose

the myristoylated moiety that docks p55^{Gag} in assembly platforms at the PM [73,86,87,93,94,98].

Although independent reports have described Ala₈₃₅ as being the calmodulin-binding site [136,137], Ala₈₃₅ is not conserved across clades, and there is a Leu, Ile, Thr or Phe at the corresponding position in some strains. Possibly, polymorphism at this position explains the diverse calmodulin-binding activity of Envs and correlates with their apoptotic potential; indeed, the A835W mutation used in many studies fully abolishes the ability of the gp41-CT to bind calmodulin. If this were the case, late, more cytopathic or apoptosis-inducing Envs would be expected to all have an Ala₈₃₅ and to bind more calmodulin, whereas the less pathogenic, early and chronic-infection viral isolates would be expected to bind less calmodulin and be more polymorphic at this position.

Prohibitin1/2

The dileucine motifs of LLP-2/LLP-3 (AA 790–800), and more specifically the dileucine motif L₇₉₉L₈₀₀ immediately upstream from the Y₈₀₂W₈₀₃ diaromatic motif, has been found to interact with prohibitin 1/2 (Phb) [249]. These proteins are produced almost ubiquitously; they are localized in numerous cellular compartments including mitochondria, the plasma membrane PM and the nucleus, and are also associated with lipid rafts. Disruption of the gp41-CT/Phb association, like gp41-CT truncation mutants, impairs cell-to-cell transmission in 'non-permissive' cells [249] (Figures 3 and 4). It is however not known whether this association genuinely contributes to the cell-to-cell transmission capacity of diverse HIV Envs or whether they are passively bound to gp41 at the time of assembly into lipid rafts. The L₇₉₉L₈₀₀ motif is not conserved between subtypes and is in many cases replaced by Val, particularly in subtype C. Nonetheless, in this work, the gp41-CT/Phb association was disrupted only when both Leu residues were mutated: single mutants were unaffected [249]. These findings suggest that this interaction, or interactions of this L₇₉₉L₈₀₀ motif with other cellular factors, is important enough to be preserved despite sequence variation. Moreover, this motif is also involved in interacting with p155-RhoGEF to block RhoA activation [250,251] (see section 'Activation of transcription' below), but there is no indication to date that these two paths converge.

Activation of transcription

NF-κB

It was recently shown that the gp41-CT of HIV-1 activates NF-κB via the canonical pathway by interacting directly with TFG-β-activated kinase 1 (TAK1) [79]. TAK1 activates the transcription factors NF-κB and AP-1. TAK1 is itself activated by cytokines and microbial pathogens, and phosphorylates IκB, routing it for proteasomal

degradation, thereby allowing NF-κB to translocate into the nucleus [252]. The gp41-CT/TAK1 interaction maps to the N-terminus of LLP-2 (C₇₆₄-L₇₇₅) and involves Y₇₆₈ in the second YxxL motif [79] (Figures 3 and 4). The HIV-1 LTR contains 2 (or 3, depending on subtype) NF-κB-binding sites [253]. The authors show that HIV mutants lacking the NF-κB-activating domains were more heavily dependent on cellular activation for replication, and thereby elegantly demonstrated that the gp41-CT contributes to viral replication in sub-optimally activated T lymphocytes by inducing the canonical NF-κB pathway [79]. In the case of SIV, the gp41-CT also activates the NF-κB pathway, but by acting upstream from TAK1, on a factor that has not yet been identified [31]. One of the functions of SIV and HIV Nef is to increase cellular activation in a manner similar to that induced by TCR engagement [90,228,254-257] in a NF-κB-independent manner [224,226,258,259]. Consequently, it has been suggested that Nef and Env may act in concert to progressively and steadily activate gene expression resulting in a positive feedback loop [79]. Alternatively, the early viral gene product Nef and the late gene product Env gp41-CT may act at different times or stages in the viral replication cycle, and induce different activation pathways [79], thereby fulfilling the requirement for two independent stimuli to fully activate T cells [260,261].

Luman

Luman (LZIP or CREB3) is a transcription factor belonging to the CREB/ATF family; it has been linked to the endoplasmic reticulum (ER) stress response. Luman activates the transcription of genes involved in ER-associated degradation of unfolded proteins [262-264]. The ER-membrane-bound form of luman is inactive; proteolytic cleavage releases an active form which enters the nucleus [263]. This activated form of luman interferes with Tat-mediated HIV transcription [265]. gp41-CT interacts with the ER-bound inactive precursor of luman, decreases its stability and targets it for degradation, thereby indirectly promoting LTR-mediated transcription (Figure 3). This interaction maps to residues 751–768, the region lying between the KE and LLP-2 [265] (Figure 4), and partly overlaps the gp41-CT region that interacts with TAK1 (C₇₆₄-L₇₇₅) [79]. Whether these two mechanisms somehow overlap or converge has not been explored.

P115-RhoGEF

Activation of RhoA through p155-RhoGEF inhibits HIV replication by affecting gene expression [251]. The gp41-CT of HIV interacts with p155-RhoGEF and thereby relieves the RhoA-induced inhibition [250,251]. This interaction involves L₇₉₉ in LLP-3. L₇₉₉ is highly conserved, and is part of the dileucine motif interacting with Phb1/2 [249] which tolerates polymorphic changes (Figure 4).

Mutations in gp41-CT leading to loss of interaction with p115-RhoGEF impair HIV-1 replication in the human Jurkat T cell line ('non-permissive' to gp41-CT truncations) [250,251]. p155-RhoGEF is a specific guanine nucleotide exchange factor (GEF) involved in actin cytoskeletal organization rearrangements [250] through activation of RhoA GTPase, which is in turn involved in actin stress fiber organization, cell cycle progress through G1 and cell proliferation. Nonetheless, the gp41-CT-mediated relief of RhoA-mediated viral inhibition seems to be independent of the stress fiber formation function of RhoA and to involve other, as yet unknown, RhoA-mediated mechanisms [251]. Furthermore, whether this viral inhibition also applies in 'permissive' T cells or whether it is restricted to 'non-permissive' is not known: both relevant studies used 'non-permissive' cells. Comparisons between 'permissive' and 'non-permissive' cells may provide some clues as to the mechanism of action of this cellular factor in the HIV replication cycle.

Immune evasion: countering intrinsic restriction/ countering antiviral restriction factors

Tetherin

Tetherin (BST-2 or CD317) is an interferon-inducible transmembrane protein that restricts the release of nascent viral particles from infected cells by tethering them to the PM [266,267] reviewed in [268,269]. HIV-1 counteracts tetherin activity through Vpu (HIV-1) [266,267] which enhances ESCRT-dependent sorting for lysosomal degradation [270,271], whereas SIV Nef [272], and the HIV-2 Env ectodomain [273] counteract tetherin by intracellular sequestration through internalization motifs. One recent study reported that a Nef-deleted SIV strain acquired compensatory changes within the gp41-CT that restored resistance to rhesus tetherin through direct gp41-CT/tetherin interaction that sequestered tetherin [272]. The alterations of the SIV gp41-CT involved a tyrosine-based internalization motif similar to the HIV-2 gp41-CT [272], underlining the importance of countering tetherin and of Env trafficking for lentiviral replication. No such evolution has been reported for HIV-1.

Open questions

Numerous cellular proteins have been described to interact with the gp41-CT. Some of these proteins and interactions have been studied in detail, particularly those involved in Env internalization and trafficking through the TGN. However, in many cases, the role or the mechanisms of these interactions have not been documented. In some cases, the results and interpretations are controversial, and raise questions about whether the proteins are really involved in the viral replication cycle as active partners, or whether they are passively incorporated into the assembly platform, where they make only

a limited if any contribution to viral replication. Different studies have been performed using different cell types and cell lines, each with their specific characteristics. Thus, there are issues about whether they reflect phenotypes of cell types relevant to the HIV replication cycle *in vivo*. Similarly, do these experimental models reflect the context of cell-to-cell transmission where the vicinity of cells and cell contacts are determinant parameters of viral infection and transmission? Some motifs involved in Env internalization and trafficking, as well as those involved in interaction with lipid rafts and in enhanced viral gene expression, are highly conserved across subtypes; others seem to display non-negligible sequence variation. It would be useful to assess whether such apparent tolerance identifies interactions that are less vital for viral replication, or whether the virus has acquired other compensatory polymorphisms to override decreased efficiency of that particular interaction.

Conclusions

Initially considered to be inessential for the HIV replication cycle, the gp41-CT has now been demonstrated to be a pleiotropic domain: it is required for viral infectivity by ensuring Env incorporation into the virion on one hand, and contributing to enhanced viral replication in target cells through many still incompletely understood interactions on the other. Work on the gp41-CT has revealed that lentiviral assembly relies on finely coordinated interactions between diverse viral components subverting cell trafficking and activation factors. The virus thereby optimizes the infectious capacity of the neo-synthesized particle, prevents immune responses and preserves the virus-producing host cell until viral release. The uncontrolled release of immature virions would not only hinder viral propagation and be deleterious to the long-term maintenance of viral infection, but would also provoke a series of bystander effects, including Env-induced apoptosis, Env-mediated disruptive effects, and sensitivity to humoral immune responses.

The Env expressed on the surface of infected cells and of virions is one of the most powerful escape mechanisms developed by HIV. Its extreme variability, dense glycosylation and its very efficient cell-to-cell spreading capacity ensure extremely effective virus shielding. This shielding is further strengthened by the only weak expression of Env at the surface of infected cells and of virions, resulting from its late expression during the viral replication cycle, intense cycling and down-regulation through the TGN. Interference with cellular trafficking factors further contributes to viral masking. These latter functions are all fulfilled by the gp41-CT. Indeed, despite some sequence divergence between subtypes, structural motifs and some sequence motifs in the gp41-CT are strongly conserved. In particular, elements involved in Env trafficking from the plasma

membrane through the endocytic and TGN compartments are conserved, underscoring their importance for viral replication and propagation. Other motifs involved in protein-protein interactions are not conserved providing evidence of subtype-related specificities. Such differences between subtypes have been poorly studied to date. The fine balance between sequence variability and highly controlled protein trafficking and dynamics allows the virus to optimize viral infectivity and viral propagation. The viral determinants that are poorly tolerant to polymorphic variation may well be interesting subjects for investigations to elucidate the subtle control of viral assembly, budding and release. Such work may lead to the identification of interactions that are possible targets for new antiviral strategies.

Abbreviations

Ab: Antibody; AP: Adaptor protein; CA: Capsid; CaM: Calmodulin; CT: Cytoplasmic tail; Env: Envelope; ER: Endoplasmic reticulum; gp: Glycoprotein; HA: Hemagglutinin; HCV: Hepatitis C virus; HIV: Human Immunodeficiency Virus; IS: Inhibitory sequence; IκB: Inhibitor κB; KE: Kennedy epitope; LLP: Lentiviral lytic peptide; MA: Matrix; MPER: Membrane proximal external region; MSD: Membrane-spanning domain; MVB: Multi vesicular bodies; NF-κB: Nuclear Factor -κB; PM: Plasma membrane; PNG: Potential N-glycosylation site; PR: Protease; RER: Rough endoplasmic reticulum; SIV: Simian Immunodeficiency virus; SP: Spacer protein; TAK-1: TGF-β-interacting factor 1; TCR: T cell receptor; TGN: trans Golgi network; TIP47: Tail-interacting Factor 47 kDa; VSV-G: Vesicular stomatitis virus-glycoprotein.

Competing interests

The authors declare no competing interests.

Authors' contributions

ESS, MM and DPB drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank Carole Devaux, Andy Chevigné and Jean Claude Schmit for critical reading of the manuscript. ESS and MM were supported by PhD scholarships from the Fonds National de la Recherche de Luxembourg. Work on the gp41-CT in the laboratory is supported by the Luxembourg Ministry of Research grant MESR-LRVT-20100604.

Received: 21 December 2012 Accepted: 1 May 2013

Published: 24 May 2013

References

1. Earl PL, Doms RW, Moss B: Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc Natl Acad Sci USA* 1990, **87**:648–652.
2. Chan DC, Fass D, Berger JM, Kim PS: Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 1997, **89**:263–273.
3. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA: Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 1998, **393**:648–659.
4. Moore JP, Doms RW: The entry of entry inhibitors: a fusion of science and medicine. *Proc Natl Acad Sci USA* 2003, **100**:10598–10602.
5. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC: Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 1997, **387**:426–430.
6. Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, Sodroski JG: The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* 1998, **393**:705–711.
7. Mao Y, Wang L, Gu C, Herschhorn A, Xiang SH, Haim H, Yang X, Sodroski J: Subunit organization of the membrane-bound HIV-1 envelope glycoprotein trimer. *Nat Struct Mol Biol* 2012, **19**:893–899.
8. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996, **272**:1955–1958.
9. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmor S, Sutton RE, Hill CM, *et al*: Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996, **381**:661–666.
10. Feng Y, Broder CC, Kennedy PE, Berger EA: HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996, **272**:872–877.
11. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, *et al*: The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 1996, **85**:1135–1148.
12. Dragic T, Litvin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA: HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996, **381**:667–673.
13. Tan K, Liu J, Wang J, Shen S, Lu M: Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc Natl Acad Sci USA* 1997, **94**:12303–12308.
14. Crise B, Buonocore L, Rose JK: CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. *J Virol* 1990, **64**:5585–5593.
15. Willey RL, Bonifacino JS, Potts BJ, Martin MA, Klausner RD: Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proc Natl Acad Sci USA* 1988, **85**:9580–9584.
16. Earl PL, Moss B, Doms RW: Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J Virol* 1991, **65**:2047–2055.
17. Courageot J, Fenouillet E, Bastiani P, Miquelis R: Intracellular degradation of the HIV-1 envelope glycoprotein. Evidence for, and some characteristics of, an endoplasmic reticulum degradation pathway. *Eur J Biochem* 1999, **260**:482–489.
18. Bernstein HB, Compans RW: Sulfation of the human immunodeficiency virus envelope glycoprotein. *J Virol* 1992, **66**:6953–6959.
19. Allan JS, Coligan JE, Barin F, McLane MF, Sodroski JG, Rosen CA, Haseltine WA, Lee TH, Essex M: Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* 1985, **228**:1091–1094.
20. Bernstein HB, Tucker SP, Hunter E, Schutzbach JS, Compans RW: Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. *J Virol* 1994, **68**:463–468.
21. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ: Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* 1990, **265**:10373–10382.
22. Earl PL, Koenig S, Moss B: Biological and immunological properties of human immunodeficiency virus type 1 envelope glycoprotein: analysis of proteins with truncations and deletions expressed by recombinant vaccinia viruses. *J Virol* 1991, **65**:31–41.
23. Schawaller M, Smith GE, Skehel JJ, Wiley DC: Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV. *Virology* 1989, **172**:367–369.
24. Fenouillet E, Jones IM: The glycosylation of human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) is important for the efficient intracellular transport of the envelope precursor gp160. *J Gen Virol* 1995, **76**(Pt 6):1509–1514.
25. Checkley MA, Luttge BG, Freed EO: HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. *J Mol Biol* 2011, **410**:582–608.
26. Bunnik EM, Pisas L, Van Nuenen AC, Schuitemaker H: Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. *J Virol* 2008, **82**:7932–7941.
27. Borggren M, Repits J, Sterjovski J, Uchtenhagen H, Churchill MJ, Karlsson A, Albert J, Achour A, Gorry PR, Fenyo EM, Jansson M: Increased sensitivity to broadly neutralizing antibodies of end-stage disease R5 HIV-1 correlates with evolution in Env glycosylation and charge. *PLoS One* 2011, **6**:e20135.
28. Fenyo EM, Esbjornsson J, Medstrand P, Jansson M: Human immunodeficiency virus type 1 biological variation and coreceptor use: from concept to clinical significance. *J Intern Med* 2011, **270**:520–531.
29. Steckbeck JD, Craigo JK, Barnes CO, Montelaro RC: Highly conserved structural properties of the C-terminal tail of HIV-1 gp41 protein despite substantial sequence variation among diverse clades: implications for functions in viral replication. *J Biol Chem* 2011, **286**:27156–27166.

30. Steckbeck JD, Kuhlmann AS, Montelaro RC: C-terminal tail of human immunodeficiency virus gp41: functionally rich and structurally enigmatic. *J Gen Virol* 2012, **94**:1–19.
31. Postler TS, Desrosiers RC: The Tale of the Long Tail: the Cytoplasmic Domain of HIV-1 gp41. *J Virol* 2012, **87**:2–15.
32. Pang HB, Hevroni L, Kol N, Eckert DM, Tsvitov M, Kay MS, Rousso I: Virion stiffness regulates immature HIV-1 entry. *Retrovirology* 2013, **10**:4.
33. Chojnacki J, Staudt T, Glass B, Bingen P, Engelhardt J, Anders M, Schneider J, Muller B, Hell SW, Krausslich HG: Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy. *Science* 2012, **338**:524–528.
34. Hunter E, Swanstrom R: Retrovirus envelope glycoproteins. *Curr Top Microbiol Immunol* 1990, **157**:187–253.
35. Buratti E, McLain L, Tisminetzky S, Cleveland SM, Dimmock NJ, Baralle FE: The neutralizing antibody response against a conserved region of human immunodeficiency virus type 1 gp41 (amino acid residues 731–752) is uniquely directed against a conformational epitope. *J Gen Virol* 1998, **79**(Pt 11):2709–2716.
36. Reading SA, Heap CJ, Dimmock NJ: A novel monoclonal antibody specific to the C-terminal tail of the gp41 envelope transmembrane protein of human immunodeficiency virus type 1 that preferentially neutralizes virus after it has attached to the target cell and inhibits the production of infectious progeny. *Virology* 2003, **315**:362–372.
37. Heap CJ, Reading SA, Dimmock NJ: An antibody specific for the C-terminal tail of the gp41 transmembrane protein of human immunodeficiency virus type 1 mediates post-attachment neutralization, probably through inhibition of virus-cell fusion. *J Gen Virol* 2005, **86**:1499–1507.
38. Cheung L, McLain L, Hollier MJ, Reading SA, Dimmock NJ: Part of the C-terminal tail of the envelope gp41 transmembrane glycoprotein of human immunodeficiency virus type 1 is exposed on the surface of infected cells and is involved in virus-mediated cell fusion. *J Gen Virol* 2005, **86**:131–138.
39. Eisenberg D, Wesson M: The most highly amphiphilic alpha-helices include two amino acid segments in human immunodeficiency virus glycoprotein 41. *Biopolymers* 1990, **29**:171–177.
40. Miller MA, Garry RF, Jaynes JM, Montelaro RC: A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. *AIDS Res Hum Retroviruses* 1991, **7**:511–519.
41. Klinger Y, Shai Y: A leucine zipper-like sequence from the cytoplasmic tail of the HIV-1 envelope glycoprotein binds and perturbs lipid bilayers. *Biochemistry* 1997, **36**:5157–5169.
42. Venable RM, Pastor RW, Brooks BR, Carson FW: Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. *AIDS Res Hum Retroviruses* 1989, **5**:7–22.
43. Dubay JW, Roberts SJ, Hahn BH, Hunter E: Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. *J Virol* 1992, **66**:6616–6625.
44. Yu X, Yuan X, McLane MF, Lee TH, Essex M: Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. *J Virol* 1993, **67**:213–221.
45. Piller SC, Dubay JW, Derdeyn CA, Hunter E: Mutational analysis of conserved domains within the cytoplasmic tail of gp41 from human immunodeficiency virus type 1: effects on glycoprotein incorporation and infectivity. *J Virol* 2000, **74**:11717–11723.
46. Kalia V, Sarkar S, Gupta P, Montelaro RC: Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. *J Virol* 2003, **77**:3634–3646.
47. Jiang J, Aiken C: Maturation-dependent human immunodeficiency virus type 1 particle fusion requires a carboxyl-terminal region of the gp41 cytoplasmic tail. *J Virol* 2007, **81**:9999–10008.
48. Miller MA, Cloyd MW, Liebmann J, Rinaldo CR Jr, Islam KR, Wang SZ, Mietzner TA, Montelaro RC: Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virology* 1993, **196**:89–100.
49. Chen SS, Lee SF, Wang CT: Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope transmembrane protein gp41. *J Virol* 2001, **75**:9925–9938.
50. Chernomordik L, Chanturiya AN, Suss-Toby E, Nora E, Zimmerberg J: An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. *J Virol* 1994, **68**:7115–7123.
51. Comardelle AM, Norris CH, Plymale DR, Gatti PJ, Choi B, Fermin CD, Haislip AM, Tencza SB, Mietzner TA, Montelaro RC, Garry RF: A synthetic peptide corresponding to the carboxy terminus of human immunodeficiency virus type 1 transmembrane glycoprotein induces alterations in the ionic permeability of *Xenopus laevis* oocytes. *AIDS Res Hum Retroviruses* 1997, **13**:1525–1532.
52. Murakami T, Freed EO: Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. *J Virol* 2000, **74**:3548–3554.
53. Gabuzda DH, Lever A, Terwilliger E, Sodroski J: Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. *J Virol* 1992, **66**:3306–3315.
54. Wyma DJ, Jiang J, Shi J, Zhou J, Lineberger JE, Miller MD, Aiken C: Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. *J Virol* 2004, **78**:3429–3435.
55. Lee SF, Wang CT, Liang JY, Hong SL, Huang CC, Chen SS: Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41. *J Biol Chem* 2000, **275**:15809–15819.
56. Zhu Y, Lu L, Chao L, Chen YH: Important changes in biochemical properties and function of mutated LLP12 domain of HIV-1 gp41. *Chem Biol Drug Des* 2007, **70**:311–318.
57. Edwards TG, Wyss S, Reeves JD, Zolla-Pazner S, Hoxie JA, Doms RW, Baribaud F: Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. *J Virol* 2002, **76**:2683–2691.
58. Wyss S, Dimitrov AS, Baribaud F, Edwards TG, Blumenthal R, Hoxie JA: Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail. *J Virol* 2005, **79**:12231–12241.
59. Abrahamyan LG, Mkrtychyan SR, Binley J, Lu M, Melikyan GB, Cohen FS: The cytoplasmic tail slows the folding of human immunodeficiency virus type 1 Env from a late prebundle configuration into the six-helix bundle. *J Virol* 2005, **79**:106–115.
60. Lu L, Zhu Y, Huang J, Chen X, Yang H, Jiang S, Chen YH: Surface exposure of the HIV-1 env cytoplasmic tail LLP2 domain during the membrane fusion process: interaction with gp41 fusion core. *J Biol Chem* 2008, **283**:16723–16731.
61. Spies CP, Compans RW: Effects of cytoplasmic domain length on cell surface expression and syncytium-forming capacity of the simian immunodeficiency virus envelope glycoprotein. *Virology* 1994, **203**:8–19.
62. Affranchino JL, Gonzalez SA: Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. *Virology* 2006, **347**:217–225.
63. Durham ND, Yewdall AW, Chen P, Lee R, Zony C, Robinson JE, Chen BK: Neutralization resistance of virological synapse-mediated HIV-1 infection is regulated by the gp41 cytoplasmic tail. *J Virol* 2012, **86**:7484–7495.
64. Davis MR, Jiang J, Zhou J, Freed EO, Aiken C: A mutation in the human immunodeficiency virus type 1 Gag protein destabilizes the interaction of the envelope protein subunits gp120 and gp41. *J Virol* 2006, **80**:2405–2417.
65. Bhakta SJ, Shang L, Prince JL, Claiborne DT, Hunter E: Mutagenesis of tyrosine and di-leucine motifs in the HIV-1 envelope cytoplasmic domain results in a loss of Env-mediated fusion and infectivity. *Retrovirology* 2011, **8**:37.
66. Haffar OK, Dowbenko DJ, Berman PW: Topogenic analysis of the human immunodeficiency virus type 1 envelope glycoprotein, gp160, in microsomal membranes. *J Cell Biol* 1988, **107**:1677–1687.
67. Kennedy RC, Henkel RD, Pauletti D, Allan JS, Lee TH, Essex M, Dreesman GR: Antiserum to a synthetic peptide recognizes the HTLV-III envelope glycoprotein. *Science* 1986, **231**:1556–1559.
68. Chanh TC, Dreesman GR, Kanda P, Linette GP, Sparrow JT, Ho DD, Kennedy RC: Induction of anti-HIV neutralizing antibodies by synthetic peptides. *EMBO J* 1986, **5**:3065–3071.
69. Cleveland SM, McLain L, Cheung L, Jones TD, Hollier M, Dimmock NJ: A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its exposure on the surface of the virion. *J Gen Virol* 2003, **84**:591–602.

70. Hollier MJ, Dimmock NJ: The C-terminal tail of the gp41 transmembrane envelope glycoprotein of HIV-1 clades A, B, C, and D may exist in two conformations: an analysis of sequence, structure, and function. *Virology* 2005, **337**:284–296.
71. Steckbeck JD, Sun C, Sturgeon TJ, Montelaro RC: Topology of the C-terminal tail of HIV-1 gp41: differential exposure of the Kennedy epitope on cell and viral membranes. *PLoS One* 2010, **5**:e15261.
72. Waheed AA, Ablan SD, Roser JD, Sowder RC, Schaffner CP, Chertova E, Freed EO: HIV-1 escape from the entry-inhibiting effects of a cholesterol-binding compound via cleavage of gp41 by the viral protease. *Proc Natl Acad Sci USA* 2007, **104**:8467–8471.
73. Waheed AA, Ablan SD, Sowder RC, Roser JD, Schaffner CP, Chertova E, Freed EO: Effect of mutations in the human immunodeficiency virus type 1 protease on cleavage of the gp41 cytoplasmic tail. *J Virol* 2010, **84**:3121–3126.
74. Futaki S, Nakase I, Suzuki T, Nameki D, Kodama E, Matsuoka M, Sugiyama Y: RNase S complex bearing arginine-rich peptide and anti-HIV activity. *J Mol Recognit* 2005, **18**:169–174.
75. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB: Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Pept Res* 2000, **56**:318–325.
76. Tung CH, Weissleder R: Arginine containing peptides as delivery vectors. *Adv Drug Deliv Rev* 2003, **55**:281–294.
77. Viard M, Ablan SD, Zhou M, Veenstra TD, Freed EO, Raviv Y, Blumenthal R: Photoinduced reactivity of the HIV-1 envelope glycoprotein with a membrane-embedded probe reveals insertion of portions of the HIV-1 Gp41 cytoplasmic tail into the viral membrane. *Biochemistry* 2008, **47**:1977–1983.
78. Postler TS, Martinez-Navio JM, Yuste E, Desrosiers RC: Evidence against extracellular exposure of a highly immunogenic region in the C-terminal domain of the simian immunodeficiency virus gp41 transmembrane protein. *J Virol* 2012, **86**:1145–1157.
79. Postler TS, Desrosiers RC: The cytoplasmic domain of the HIV-1 glycoprotein gp41 induces NF-kappaB activation through TGF-beta-activated kinase 1. *Cell Host Microbe* 2012, **11**:181–193.
80. Jouvenet N, Neil SJ, Bess C, Johnson MC, Virgen CA, Simon SM, Bieniasz PD: Plasma membrane is the site of productive HIV-1 particle assembly. *PLoS Biol* 2006, **4**:e435.
81. Welsch S, Keppler OT, Habermann A, Allespach I, Krijnse-Locker J, Krausslich HG: HIV-1 buds predominantly at the plasma membrane of primary human macrophages. *PLoS Pathog* 2007, **3**:e36.
82. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M: In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J Cell Biol* 2007, **177**:329–341.
83. Adamson CS, Freed EO: Human immunodeficiency virus type 1 assembly, release, and maturation. *Adv Pharmacol* 2007, **55**:347–387.
84. Bieniasz PD: The cell biology of HIV-1 virion genesis. *Cell Host Microbe* 2009, **5**:550–558.
85. Ono A, Freed EO: Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc Natl Acad Sci USA* 2001, **98**:13925–13930.
86. Ono A, Freed EO: Role of lipid rafts in virus replication. *Adv Virus Res* 2005, **64**:311–358.
87. Waheed AA, Freed EO: Lipids and membrane microdomains in HIV-1 replication. *Virus Res* 2009, **143**:162–176.
88. Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO: Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc Natl Acad Sci USA* 2004, **101**:14889–14894.
89. Chukkappalli V, Hogue IB, Boyko V, Hu WS, Ono A: Interaction between the human immunodeficiency virus type 1 Gag matrix domain and phosphatidylinositol-(4,5)-bisphosphate is essential for efficient gag membrane binding. *J Virol* 2008, **82**:2405–2417.
90. Simmons A, Aluvihare V, McMichael A: Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* 2001, **14**:763–777.
91. Zhou W, Parent LJ, Wills JW, Resh MD: Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J Virol* 1994, **68**:2556–2569.
92. Ono A, Orenstein JM, Freed EO: Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J Virol* 2000, **74**:2855–2866.
93. Chow JY, Jeffries CM, Kwan AH, Guss JM, Trewella J: Calmodulin disrupts the structure of the HIV-1 MA protein. *J Mol Biol* 2010, **400**:702–714.
94. Ghanam RH, Fernandez TF, Fledderman EL, Saad JS: Binding of calmodulin to the HIV-1 matrix protein triggers myristate exposure. *J Biol Chem* 2010, **285**:41911–41920.
95. Zhou W, Resh MD: Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J Virol* 1996, **70**:8540–8548.
96. Spearman P, Horton R, Ratner L, Kuli-Zade I: Membrane binding of human immunodeficiency virus type 1 matrix protein *in vivo* supports a conformational myristyl switch mechanism. *J Virol* 1997, **71**:6582–6592.
97. Scarlata S, Carter C: Role of HIV-1 Gag domains in viral assembly. *Biochim Biophys Acta* 2003, **1614**:62–72.
98. Ono A, Waheed AA, Joshi A, Freed EO: Association of human immunodeficiency virus type 1 gag with membrane does not require highly basic sequences in the nucleocapsid: use of a novel Gag multimerization assay. *J Virol* 2005, **79**:14131–14140.
99. Wright ER, Schooler JB, Ding HJ, Kieffer C, Fillmore C, Sundquist WI, Jensen GJ: Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells. *EMBO J* 2007, **26**:2218–2226.
100. Jouvenet N, Bieniasz PD, Simon SM: Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* 2008, **454**:236–240.
101. Hill CP, Worthylake D, Bancroft DP, Christensen AM, Sundquist WI: Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc Natl Acad Sci USA* 1996, **93**:3099–3104.
102. Briggs JA, Riches JD, Glass B, Bartonova V, Zanetti G, Krausslich HG: Structure and assembly of immature HIV. *Proc Natl Acad Sci USA* 2009, **106**:11090–11095.
103. Alfadhi A, Barklis RL, Barklis E: HIV-1 matrix organizes as a hexamer of trimers on membranes containing phosphatidylinositol-(4,5)-bisphosphate. *Virology* 2009, **387**:466–472.
104. Murakami T: Retroviral env glycoprotein trafficking and incorporation into virions. *Mol Biol Int* 2012, **2012**:682850.
105. Ghanam RH, Samal AB, Fernandez TF, Saad JS: Role of the HIV-1 matrix protein in gag intracellular trafficking and targeting to the plasma membrane for virus assembly. *Front Microbiol* 2012, **3**:55.
106. Sandrin V, Cosset FL: Intracellular versus cell surface assembly of retroviral pseudotypes is determined by the cellular localization of the viral glycoprotein, its capacity to interact with Gag, and the expression of the Nef protein. *J Biol Chem* 2006, **281**:528–542.
107. Sandrin V, Russell SJ, Cosset FL: Targeting retroviral and lentiviral vectors. *Curr Top Microbiol Immunol* 2003, **281**:137–178.
108. Leung K, Kim JO, Ganesh L, Kabat J, Schwartz O, Nabel GJ: HIV-1 assembly: viral glycoproteins segregate quantally to lipid rafts that associate individually with HIV-1 capsids and virions. *Cell Host Microbe* 2008, **3**:285–292.
109. Jorgenson RL, Vogt VM, Johnson MC: Foreign glycoproteins can be actively recruited to virus assembly sites during pseudotyping. *J Virol* 2009, **83**:4060–4067.
110. Lee YM, Tang XB, Cimackasky LM, Hildreth JE, Yu XF: Mutations in the matrix protein of human immunodeficiency virus type 1 inhibit surface expression and virion incorporation of viral envelope glycoproteins in CD4+ T lymphocytes. *J Virol* 1997, **71**:1443–1452.
111. Owens RJ, Dubay JW, Hunter E, Compans RW: Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. *Proc Natl Acad Sci USA* 1991, **88**:3987–3991.
112. Cosson P: Direct interaction between the envelope and matrix proteins of HIV-1. *EMBO J* 1996, **15**:5783–5788.
113. Hourieux C, Brand D, Sizaret PY, Lemiale F, Lebigot S, Barin F, Roingard P: Identification of the glycoprotein 41(TM) cytoplasmic tail domains of human immunodeficiency virus type 1 that interact with Pr55Gag particles. *AIDS Res Hum Retroviruses* 2000, **16**:1141–1147.
114. Freed EO, Martin MA: Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. *J Virol* 1995, **69**:1984–1989.
115. Mammano F, Kondo E, Sodroski J, Bukovsky A, Gottlinger HG: Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains. *J Virol* 1995, **69**:3824–3830.
116. Wyma DJ, Kotov A, Aiken C: Evidence for a stable interaction of gp41 with Pr55(Gag) in immature human immunodeficiency virus type 1 particles. *J Virol* 2000, **74**:9381–9387.

117. Celma CC, Manrique JM, Affranchino JL, Hunter E, Gonzalez SA: Domains in the simian immunodeficiency virus gp41 cytoplasmic tail required for envelope incorporation into particles. *Virology* 2001, **283**:253–261.
118. Freed EO, Martin MA: Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *J Virol* 1996, **70**:341–351.
119. Murakami T, Freed EO: The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. *Proc Natl Acad Sci USA* 2000, **97**:343–348.
120. Akari H, Fukumori T, Adachi A: Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions. *J Virol* 2000, **74**:4891–4893.
121. Lodge R, Gottlinger H, Gabuzda D, Cohen EA, Lemay G: The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells. *J Virol* 1994, **68**:4857–4861.
122. Lodge R, Lalonde JP, Lemay G, Cohen EA: The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. *EMBO J* 1997, **16**:695–705.
123. LaBranche CC, Sauter MM, Haggarty BS, Vance PJ, Romano J, Hart TK, Bugelski PJ, Marsh M, Hoxie JA: A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. *J Virol* 1995, **69**:5217–5227.
124. Yu X, Yuan X, Matsuda Z, Lee TH, Essex M: The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J Virol* 1992, **66**:4966–4971.
125. Dorfman T, Mammano F, Haseltine WA, Gottlinger HG: Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. *J Virol* 1994, **68**:1689–1696.
126. Gonzalez SA, Burny A, Affranchino JL: Identification of domains in the simian immunodeficiency virus matrix protein essential for assembly and envelope glycoprotein incorporation. *J Virol* 1996, **70**:6384–6389.
127. Beaumont E, Vendrame D, Verrier B, Roch E, Biron F, Barin F, Mammano F, Brand D: Matrix and envelope coevolution revealed in a patient monitored since primary infection with human immunodeficiency virus type 1. *J Virol* 2009, **83**:9875–9889.
128. Owens RJ, Rose JK: Cytoplasmic domain requirement for incorporation of a foreign envelope protein into vesicular stomatitis virus. *J Virol* 1993, **67**:360–365.
129. Murakami T, Ablan S, Freed EO, Tanaka Y: Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. *J Virol* 2004, **78**:1026–1031.
130. Kol N, Shi Y, Tsvitov M, Barlam D, Shneck RZ, Kay MS, Rousso I: A stiffness switch in human immunodeficiency virus. *Biophys J* 2007, **92**:1777–1783.
131. Pettit SC, Moody MD, Wehbie RS, Kaplan AH, Nantermet PV, Klein CA, Swanstrom R: The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. *J Virol* 1994, **68**:8017–8027.
132. Muranyi W, Malkusch S, Muller B, Heilemann M, Krausslich HG: Super-resolution microscopy reveals specific recruitment of HIV-1 envelope proteins to viral assembly sites dependent on the envelope C-terminal tail. *PLoS Pathog* 2013, **9**:e1003198.
133. Egan MA, Carruth LM, Rowell JF, Yu X, Siliciano RF: Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. *J Virol* 1996, **70**:6547–6556.
134. Micoli KJ, Pan G, Wu Y, Williams JP, Cook WJ, McDonald JM: Requirement of calmodulin binding by HIV-1 gp160 for enhanced FAS-mediated apoptosis. *J Biol Chem* 2000, **275**:1233–1240.
135. Radding W, Pan ZQ, Hunter E, Johnston P, Williams JP, McDonald JM: Expression of HIV-1 envelope glycoprotein alters cellular calmodulin. *Biochem Biophys Res Commun* 1996, **218**:192–197.
136. Ishikawa H, Sasaki M, Noda S, Koga Y: Apoptosis induction by the binding of the carboxyl terminus of human immunodeficiency virus type 1 gp160 to calmodulin. *J Virol* 1998, **72**:6574–6580.
137. Micoli KJ, Mamaeva O, Piller SC, Barker JL, Pan G, Hunter E, McDonald JM: Point mutations in the C-terminus of HIV-1 gp160 reduce apoptosis and calmodulin binding without affecting viral replication. *Virology* 2006, **344**:468–479.
138. Rein A, Mirro J, Haynes JG, Ernst SM, Nagashima K: Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. *J Virol* 1994, **68**:1773–1781.
139. Brody BA, Rhee SS, Hunter E: Postassembly cleavage of a retroviral glycoprotein cytoplasmic domain removes a necessary incorporation signal and activates fusion activity. *J Virol* 1994, **68**:4620–4627.
140. Wilk T, Pfeiffer T, Bosch V: Retained *in vitro* infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. *Virology* 1992, **189**:167–177.
141. Emerson V, Haller C, Pfeiffer T, Fackler OT, Bosch V: Role of the C-terminal domain of the HIV-1 glycoprotein in cell-to-cell viral transmission between T lymphocytes. *Retrovirology* 2010, **7**:43.
142. Iwatani Y, Ueno T, Nishimura A, Zhang X, Hattori T, Ishimoto A, Ito M, Sakai H: Modification of virus infectivity by cytoplasmic tail of HIV-1 TM protein. *Virus Res* 2001, **74**:75–87.
143. Zhu P, Chertova E, Bess J Jr, Lifson JD, Arthur LO, Liu J, Taylor KA, Roux KH: Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc Natl Acad Sci USA* 2003, **100**:15812–15817.
144. Yuste E, Reeves JD, Doms RW, Desrosiers RC: Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J Virol* 2004, **78**:6775–6785.
145. Chertova E, Bess JW Jr, Crise BJ, Sowder IR, Schaden TM, Hilburn JM, Hoxie JA, Benveniste RE, Lifson JD, Henderson LE, Arthur LO: Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol* 2002, **76**:5315–5325.
146. Tsujimoto H, Cooper RW, Kodama T, Fukasawa M, Miura T, Ohta Y, Ishikawa K, Nakai M, Frost E, Roelants GE, et al: Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. *J Virol* 1988, **62**:4044–4050.
147. Chakrabarti L, Emerman M, Tiollais P, Sonigo P: The cytoplasmic domain of simian immunodeficiency virus transmembrane protein modulates infectivity. *J Virol* 1989, **63**:4395–4403.
148. Hirsch VM, Edmondson P, Murphey-Corb M, Arbeille B, Johnson PR, Mullins JI: SIV adaptation to human cells. *Nature* 1989, **341**:573–574.
149. Kodama T, Wooley DP, Naidu YM, Kestler HW 3rd, Daniel MD, Li Y, Desrosiers RC: Significance of premature stop codons in env of simian immunodeficiency virus. *J Virol* 1989, **63**:4709–4714.
150. Ritter GD Jr, Mulligan MJ, Lydy SL, Compans RW: Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. *Virology* 1993, **197**:255–264.
151. Zingler K, Littman DR: Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases env incorporation into particles and fusogenicity and infectivity. *J Virol* 1993, **67**:2824–2831.
152. Shacklett BL, Weber CJ, Shaw KE, Keddie EM, Gardner MB, Sonigo P, Luciw PA: The intracytoplasmic domain of the Env transmembrane protein is a locus for attenuation of simian immunodeficiency virus SIVmac in rhesus macaques. *J Virol* 2000, **74**:5836–5844.
153. Mulligan MJ, Yamshchikov GV, Ritter GD Jr, Gao F, Jin MJ, Nail CD, Spies CP, Hahn BH, Compans RW: Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. *J Virol* 1992, **66**:3971–3975.
154. Nilsson T, Warren G: Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr Opin Cell Biol* 1994, **6**:517–521.
155. Munro S: An investigation of the role of transmembrane domains in Golgi protein retention. *EMBO J* 1995, **14**:4695–4704.
156. Bultmann A, Muranyi W, Seed B, Haas J: Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein that inhibit cell surface expression. *J Virol* 2001, **75**:5263–5276.
157. Rowell JF, Stanhope PE, Siliciano RF: Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. *J Immunol* 1995, **155**:473–488.
158. Ohno H, Fournier MC, Poy G, Bonifacino JS: Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. *J Biol Chem* 1996, **271**:29009–29015.

159. Ohno H, Aguilar RC, Fournier MC, Hennecke S, Cosson P, Bonifacino JS: **Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family.** *Virology* 1997, **238**:305–315.
160. Batonick M, Favre M, Boge M, Spearman P, Honing S, Thali M: **Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2.** *Virology* 2005, **342**:190–200.
161. Camus G, Segura-Morales C, Molle D, Lopez-Verges S, Begon-Pescia C, Cazeville C, Schu P, Bertrand E, Berlioz-Torrent C, Basyuk E: **The clathrin adaptor complex AP-1 binds HIV-1 and MLV Gag and facilitates their budding.** *Mol Biol Cell* 2007, **18**:3193–3203.
162. Dong X, Li H, Derdowski A, Ding L, Burnett A, Chen X, Peters TR, Dermody TS, Woodruff E, Wang JJ, Spearman P: **AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly.** *Cell* 2005, **120**:663–674.
163. Liu L, Sutton J, Woodruff E, Villalta F, Spearman P, Dong X: **Defective HIV-1 particle assembly in AP-3-deficient cells derived from patients with Hermansky-Pudlak syndrome type 2.** *J Virol* 2012, **86**:11242–11253.
164. Berlioz-Torrent C, Shacklett BL, Erdtmann L, Delamarre L, Bouchaert I, Sonigo P, Dokhelar MC, Benarous R: **Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins.** *J Virol* 1999, **73**:1350–1361.
165. Lopez-Verges S, Camus G, Blot G, Beauvoir R, Benarous R, Berlioz-Torrent C: **Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions.** *Proc Natl Acad Sci USA* 2006, **103**:14947–14952.
166. Bowers K, Pelchen-Matthews A, Honing S, Vance PJ, Creary L, Haggarty BS, Romano J, Ballensiefen W, Hoxie JA, Marsh M: **The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis.** *Traffic* 2000, **1**:661–674.
167. Wyss S, Berlioz-Torrent C, Boge M, Blot G, Honing S, Benarous R, Thali M: **The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter].** *J Virol* 2001, **75**:2982–2992.
168. Byland R, Vance PJ, Hoxie JA, Marsh M: **A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein.** *Mol Biol Cell* 2007, **18**:414–425.
169. Dingwell KS, Johnson DC: **The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions.** *J Virol* 1998, **72**:8933–8942.
170. Johnson DC, Huber MT: **Directed egress of animal viruses promotes cell-to-cell spread.** *J Virol* 2002, **76**:1–8.
171. Orsini MJ, Parent JL, Mundell SJ, Marchese A, Benovic JL: **Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization.** *J Biol Chem* 1999, **274**:31076–31086.
172. Blot G, Janvier K, Le Panse S, Benarous R, Berlioz-Torrent C: **Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity.** *J Virol* 2003, **77**:6931–6945.
173. Murray JL, Mavrakis M, McDonald NJ, Yilla M, Sheng J, Bellini WJ, Zhao L, Le Doux JM, Shaw MW, Luo CC, *et al*: **Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses, and measles virus.** *J Virol* 2005, **79**:11742–11751.
174. Lambele M, Labrosse B, Roch E, Moreau A, Verrier B, Barin F, Roingard P, Mammano F, Brand D: **Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of envelope glycoproteins and viral assembly.** *J Virol* 2007, **81**:125–140.
175. Bauby H, Lopez-Verges S, Hoeffel G, Delcroix-Genete D, Janvier K, Mammano F, Hosmalin A, Berlioz-Torrent C: **TIP47 is required for the production of infectious HIV-1 particles from primary macrophages.** *Traffic* 2010, **11**:455–467.
176. Feng Y, Press B, Wandinger-Ness A: **Rab 7: an important regulator of late endocytic membrane traffic.** *J Cell Biol* 1995, **131**:1435–1452.
177. Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M: **Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments.** *Cell* 1990, **62**:317–329.
178. Vitelli R, Santillo M, Lattero D, Chiariello M, Bifulco M, Bruni CB, Bucci C: **Role of the small GTPase Rab7 in the late endocytic pathway.** *J Biol Chem* 1997, **272**:4391–4397.
179. Vieira OV, Bucci C, Harrison RE, Trimble WS, Lanzetti L, Gruenberg J, Schreiber AD, Stahl PD, Grinstein S: **Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase.** *Mol Cell Biol* 2003, **23**:2501–2514.
180. Wang T, Ming Z, Xiaochun W, Hong W: **Rab7: role of its protein interaction cascades in endo-lysosomal traffic.** *Cell Signal* 2011, **23**:516–521.
181. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, Lieberman J, Elledge SJ: **Identification of host proteins required for HIV infection through a functional genomic screen.** *Science* 2008, **319**:921–926.
182. Caillet M, Janvier K, Pelchen-Matthews A, Delcroix-Genete D, Camus G, Marsh M, Berlioz-Torrent C: **Rab7A is required for efficient production of infectious HIV-1.** *PLoS Pathog* 2011, **7**:e1002347.
183. Bucci C, Thomsen P, Nicoziani P, McCarthy J, Van Deurs B: **Rab7: a key to lysosome biogenesis.** *Mol Biol Cell* 2000, **11**:467–480.
184. Lebrand C, Corti M, Goodson H, Cosson P, Cavalli V, Mayran N, Faure J, Gruenberg J: **Late endosome motility depends on lipids via the small GTPase Rab7.** *EMBO J* 2002, **21**:1289–1300.
185. Ceresa BP, Bahr SJ: **rab7 activity affects epidermal growth factor: epidermal growth factor receptor degradation by regulating endocytic trafficking from the late endosome.** *J Biol Chem* 2006, **281**:1099–1106.
186. Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL: **Role for Rab7 in maturation of late autophagic vacuoles.** *J Cell Sci* 2004, **117**:4837–4848.
187. Gutierrez MG, Munafò DB, Beron W, Colombo MI: **Rab7 is required for the normal progression of the autophagic pathway in mammalian cells.** *J Cell Sci* 2004, **117**:2687–2697.
188. Schiavoni I, Muratori C, Piacentini V, Giammaroli AM, Federico M: **The HIV-1 Nef protein: how an AIDS pathogenic factor turns to a tool for combating AIDS.** *Curr Drug Targets Immune Endocr Metabol Disord* 2004, **4**:19–27.
189. Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, Dong C, Wu L, Kominami E, Ueno T, Yamamoto A, *et al*: **Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages.** *J Cell Biol* 2009, **186**:255–268.
190. Dinkins C, Arko-Mensah J, Deretic V: **Autophagy and HIV.** *Semin Cell Dev Biol* 2010, **21**:712–718.
191. Evans DT, Tillman KC, Desrosiers RC: **Envelope glycoprotein cytoplasmic domains from diverse lentiviruses interact with the prenylated Rab acceptor.** *J Virol* 2002, **76**:327–337.
192. Blancou P, Evans DT, Desrosiers RC: **PRA1 co-localizes with envelope but does not influence primate lentivirus production, infectivity or envelope incorporation.** *J Gen Virol* 2005, **86**:1785–1790.
193. Brown DA, London E: **Structure and function of sphingolipid- and cholesterol-rich membrane rafts.** *J Biol Chem* 2000, **275**:17221–17224.
194. Simons K, Gerl MJ: **Revitalizing membrane rafts: new tools and insights.** *Nat Rev Mol Cell Biol* 2010, **11**:688–699.
195. Munro S: **Lipid rafts: elusive or illusive?** *Cell* 2003, **115**:377–388.
196. Aloia RC, Tian H, Jensen FC: **Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes.** *Proc Natl Acad Sci USA* 1993, **90**:5181–5185.
197. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG: **The HIV lipidome: a raft with an unusual composition.** *Proc Natl Acad Sci USA* 2006, **103**:2641–2646.
198. Bhattacharya J, Peters PJ, Clapham PR: **Human immunodeficiency virus type 1 envelope glycoproteins that lack cytoplasmic domain cysteines: impact on association with membrane lipid rafts and incorporation onto budding virus particles.** *J Virol* 2004, **78**:5500–5506.
199. Campbell S, Oshima M, Mirro J, Nagashima K, Rein A: **Reversal by dithiothreitol treatment of the block in murine leukemia virus maturation induced by disulfide cross-linking.** *J Virol* 2002, **76**:10050–10055.
200. Guyader M, Kiyokawa E, Abrami L, Turelli P, Trono D: **Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization.** *J Virol* 2002, **76**:10356–10364.
201. Graham DR, Chertova E, Hilburn JM, Arthur LO, Hildreth JE: **Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts.** *J Virol* 2003, **77**:8237–8248.
202. Liao Z, Graham DR, Hildreth JE: **Lipid rafts and HIV pathogenesis: virion-associated cholesterol is required for fusion and infection of susceptible cells.** *AIDS Res Hum Retroviruses* 2003, **19**:675–687.

203. Campbell S, Gaus K, Bittman R, Jessup W, Crowe S, Mak J: **The raft-promoting property of virion-associated cholesterol, but not the presence of virion-associated Brij 98 rafts, is a determinant of human immunodeficiency virus type 1 infectivity.** *J Virol* 2004, **78**:10556–10565.
204. Yang P, Ai LS, Huang SC, Li HF, Chan WE, Chang CW, Ko CY, Chen SS: **The cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein gp41 harbors lipid raft association determinants.** *J Virol* 2010, **84**:59–75.
205. Rouso I, Mixon MB, Chen BK, Kim PS: **Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity.** *Proc Natl Acad Sci USA* 2000, **97**:13523–13525.
206. Bhattacharya J, Repik A, Clapham PR: **Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes.** *J Virol* 2006, **80**:5292–5300.
207. Chan WE, Lin HH, Chen SS: **Wild-type-like viral replication potential of human immunodeficiency virus type 1 envelope mutants lacking palmitoylation signals.** *J Virol* 2005, **79**:8374–8387.
208. Bhatia AK, Kaushik R, Campbell NA, Pontow SE, Ratner L: **Mutation of critical serine residues in HIV-1 matrix result in an envelope incorporation defect which can be rescued by truncation of the gp41 cytoplasmic tail.** *Virology* 2009, **384**:233–241.
209. Ali A, Avalos RT, Ponimaskin E, Nayak DP: **Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein.** *J Virol* 2000, **74**:8709–8719.
210. Henderson G, Murray J, Yeo RP: **Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins.** *Virology* 2002, **300**:244–254.
211. Day JR, Munk C, Guatelli JC: **The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity.** *J Virol* 2004, **78**:1069–1079.
212. Schiavoni I, Trapp S, Santarcangelo AC, Piacentini V, Pugliese K, Baur A, Federico M: **HIV-1 Nef enhances both membrane expression and virion incorporation of Env products. A model for the Nef-dependent increase of HIV-1 infectivity.** *J Biol Chem* 2004, **279**:22996–23006.
213. Bresnahan PA, Yonemoto W, Ferrell S, Williams-Herman D, Geleziunas R, Greene WC: **A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor.** *Curr Biol* 1998, **8**:1235–1238.
214. Adnan S, Balamurugan A, Trocha A, Bennett MS, Ng HL, Ali A, Brander C, Yang OO: **Nef interference with HIV-1-specific CTL antiviral activity is epitope specific.** *Blood* 2006, **108**:3414–3419.
215. Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL: **HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells.** *PLoS Pathog* 2008, **4**:e1000131.
216. El-Far M, Isabelle C, Chomont N, Bourbonniere M, Fonseca S, Ancuta P, Peretz Y, Chouikh Y, Halwani R, Schwartz O, *et al*: **Down-Regulation of CTLA-4 by HIV-1 Nef Protein.** *PLoS One* 2013, **8**:e54295.
217. Stolp B, Fackler OT: **How HIV takes advantage of the cytoskeleton in entry and replication.** *Viruses* 2011, **3**:293–311.
218. Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB: **The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages.** *J Exp Med* 1994, **179**:101–113.
219. Roeth JF, Collins KL: **Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways.** *Microbiol Mol Biol Rev* 2006, **70**:548–563.
220. Zhou J, Aiken C: **Nef enhances human immunodeficiency virus type 1 infectivity resulting from intervirion fusion: evidence supporting a role for Nef at the virion envelope.** *J Virol* 2001, **75**:5851–5859.
221. Stumptner-Cuvelette P, Jouve M, Helft J, Dugast M, Glouzman AS, Jooss K, Raposo G, Benaroch P: **Human immunodeficiency virus-1 Nef expression induces intracellular accumulation of multivesicular bodies and major histocompatibility complex class II complexes: potential role of phosphatidylinositol 3-kinase.** *Mol Biol Cell* 2003, **14**:4857–4870.
222. Zheng YH, Plemenitas A, Fielding CJ, Peterlin BM: **Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions.** *Proc Natl Acad Sci USA* 2003, **100**:8460–8465.
223. St Gelais C, Coleman CM, Wang JH, Wu L: **HIV-1 Nef Enhances Dendritic Cell-Mediated Viral Transmission to CD4(+) T Cells and Promotes T-Cell Activation.** *PLoS One* 2012, **7**:e34521.
224. Wolf D, Witte V, Clark P, Blume K, Lichtenheld MG, Baur AS: **HIV Nef enhances Tat-mediated viral transcription through a hnRNP-K-nucleated signaling complex.** *Cell Host Microbe* 2008, **4**:398–408.
225. Omoto S, Fujii YR: **Regulation of human immunodeficiency virus 1 transcription by nef microRNA.** *J Gen Virol* 2005, **86**:751–755.
226. Witte V, Laffert B, Gintschel P, Krautkramer E, Blume K, Fackler OT, Baur AS: **Induction of HIV transcription by Nef involves Lck activation and protein kinase C theta raft recruitment leading to activation of ERK1/2 but not NF kappa B.** *J Immunol* 2008, **181**:8425–8432.
227. Neri F, Giolo G, Potesta M, Petrini S, Doria M: **The HIV-1 Nef protein has a dual role in T cell receptor signaling in infected CD4+ T lymphocytes.** *Virology* 2011, **410**:316–326.
228. Arold ST, Baur AS: **Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein.** *Trends Biochem Sci* 2001, **26**:356–363.
229. Chazal N, Singer G, Aiken C, Hammarskjold ML, Rekosh D: **Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity.** *J Virol* 2001, **75**:4014–4018.
230. Perugi F, Muriaux D, Ramirez BC, Chabani S, Decroly E, Darlix JL, Blot V, Pique C: **Human Discs Large is a new negative regulator of human immunodeficiency virus-1 infectivity.** *Mol Biol Cell* 2009, **20**:498–508.
231. Coleman SH, Van Damme N, Day JR, Noviello CM, Hitchin D, Madrid R, Benichou S, Guatelli JC: **Leucine-specific, functional interactions between human immunodeficiency virus type 1 Nef and adaptor protein complexes.** *J Virol* 2005, **79**:2066–2078.
232. Madrid R, Janvier K, Hitchin D, Day J, Coleman S, Noviello C, Bouchet J, Benmerah A, Guatelli J, Benichou S: **Nef-induced alteration of the early/recycling endosomal compartment correlates with enhancement of HIV-1 infectivity.** *J Biol Chem* 2005, **280**:5032–5044.
233. Costa LJ, Chen N, Lopes A, Aguiar RS, Tanuri A, Plemenitas A, Peterlin BM: **Interactions between Nef and AIP1 proliferate multivesicular bodies and facilitate egress of HIV-1.** *Retrovirology* 2006, **3**:33.
234. Costa LJ, Zheng YH, Sabotic J, Mak J, Fackler OT, Peterlin BM: **Nef binds p6* in GagPol during replication of human immunodeficiency virus type 1.** *J Virol* 2004, **78**:5311–5323.
235. Leihener A, Ludwig C, Wagner R: **Influence of extended mutations of the HIV-1 transframe protein p6 on Nef-dependent viral replication and infectivity in vitro.** *Virology* 2009, **387**:200–210.
236. He C, Klionsky DJ: **Regulation mechanisms and signaling pathways of autophagy.** *Annu Rev Genet* 2009, **43**:67–93.
237. Fader NC, Colombo MI: **Autophagy and multivesicular bodies: two closely related partners.** *Cell Death Differ* 2009, **16**:70–78.
238. Noda T, Yoshimori T: **Molecular basis of canonical and bactericidal autophagy.** *Int Immunol* 2009, **21**:1199–1204.
239. Welsch S, Muller B, Krausslich HG: **More than one door - Budding of enveloped viruses through cellular membranes.** *FEBS Lett* 2007, **581**:2089–2097.
240. Schibli DJ, Montelaro RC, Vogel HJ: **The membrane-proximal tryptophan-rich region of the HIV glycoprotein, gp41, forms a well-defined helix in dodecylphosphocholine micelles.** *Biochemistry* 2001, **40**:9570–9578.
241. Sun ZY, Oh KJ, Kim M, Yu J, Brusci V, Song L, Qiao Z, Wang JH, Wagner G, Reinherz EL: **HIV-1 broadly neutralizing antibody extracts its epitope from a kinked gp41 ectodomain region on the viral membrane.** *Immunity* 2008, **28**:52–63.
242. Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W: **Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions.** *PLoS Pathog* 2010, **6**:e1000880.
243. Lai RP, Yan J, Heeney J, McClure MO, Gottlinger H, Luban J, Pizzato M: **Nef decreases HIV-1 sensitivity to neutralizing antibodies that target the membrane-proximal external region of TMgp41.** *PLoS Pathog* 2011, **7**:e1002442.
244. Tokunaga K, Kojima A, Kurata T, Ikuta K, Inubushi R, Shimano R, Kawamura M, Akari H, Koyama AH, Adachi A: **Producer cell-dependent requirement of the Nef protein for efficient entry of HIV-1 into cells.** *Biochem Biophys Res Commun* 1998, **250**:565–568.
245. Tokunaga K, Kojima A, Kurata T, Ikuta K, Akari H, Koyama AH, Kawamura M, Inubushi R, Shimano R, Adachi A: **Enhancement of human immunodeficiency virus type 1 infectivity by Nef is producer cell-dependent.** *J Gen Virol* 1998, **79**(Pt 10):2447–2453.

246. Srinivas SK, Srinivas RV, Anantharamaiah GM, Compans RW, Segrest JP: Cytosolic domain of the human immunodeficiency virus envelope glycoproteins binds to calmodulin and inhibits calmodulin-regulated proteins. *J Biol Chem* 1993, **268**:22895–22899.
247. Tencza SB, Mietzner TA, Montelaro RC: Calmodulin-binding function of LLP segments from the HIV type 1 transmembrane protein is conserved among natural sequence variants. *AIDS Res Hum Retroviruses* 1997, **13**:263–269.
248. Pan Z, Radding W, Zhou T, Hunter E, Mountz J, McDonald JM: Role of calmodulin in HIV-potentiated Fas-mediated apoptosis. *Am J Pathol* 1996, **149**:903–910.
249. Emerson V, Holtkotte D, Pfeiffer T, Wang IH, Schnolzer M, Kempf T, Bosch V: Identification of the cellular prohibitin 1/prohibitin 2 heterodimer as an interaction partner of the C-terminal cytoplasmic domain of the HIV-1 glycoprotein. *J Virol* 2010, **84**:1355–1365.
250. Zhang H, Wang L, Kao S, Whitehead IP, Hart MJ, Liu B, Duus K, Burridge K, Der CJ, Su L: Functional interaction between the cytoplasmic leucine-zipper domain of HIV-1 gp41 and p115-RhoGEF. *Curr Biol* 1999, **9**:1271–1274.
251. Wang L, Zhang H, Solski PA, Hart MJ, Der CJ, Su L: Modulation of HIV-1 replication by a novel RhoA effector activity. *J Immunol* 2000, **164**:5369–5374.
252. Adhikari A, Xu M, Chen ZJ: Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* 2007, **26**:3214–3226.
253. Nabel G, Baltimore D: An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 1987, **326**:711–713.
254. Saksela K, Cheng G, Baltimore D: Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J* 1995, **14**:484–491.
255. Alexander L, Du Z, Rosenzweig M, Jung JU, Desrosiers RC: A role for natural simian immunodeficiency virus and human immunodeficiency virus type 1 nef alleles in lymphocyte activation. *J Virol* 1997, **71**:6094–6099.
256. Wang JK, Kiyokawa E, Verdin E, Trono D: The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc Natl Acad Sci USA* 2000, **97**:394–399.
257. Fenard D, Yonemoto W, De Noronha C, Cavois M, Williams SA, Greene WC: Nef is physically recruited into the immunological synapse and potentiates T cell activation early after TCR engagement. *J Immunol* 2005, **175**:6050–6057.
258. Biggs TE, Cooke SJ, Barton CH, Harris MP, Saksela K, Mann DA: Induction of activator protein 1 (AP-1) in macrophages by human immunodeficiency virus type-1 NEF is a cell-type-specific response that requires both hck and MAPK signaling events. *J Mol Biol* 1999, **290**:21–35.
259. Schragar JA, Minassian V, Marsh JW: HIV Nef increases T cell ERK MAP kinase activity. *J Biol Chem* 2002, **277**:6137–6142.
260. Janeway CA Jr, Bottomly K: Signals and signs for lymphocyte responses. *Cell* 1994, **76**:275–285.
261. Linsley PS, Ledbetter JA: The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 1993, **11**:191–212.
262. DenBoer LM, Hardy-Smith PW, Hogan MR, Cockram GP, Audas TE, Lu R: Luman is capable of binding and activating transcription from the unfolded protein response element. *Biochem Biophys Res Commun* 2005, **331**:113–119.
263. Liang G, Audas TE, Li Y, Cockram GP, Dean JD, Martyn AC, Kokame K, Lu R: Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Mol Cell Biol* 2006, **26**:7999–8010.
264. Audas TE, Li Y, Liang G, Lu R: A novel protein, Luman/CREB3 recruitment factor, inhibits Luman activation of the unfolded protein response. *Mol Cell Biol* 2008, **28**:3952–3966.
265. Blot G, Lopez-Verges S, Treand C, Kubat NJ, Delcroix-Genete D, Emiliani S, Benarous R, Berlioz-Torrent C: Luman, a new partner of HIV-1 TMgp41, interferes with Tat-mediated transcription of the HIV-1 LTR. *J Mol Biol* 2006, **364**:1034–1047.
266. Neil SJ, Zang T, Bieniasz PD: Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008, **451**:425–430.
267. Jouvenet N, Neil SJ, Zhadina M, Zang T, Kratovac Z, Lee Y, McNatt M, Hatzioannou T, Bieniasz PD: Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. *J Virol* 2009, **83**:1837–1844.
268. Douglas JL, Gustin JK, Viswanathan K, Mansouri M, Moses AV, Fruh K: The great escape: viral strategies to counter BST-2/tetherin. *PLoS Pathog* 2010, **6**:e1000913.
269. Evans DT, Serra-Moreno R, Singh RK, Guatelli JC: BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends Microbiol* 2010, **18**:388–396.
270. Janvier K, Pelchen-Matthews A, Renaud JB, Caillet M, Marsh M, Berlioz-Torrent C: The ESCRT-0 component HRS is required for HIV-1 Vpu-mediated BST-2/tetherin down-regulation. *PLoS Pathog* 2011, **7**:e1001265.
271. Kuhl BD, Sloan RD, Donahue DA, Bar-Magen T, Liang C, Wainberg MA: Tetherin restricts direct cell-to-cell infection of HIV-1. *Retrovirology* 2010, **7**:115.
272. Serra-Moreno R, Jia B, Breed M, Alvarez X, Evans DT: Compensatory changes in the cytoplasmic tail of gp41 confer resistance to tetherin/BST-2 in a pathogenic nef-deleted SIV. *Cell Host Microbe* 2011, **9**:46–57.
273. Le Tortorec A, Neil SJ: Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *J Virol* 2009, **83**:11966–11978.

doi:10.1186/1742-4690-10-54

Cite this article as: Santos da Silva *et al.*: The frantic play of the concealed HIV envelope cytoplasmic tail. *Retrovirology* 2013 **10**:54.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit



2. Polymorphisms in the gp41 cytoplasmic tail of HIV-1 subtype C viruses impair replication capacity in CD4+ T lymphocytes but not in macrophages

Santos da Silva E, Mulinge M, Rybicki A, Lemaire M, Masquelier C, Servais JY, Schmit JC, Seguin-Devaux C, Perez Bercoff D.

Soumis à *Retrovirology*

1.1. Résumé

Introduction

L'enveloppe (Env) du virus de l'immunodéficience humaine (VIH) est extrêmement variable et elle est primordiale dans le processus d'entrée dans la cellule cible. La queue intracytoplasmique de l'enveloppe (gp41CT) est responsable de l'incorporation de l'enveloppe dans les virions bourgeonnants, mais joue également un rôle important dans l'infectivité du virus et régule le trafic intracellulaire de Env. Cette région a été étudiée principalement à l'aide de mutants ponctuels introduits dans le clone de référence NL4.3 ou de mutants de délétion. Des polymorphismes existent dans gp41CT mais leur impact n'a jamais été décrit. Dans ce travail, nous avons étudié l'impact de polymorphismes dans gp41CT sur la réplication virale dans les cellules cibles naturelles du virus.

Résultats

Des lymphocytes T CD4+ (LT CD4+) activés ainsi que des macrophages ont été infectés par des paires de virus recombinants. Ces virus possèdent un génome constant issu du clone de référence (NL4.3), dans lesquels soit toute l'enveloppe (gp160), soit l'ectodomaine de l'enveloppe sont remplacées par les séquences de virus primaires correspondantes. Ainsi ces virus possèdent soit la gp41CT primaire (recombinants Env), soit la gp41CT de la souche de référence fusionnée à l'ectodomaine primaire (recombinants EnvEC). Dans les LT CD4+ activés, la réplication des recombinants Env et EnvEC de sous-type C est plus faible que celle des recombinants avec des enveloppes de sous-type B. De

plus, les virus recombinants Env répliquent moins bien que leur homologue EnvEC, alors que les virus Env et EnvEC de sous-type B répliquent de façon comparable dans les LT CD4+. Ces différences d'infectivité (Env B = EnvEC B > EnvEC C > Env C) n'ont toutefois pas été observées au niveau des macrophages (MDMs) où le niveau de répllication était semblable quel que soit le sous-type de l'enveloppe et quel que soit le domaine gp41CT. L'ajout de nucleosides exogènes aux cultures de MDMs augmente le niveau de répllication mais n'affecte pas l'issue de l'infection. La présentation des virus Env et EnvEC aux LT CD4+ par des cellules dendritiques matures dérivées de monocytes (MDDCs) montre des phénotypes de répllication semblables, suggérant que ces enveloppes ne présentent pas un avantage de transmission et que la faible répllication virale est spécifique aux lymphocytes T et est en partie due au domaine gp41CT.

L'observation de la distribution de l'enveloppe et du précurseur Pr55^{Gag} dans les LT CD4+ indique que la distribution de Env est comparable quelle que soit l'origine de gp41CT. Ceci est en accord avec la conservation des motifs impliqués dans le trafic intracellulaire. En revanche, dans les LT CD4+ infectés par des virus recombinants Env de sous-type C, les agrégats Pr55^{Gag} polarisés étaient absents ou peu visibles, alors qu'ils étaient bien visibles dans les LT CD4+ infectés par les recombinants EnvEC correspondants ou dans les cellules infectées par des virus portant une enveloppe de sous-type B. A l'inverse, dans les MDMs on n'observe aucune différence entre les cultures infectées par les recombinants Env ou les recombinants EnvEC, il y a bien présence et colocalisation d'enveloppe et de Pr55^{Gag}. Les analyses d'incorporation d'enveloppe dans les virions formés dans les LT CD4+ par Western Blot et par ELISA suggèrent qu'il n'y a pas de différence d'incorporation entre virus Env et EnvEC.

L'analyse des séquences des gp41CT de sous-type C a permis de mettre en évidence plusieurs polymorphismes spécifiques du sous-type C. Certains polymorphismes étaient souvent associés aux Env présentant les plus faibles niveaux de répllication : LLL₇₇₆→FIL₇₇₆ et une insertion de sept acides aminés dans le domaine LLP-2, et la mutation LL₈₅₆→LQ₈₅₆ à l'extrémité C-terminale de LLP-1. Ces motifs ont été révertés dans deux enveloppes de sous-type C et introduits dans gp41CT de NLAD8 (qui contient l'ectodomaine du clone R5 ADA et gp41CT de NL4.3). Chacune des mutations introduite seule affecte la répllication virale différemment selon le contexte génétique de Env. En revanche, les double ou triples mutants répliquent mieux que les simples mutants et parfois mieux que l'enveloppe

originale. Ces observations suggèrent que les polymorphismes dans LLP-2 et dans LLP-1 sont liés, peut-être pour préserver la conformation globale de ce domaine.

Conclusions

Des polymorphismes dans la gp41CT sous-type C influencent la capacité répliquative des virions dans les LT CD4+, mais pas dans les MDMs, en modulant la distribution intracellulaire du précurseur Pr55^{Gag}, sans toutefois influencer l'incorporation de l'enveloppe. Ces résultats suggèrent un défaut d'assemblage probablement lié à la rencontre de Pr55^{Gag} et Env, et qui diminuerait la quantité de virions produits par les LT CD4+ infectés par des virus avec gp41CT de sous-type C, mais n'affecterait pas l'incorporation de l'enveloppe par virion. Il est possible qu'un facteur cellulaire présent ou actif dans les LT CD4+ et absent des MDMs soit à l'origine des différents phénotypes observés entre les LT CD4+ et les MDMs. Les polymorphismes caractéristiques de LLP-2 et LLP-1 de sous-type C pourraient moduler l'interaction ou l'induction d'un tel facteur dans les LT CD4+.

Polymorphisms in the gp41 cytoplasmic tail of HIV-1 subtype C viruses impair replication capacity in CD4+ T lymphocytes but not in macrophages

Eveline Santos da Silva^{1‡}, Martin Mulinge^{1‡}, Arkadiusz Rybicki¹, Morgane Lemaire¹, Cécile Masquelier¹, Jean-Yves Servais¹, Jean-Claude Schmit^{1,2}, Carole Seguin-Devaux¹, Danielle Perez Bercoff^{1#}

[‡]Both authors contributed equally to this work

¹ Laboratory of Retrovirology, CRP-Santé, 84, Val Fleuri, L-1526 Luxembourg, Luxembourg

² Centre Hospitalier de Luxembourg, Service National des Maladies Infectieuses, 4 Rue Ernest Barblé L-1210 Luxembourg, Luxembourg

Corresponding author:

Danielle Perez Bercoff

Laboratory of Retrovirology, CRP-Santé

84, Val Fleuri

L-1526 Luxembourg

Luxembourg

Phone: +352 26-970-318

Fax: +352 26-970-221

Mail: danielle.perezbercoff@crp-sante.lu

Abstract

Background:

The unusually long gp41 cytoplasmic tail (gp41CT) of lentiviruses ensures multiple functions, including envelope (Env) incorporation into virions, polarized viral budding, Env clustering in the mature virion and at the viral synapse, Env intracellular trafficking, and cell activation. Most studies to date have investigated the role of the gp41CT using laboratory-adapted strains, mutants and truncations thereof, and reported cell-type dependent phenotypes. Here we investigated the functional impact of naturally-occurring polymorphisms within the gp41CT of 12 subtype C and 10 subtype B primary Envs on replication in CD4⁺ T cells and monocyte-derived-macrophages (MDMs), using recombinant virus pairs harboring full patient-derived Envs (gp160) or chimeric Envs comprising the same patient-derived Env ectodomain (gp140, EnvEC) fused to the NL4.3 gp41CT.

Results:

In CD4⁺ T cells, subtype B recombinant pairs replicated similarly regardless of the gp41CT (patient-derived or NL4.3-derived), whereas for subtype C, viruses containing subtype C gp41CTs displayed lower replication capacity than their chimeric counterpart containing the gp41CT of NL4.3. In MDMs, in contrast, subtype B and subtype C recombinant viruses had similar replication capacities, regardless of the gp41CT. Confocal microscopy showed similar Env expression, intracellular distribution and localization patterns for all Envs. In contrast, p55Gag assembly in localized clusters was decreased or undetectable in CD4⁺ T cells infected with subtype C Env recombinant viruses, whereas such clusters were readily visualized in CD4⁺ T cells infected with subtype B viruses or with viruses expressing chimeric Envs (subtype C ectodomain fused to the gp41CT of NL4.3). Env incorporation into virions was not impacted by the defect in p55Gag clustering. Mutational analysis of subtype C-specific polymorphisms indicated that the C-terminal LQ₈₅₆ polymorphism contributes to viral replication in subtype C. Further, the absence of other polymorphisms within LLP-2 could be rescued by concomitantly reverting the C-terminal LQ₈₅₆ polymorphism, implying a link between these polymorphisms. Presentation of subtype C viruses by monocyte-derived dendritic cells did not improve replication, arguing against an advantage of these Envs in DC-mediated transmission.

Conclusions:

The lower replication capacity of subtype C strains in CD4⁺ T cells maps partly to the viral Env ectodomain and partly to the gp41CT, which fails to induce p55Gag clustering. The cell-type

specificity of this phenotype suggests the involvement of a cell factor present in CD4+ T cells but lacking in MDMs. Identifying the underlying factor(s) and mechanisms could open new leads to interfere with viral replication in activated CD4+ T cells.

Introduction

Spread of Human Immunodeficiency Virus (HIV-1) to new target cells *in vitro* and *in vivo* occurs via infection with free virions or by direct transmission of newly synthesized virions budding from an infected “donor” cell to a nearby target cell across close conjugates and membrane bridges termed Virological Synapses [1-7] reviewed in [8, 9]. Cell-to-cell transmission is documented to be far more efficient than infection with released cell-free virion [10-13]. Both modes of infection are mediated by the viral envelope (Env). Env is a highly glycosylated trimeric complex composed of a surface subunit (gp120) and a transmembrane anchoring subunit (gp41) which are non-covalently linked. gp120 consists of five variable regions (V1-V5) interposed with five relatively conserved regions (C1-C5) [14] and ensures viral adsorption and binding to the CD4 receptor and a coreceptor (CCR5 or CXCR4) [15-18]. gp41 consists of three domains: the ectodomain, comprising the hydrophobic, glycine-rich fusion peptide at the N-terminus, the membrane-spanning-domain (MSD) and the cytoplasmic tail (gp41CT). Binding of gp120 to CD4 induces conformational changes within Env exposing the V3-loop which interacts with the coreceptor [19-22]. This interaction in turn induces further conformational changes leading to the insertion of the gp41 N-terminal fusion peptide into the target cell membrane and to fusion of the viral and cellular membranes [23, 24].

The Env surface subunit gp120 and the extracellular portion of gp41 have been extensively studied. However, the intracellular gp41CT has been granted far less investigations. The HIV-1 gp41CT is unusually long (~150 amino acids (AA)) in comparison to other retroviruses (< 50 AA) [25]. Despite considerable sequence variation, its physicochemical and structural properties are conserved across HIV types and HIV-1 subtypes [26]. Immediately downstream of the MSD is the immunodominant Kennedy polypeptide sequence which is not thought to adopt any particular conformation but contains three immunogenic epitopes conferring susceptibility to antibody neutralization [27-30]. The Kennedy sequence is followed by three highly conserved α -helix domains referred to as the lentivirus lytic peptides (LLP): LLP-2 (AA 773–793) which overlaps the LLP-3 leucine zipper domain (AA 785–807), and LLP-1 (AA 833–856) [31]. Several crucial functions are associated with the gp41CT (recently reviewed in [32-35]). These include polarized viral assembly and budding [36-38], Env incorporation into virions by interacting with the matrix protein (MA) of the p55Gag precursor, viral fusion capacity and cell-to-cell transmission [39-43] and Env clustering at the surface of the virion [44-48]. The MA/gp41CT interaction involves LLP-2, and charged AA within LLP-2 and LLP-1 (P_{T/R}RIR domain)

[49-55]. The gp41CT also contains a number of motifs regulating Env intracellular trafficking between the plasma membrane (PM) and the Trans Golgi Network (TGN) via interaction with numerous cellular factors. The highly conserved Y₇₁₂SPL motif binds adaptor proteins (AP) of the endocytic trafficking network: binding to clathrin adaptor AP-2 μ (medium) chains mediates Env endocytosis [56-58]; binding with the AP-1 and AP-3 adaptor complexes regulates Env trafficking through the TGN and addresses Env to the basolateral PM in polarized cells [57, 59]. The Y₈₀₂W₈₀₃ diaromatic motif is also involved in the retrograde transport of Env from the endosome through the TGN [60-64]. The C-terminus dileucine LL₈₅₆ interacts with AP-1 and AP-2 and thereby contributes to Env internalization and proper routing through the TGN in concert with Y₇₁₂SPL [65, 66]. Recently, the gp41CT of HIV and of SIV have been shown to contribute to cell activation by inducing [67] the canonical NF- κ B pathway through Y₇₆₈ within the YHRL motif of LLP-2.

gp41CT truncated viruses display a cell-type dependent phenotype and are unable to replicate in some T cell lines (H9, MT-2, Jurkat and CEMx174) and primary target cells of HIV (primary CD4+ T cells and macrophages), referred to as “non-permissive cells”, whereas the impact of such truncations is limited in MT-4 and C8166, and in several laboratory cell lines (HEK293T, HeLa, and COS) (“permissive cells”) [55, 68-74]. The non-permissive phenotype has been associated with decreased Env incorporation into virions [69, 72] and/or failure of the viral p55Gag precursor to accumulate and polarize at the viral synapse [69, 72], whereas Env levels at the PM were unaffected [52, 68, 69, 72]. Others report gp41CT truncations impair infectivity of free virions as well as cell-to-cell transmission of viruses, despite comparable Env incorporation levels [43]. These observations further underscore the fact that cell-type specific factors regulating Env trafficking or incorporation into virions play a chief role, but these remain poorly understood.

Although Env variability and subtype-related specificities are acknowledged in studies investigating the Env ectodomain, most mutational or biochemical studies addressing the role of the gp41CT have been performed using laboratory-adapted strains with mutations in charged residues or disrupted dileucine motifs. Two studies have directly addressed polymorphisms in the gp41CT, one through a biochemical approach, showing the conservation of the biophysical properties despite type and subtype specific polymorphisms [26] and one investigating the impact of 4 patient-derived clones harboring specific mutations in the Y₇₁₂ and Y₈₀₂W₈₀₃ trafficking motifs [62]. Studies addressing the functional impact of non-B polymorphisms on viral replication are missing. In this study, we

investigated the contribution of polymorphisms in the gp41CT to viral propagative infection in primary CD4⁺ T cells and monocyte-derived-macrophages (MDMs), the main target cells for HIV infection *in vivo*, and their transmission potential by dendritic cells (DCs). We focused on subtype C because it is the most widely spread worldwide [75], despite its lower replication capacity *in vitro* [76, 77] and subtype B because it is the most studied via reference strains. Using recombinant virus pairs containing patient-derived gp140 (Env ectodomain, hereafter EnvEC) or gp160 (full Env), we found that recombinant viruses with subtype C Envs had lower replication capacity than those with subtype B Envs in CD4⁺ T cells but not in MDMs, and that both the ectodomain and the gp41CT directly impacted replication capacity. This phenotype was not associated with altered Env intracellular distribution or incorporation into virions but with lower or undetectable polarized p55Gag clusters in CD4⁺ T lymphocytes. Mutational analysis highlighted that the C-terminal LQ₈₅₆ polymorphism is chief for replication of this subtype and was suggestive of strong structural constraints necessary for interaction of Env with a cellular factor expressed in CD4⁺ T cells but not in MDMs.

Results

The gp41CT influences virus replication in CD4⁺ T cells but not in MDMs.

To assess the impact of naturally occurring polymorphisms in the gp41CT on viral replication in comparison to the more studied gp41CT of NL4.3, we used a comparative approach based on NL4.3-derived recombinant virus pairs carrying patient-derived full-Envs (gp160) (Env recombinant viruses) (Fig 1A) or chimeric Envs comprising the same patient-derived ectodomain (gp140) fused to the NL4.3 gp41CT (EnvEC recombinant viruses) (Fig 1B) in a constant NL4.3-derived backbone. Infection of CD4⁺ T cells and of MDMs with Env and EnvEC recombinant virus pairs carrying the NL4.3 and NLAD8 Env or EnvEC was comparable, as expected since in both cases, the gp41CT originates from NL4.3 (Fig 2A and 2C) and validated the use of recombinant virus pairs.

Subtype B Env recombinant viruses reached higher replication levels than subtype C strains in CD4⁺ T cells (364 ng/ml p24 in supernatants vs 29 ng/ml p24 at day 5 post infection respectively, $p < 0.001$) (Fig 2A) whereas no difference between subtypes was recorded in MDMs (3.9 ng/ml and 5.2 ng/ml p24 for subtypes B and C respectively) (Fig 2C). Differences in viral replication followed a similar pattern at day 7 for CD4⁺ T cells and days 4 and 10 in MDMs (data not shown). When Env and EnvEC recombinant virus pairs were compared, subtype C full Env recombinants featured lower

replication than the chimeric EnvEC recombinant viruses carrying the gp41CT of NL4.3 in CD4+ T cells (mean p24 at day 5: 29 ng/ml vs 148 ng/ml, $p < 0.0001$) (Fig 2A and 2B). In 6/12 cases, replication of the Env recombinant viruses was 4-10-fold lower than its chimeric EnvEC counterpart, and in 5/12 cases it was 10-100 fold lower (Fig 2B). In one case, replication was similar for the full Env and its isogenic chimeric recombinant EnvEC (Fig 2B). For subtype B strains, in contrast, replacing the wild type (wt) gp41CT with the gp41CT of NL4.3 had no impact on viral replication ($p > 0.05$) (Fig 2A and 2B). In MDMs, viral replication of subtype B and of subtype C recombinant virus pairs was comparable, regardless of subtype and regardless of the gp41CT (Fig 2C and 2D).

These observations indicate that both the ectodomain, which is directly involved in fusion, but also the intraviral gp41CT contribute to viral replication capacity in CD4+ T cells, but not in the terminally differentiated macrophage, suggesting the involvement of a cell-type specific factor or of other cell-type specific mechanisms.

Subtype C Envs are not more easily transmitted by DCs than free virions

We next inquired whether the poor replication capacity of subtype C Envs in CD4+ T cells could be compensated for by an advantageous transmission potential. Presentation of subtype C Env and EnvEC recombinant pairs by MDDCs led to similar infection profiles of CD4+ T cells, although the difference between Env and EnvEC recombinant viruses was slightly attenuated compared to infection of CD4+ T cells by free virus (Fig 3). Importantly, this attenuation was not due to higher levels of replication of MDDC-presented Env recombinant viruses, as presentation of Env recombinant viruses by MDDCs resulted in similar infection levels as infection of PHA-stimulated CD4+ T cells by free virions ($p > 0.05$), but to lower replication of MDDC-presented EnvEC recombinant virions compared to infection of PHA-stimulated CD4+ cells ($p = 0.0057$) (Fig 3). Because there was no difference between the level of infection reached with MDDC-presented and free virus for the reference strains, it is unlikely that this result reflects different activation routes of CD4+ T cells, i.e MDDC vs PHA. These results rather indicate that the poor viral replication of subtype C Envs in CD4+ T cells is not balanced by an advantage in transmission by MDDCs and argue against the hypothesis that subtype C strains are more easily transmitted at the cost of fitness.

The gp41CT of subtype C decreases p55Gag clustering but not Env distribution in CD4+ T cells

We then asked whether Env expression, intracellular or PM expression could be responsible for different replication patterns observed in infected CD4+ T cells and MDMs. Using confocal microscopy, Env was detected at the PM of CD4+ T cells and of MDMs infected with Env and EnvEC recombinant viruses and there was no apparent difference in Env expression levels or localization, indicating that Env is expressed and traffics similarly to the PM regardless of the gp41CT sequence (Fig 4). Polarized p55Gag clusters were readily observed in CD4+ T cells infected with subtype B recombinant viruses (Fig 4A) as well as with subtype C EnvEC recombinants (Fig 4B), and some colocalization with Env was observed, as expected. In contrast, p55Gag clusters were scarce in CD4+ T cells infected with subtype C Env recombinants (Fig 4B), revealing the gp41CT of subtype C Envs did not induce p55Gag polarization. Furthermore, p55Gag polarized clusters were very rarely or never detected in CD4+ T cells infected with subtype C Env recombinants with very poor replication capacity (e.g. Env 0266, 0487, 0148, 0097) (Fig 4B), whereas in cultures of CD4+ T cells infected with subtype C Env recombinants with intermediate replication capacity (e.g. Env 1671, 1677), some p55Gag clusters could be detected (Fig 4B). The number of cells presenting such clusters remained scarce compared to CD4+ T cell cultures infected with subtype B Envs.

In MDMs, p55Gag clusters were readily detected, free or as part of bright p55Gag/Env colocalized assemblies (Fig 4C and 4D). No difference was observed between MDMs infected with Env or with EnvEC recombinant viruses (Fig 4C and 4D). Further, similar p55Gag, Env and p55Gag/Env colocalized assemblies were observed in cells infected with subtype B and subtype C viruses, in line with the comparable replication capacities recorded for all Env and EnvEC recombinants in this cell type.

These data indicate that the gp41CT of subtype C fails to mediate p55Gag polarized clustering in CD4+ T cells, leading to poor replication capacity. The cell-type specificity of this phenotype suggests that activated CD4+ T cells express a factor involved in gp41CT-dependent p55Gag clustering which is not expressed in macrophages.

Env incorporation into virions is not impaired

Env expression at the surface of cells does not directly reflect the level of Env incorporation into virions and viral infectivity [42, 52, 68, 69, 78]. Furthermore, p55Gag polarized clustering is Env-dependent [36-38] and gp41CT truncations affect Env incorporation into virions produced in non-permissive cells, including CD4⁺ T cells and macrophages [40, 49, 50, 54, 55]. We therefore investigated whether the defect in p55Gag clustering impacted Env incorporation into virions produced by CD4⁺ T cells by assessing the relative amounts of gp120 and p24 in purified virions produced from infected CD4⁺ T cells. Western Blot and ELISA showed similar gp120:p24 ratios for the reference strains NL4.3 and NLAD8 and for most subtype B Env and EnvEC recombinants (Fig 5A and 5B), in line with comparable viral replication levels and with normal p55Gag clustering. For some subtype B and for most subtype C, however, the low viral production in CD4⁺ T cell supernatants translated into very faint or undetectable gp120 and sometimes even in p24 by Western Blot for most Env recombinant viruses, and even for some EnvEC recombinant viruses. This held true for CD4⁺ T cell supernatants collected at days 7, 9-10 post infection. Quantification by ELISA led to detectable gp120 for 6 subtype C pairs only when performed on raw culture supernatants to avoid losing virus during the purification step. Available ELISA tests recorded comparable Env levels in Env and EnvEC recombinant virus pairs (Fig 5B), indicating that Envs containing the gp41CT of NL4.3 were not incorporated more efficiently into virions than their wt subtype B or subtype C counterpart. Of note, the gp120:p24 ratio by ELISA seemed lower for subtype C than for subtype B, for both Env and EnvECs, although there was no statistical support. One reason could be this reflects differences in binding affinity of the antibody against gp120. Therefore the gp41CT-dependent defect in p55Gag clustering likely diminishes the total number of progeny virions produced and/or transmitted by infected CD4⁺ T cells, rather than the intrinsic infectious potential of each individual virion produced by CD4⁺ T cells.

The observations that subtype C Env and EnvEC recombinant pairs were similarly incorporated by a NL4.3-derived MA, and that no difference in replication capacity was observed in MDMs argue against an intrinsic bias linked to the use of a subtype B backbone with subtype C gp41CTs. Nonetheless, to rule out that the use of a recombinant viral assay based on a subtype B backbone could artificially affect the p55Gag/gp41CT interaction, we sequenced all MAs from all samples included. All residues known to be involved in the interaction of MA with Env and in Env incorporation into virions (i.e. residues L₈ [47, 79], L₁₂, L₃₀, V₃₄ [78, 80], K₃₂ [52], L₄₉ [81], the basic domain of MA

(AA 17-21) [82]) were 100% conserved in all subtype C strains, with the exception of S₉ [47, 79], which was replaced by an Arg in all subtype C sequences, and of residue L₃₀, which was conserved in 8/12 of strains and was replaced by a Met in the remaining 4 viruses (Fig 6A). R₉ was present in all subtype C strains, regardless of replication capacity, and the role of this specific polymorphism is not known. The presence of the L30M mutation did not correlate with poor replication or with lower Env incorporation levels. Basic residues 17-21 mediating MA interaction with Env [40, 52, 53, 55, 70, 74, 83] or AA involved in p55Gag trafficking via adaptor proteins, such as Y₁₃₂ and V₁₃₅ at the MA/CA junction [84-87] were also conserved (Fig 6A). Other subtype C specific polymorphisms (Fig 6A) were generally found in all sequences and we could not identify polymorphisms that were only present in strains with very poor replication capacity or that were associated with the presence of subtype C polymorphisms within the gp41CT (see below).

Gp41CT sequence analysis

gp41CT sequence analysis highlighted that subtype B strains closely resembled the NL4.3 reference, whereas a number of subtype C-specific polymorphisms were identified (Fig 6B and 6C). The main Y₇₁₂SPL endocytic motif, the Y₈₀₂W₈₀₃ diaromatic motif, the second Y₇₆₈XXL motif as well as all but one Arg spanning the LLP α -helices, the Arg-rich P_{T/R}RIR motif and Cys residues within LLP-1 were highly conserved in all samples, underscoring their chief role in Env trafficking, incorporation and viral replication. The Kennedy sequence contained a number of subtype-specific mutations, including a R \rightarrow Q and D \rightarrow N/S/G mutations in the E₇₃₉RDRD₇₄₃ epitope (Fig 6B). Polymorphisms involving polar/charged residues (WN₇₉₈ \rightarrow GS₇₉₈, SQ₈₀₅ \rightarrow GL₈₀₅, N₈₀₉ \rightarrow K, NA₈₁₇ \rightarrow DT₈₁₇ and R₈₅₃ \rightarrow A in LLP-1) were detected in nearly all subtype C strains, regardless of viral replication level, perhaps because they are unlikely to interfere with the α -helical structure of the LLP domains. A conserved seven AA insertion (SSLRGLQ) (2 α -helical turns) was found between R₇₈₇ and R₇₈₈ in 8/12 subtype C Envs but never in subtype B Envs (Fig 6B and 6C). Other subtype C-specific polymorphisms involved the dileucine motifs spanning the gp41CT LLP-2/3 α -helices (LLL₇₇₆ \rightarrow FIL₇₇₆ and LL₈₀₀ \rightarrow LV₈₀₀) as well as the terminal dileucine motif LL₈₅₆ within LLP-1 which was replaced by LQ₈₅₆ in 10/12 subtype C Envs (8 pure, and 2 LL/LQ₈₅₆ mixtures).

When sequences were sorted according to whether their gp41CT were associated with intermediate or poor replication capacity in CD4+ T cells, polymorphisms at dileucine motifs and the seven AA insertion in LLP-2 were often, but not always, present in Env recombinants with poor replication capacity.

Polymorphisms in the gp41CT are linked and the LQ₈₅ polymorphism is important for viral replication of subtype C Envs in CD4+ T cells

To investigate the role of these motifs, we inserted these motifs into the NLAD8Env because it is R5, as most Envs included in this study. In parallel, we cloned two patient-derived subtype C Envs mediating mildly impaired (1671) or poor (0266) replication capacity and reverted these three polymorphisms to the canonical motifs typically found in subtype B, generating a panel of single, double and triple mutant NLAD8, 1671 and 0266 clones (see Materials and Methods) which were used to produce Env^{mut} recombinant viruses.

As shown in Figures 7B and 7C, removal of the LQ₈₅₆ polymorphism at the C-terminal end of the gp41CT decreased viral replication capacity by over 96% for 1671 Env recombinant virions and by 73-93% for 0266 Env recombinants, revealing that the LQ₈₅₆ polymorphism contributes substantially to replication capacity in subtype C Envs and could not be reverted.

In the 1671 Env recombinant virus, polymorphisms within LLP-2 (i.e. the FIL₇₇₆ and the 7 AA insertion) could be reverted without impacting viral replication (Fig 7B). Further, removing these polymorphisms restored replication of the replication incompetent 1671^{LL856} mutant to levels comparable to the wt 1671 Env (Fig 7A). In the 0266 Env recombinant viruses, each one of the single mutations in LLP-2 (FIL₇₇₆ → LLL₇₇₆ and deletion of the 7 AA) alone or together, nearly fully abrogated replication capacity in CD4+ T cells (Fig 7B), although viral production by transfection of HEK cells was unaffected (not shown). Importantly, the double mutants containing the LQ₈₅₆ → LL₈₅₆ had slightly higher replication capacities, and the triple mutant 0266^{-7AA+LLL776+LL856} recovered replication capacity to ~40% of wt 0266 Env (Fig 7C).

Single polymorphisms inserted alone in the NLAD8 Env did not impact viral replication capacity, but the NLAD8^{+7AA+LQ} double mutant and the NLAD8^{+7AA+FIL+LQ} had lower replication capacities than the wt in 2/3 experiments (Fig 7A).

Taken together, these observations suggest that the LQ₈₅₆ polymorphism is linked to polymorphisms within LLP-2 and that each single mutation may affect the overall gp41CT structure and impair replication, while cumulative mutations restore a replication-prone structure.

Western Blot analyses of purified NLAD8 mutants (Fig 7D) and of 1671 revertant virions (Fig 7E) and quantification of the gp120:p24 ratio by ELISA (not shown) showed that Env incorporation levels were not substantially impacted by the subtype C polymorphisms into NLAD8 Env or by their suppression from Env 1671 (Fig 7D and E). The 0266 revertants could not be visualized by Western Blot nor quantified by ELISA due to the undetectable replication levels.

Altogether, these results show that the presence of the LQ₈₅₆ polymorphism substantially contributes to viral replication of subtype C viruses through mechanisms unrelated to Env incorporation levels. The fact that the impact of these polymorphisms was Env dependent and that the concomitant mutation of the three polymorphisms partially restores or improves viral replication suggests that these polymorphisms are related and that gp41CT does not tolerate mutations unless the overall structure of the gp41CT is preserved.

Discussion

In this study, we addressed the functional impact of naturally-occurring polymorphisms in the gp41CT of HIV-1 on viral replication in CD4+ T cells and in MDMs, the main target cells for HIV *in vivo*. We used an approach based on paired recombinant viruses differing solely by their gp41CT in order to assess the impact of the gp41CT in its autologous Env genetic context (Fig 1). We found that subtype C Envs have lower replication capacity than subtype B Envs, in agreement with previous studies mapping the lower replication capacity of subtype C strains [76, 77] to the viral Env [88, 89]. Further, the relative replication capacities (subtype B Env = subtype B EnvEC > Subtype C EnvEC > subtype C Env) indicate that both the Env ectodomain and the Env gp41CT directly contribute to the lower replication capacity of subtype C Envs (Fig 2A and 2B). Tropism of subtype B and C Envs (not shown) was not different and was not related to the level of replication in either cell type. Strikingly, decreased viral replication was observed in CD4+ T cells, but not in MDMs (Fig 2C and 2D), in contrast to the impact of gp41CT truncations, which similarly impair viral replication in CD4+ T cells and in macrophages [72]. Adding extracellular deoxynucleoside (dNs) to MDM cultures increased viral replication but did not result in differences between Env and EnvEC recombinant viruses (data not

shown), arguing against a mechanism involving the levels of replication or the low dNTP pools typically found in MDMs [90-92], and, and rather pointing to later steps of the viral cycle. This is, to our knowledge, the first report showing a functional impact of natural polymorphisms in the gp41CT on viral replication.

Confocal microscopy showed that Envs reached the PM in CD4⁺ T cells and in MDMs and clustered similarly to their chimeric counterpart containing the gp41CT of NL4.3, even for subtype C Envs featuring very poor replication capacity in CD4⁺ T cells, such as 0487, 0148 and 0097 (Fig 4B and 4D). This is in line with the conservation of domains involved in Env intracellular trafficking, such as the Y₇₁₂SPL main endocytic motif [57, 58, 93, 94] and the Y₈₀₂W₈₀₃ intracellular trafficking motif [62, 95-98] (Fig 6B and C). Poor replication capacity of subtype C Env recombinants in CD4⁺ T cells was associated with decreased or undetectable polarized p55Gag clusters at the PM of CD4⁺ T cells and of Env/p55Gag assemblies (Fig 4B). It has been reported previously that gp41CT truncation alters the localization of p55Gag and affects polarization of the viral capsid at the viral synapse (VS) in some cell types and that this requires an interaction between p55Gag and Env [36-38]. As the Y₇₁₂SPL motif responsible for specific targeting to the basolateral membrane surface in polarized cells [37, 99] was 100% conserved, it is likely that suggesting that the p55Gag/Env interaction and gp41CT-mediated p55Gag clustering is impaired in CD4⁺ T cells infected with subtype C Env recombinants, and that polymorphisms in the gp41CT modulate the efficiency of this interaction. In MDMs in contrast, few but bright p55Gag clusters were detected, and these were generally at the PM and colocalized with Env in intense, well-defined spots, for both subtype B and subtype C Envs, and independently of the gp41CT (Fig 4C and D). In CD4⁺ T cells, the formation of the VS is mediated by Env-receptor interactions [8]; in contrast, in the macrophage, Env expression is not required for recruitment of Gag to the macrophage-macrophage or macrophage-T cell VS [100]. This is in line with our observations and could be one reason why polymorphisms in the gp41CT did not affect propagative infection in MDMs. The viral assembly process in macrophages involves deep and dense invaginations connected to the PM, and the virus remains undercover in these PM-related invaginations or in Virus Containing Compartments where it is concentrated [101-104], reflecting the lesser dependence of viral transmission on Env in this cell type owing to a favorable cell-to-cell transmission process.

Four models of viral assembly and budding have been proposed (reviewed in [32, 105, 106]) and there is evidence in support of each. The “passive incorporation model” posits that p55Gag and Env

colocalize randomly at the cell membrane. The three other models, in contrast, propose that the p55Gag/Env encounter is a regulated phenomenon and that Env incorporation into virions results from (i) a regulated co-targeting of both to assembly platforms, (ii) direct p55Gag/gp41CT interaction, or (iii) an indirect interaction mediated by other cellular factors (detailed in [32]), such as lipid rafts, AP proteins, TIP47 and Rab11-FIP1C for example [60-64, 86, 107]. Our data are in support of a model by which two of these mechanisms co-exist, i.e. there could be a “basal level” of p55Gag/gp41CT interaction which ensures sufficient p55Gag clustering and Env incorporation into nascent virions, as seen in MDMs or in CD4+ T cells infected with the subtype C Env recombinant viruses. This direct interaction could be favored or stabilized by a cellular factor that is present in activated CD4+ T cells but not in macrophages. In other words, the p55Gag/gp41CT interaction would thus be direct or indirect, depending on the cell-type, involving a cellular factor that is present in (activated) CD4+ T cells but not in macrophages. The presence of some p55Gag clusters in CD4+ T cells infected with Env recombinants with intermediate replication capacity and the absence of such clusters upon infection with poorly replicative Env recombinants supports the view that determinants within these Envs affect its interaction with this factor. In this scenario, the absence of this cell factor in macrophages could partly account for the lower viral replication level observed in macrophages, in conjunction with other factors, such as SAMHD1 [91]. Identifying this factor could open new leads to control viral replication by targeting this factor in CD4+ T cells.

Why subtype C strains would have evolved to modify the Env and the gp41CT to reach lower replication levels, and how polymorphisms in this region would benefit the virus remains to be established. One possibility is that these polymorphisms reduce the immunogenic or cytopathic potential of Env, and therefore represent an advantage in terms of immune escape thereby favoring the long term persistence of infection. In our experiments, CD4+ T cell death was related to the level of infectivity but not to a specific Env or Env gp41CT (data not shown), arguing against this hypothesis. Alternatively, other compensatory mechanisms may exist elsewhere in the subtype C viral genome, such as a third NF- κ B site typically found in the LTR of subtype C strains [108]. The gp41CT has been shown to affect viral transcription by directly activating the NF- κ B pathway [67] or by indirectly promoting LTR-driven transcription by repressing I κ B [109]. Residues involved in NF- κ B activation [110] were conserved in subtype C gp41CTs. Furthermore, in our system, viral replication was impaired in DC- or PHA-stimulated CD4+ T cells i.e. in fully activated lymphocytes, in which the

impact of gp41CT mediated NF- κ B activation may be overrode by high levels of activation [67]. Moreover, Env and EnvEC expression levels were comparable in all infected cell cultures. If CD4+ T cell activation levels were involved, both p55Gag and Env expression levels in CD4+ T cells and in MDMs would likely have been lower, or some p55Gag expression would have been detected in the absence of Env, as recorded in latency [111]. Altogether, these observations point to a gp41CT-mediated defect in p55Gag clustering.

Despite the defect in p55Gag clustering, when they were produced, Env and EnvEC virion pairs contained similar levels of gp120 relative to p24 (Fig 5 and Fig 7D and 7E). Deletions downstream of the KE (ablating the LLP domains) or small deletions within LLP-2 have been reported to severely impair Env incorporation into virions [40, 49, 50, 54, 55]. Because polymorphisms in subtype C strains did not affect Env incorporation into virions, some degree of sequence flexibility must be tolerated. The observation that Env and EnvEC virus pairs contained comparable levels of Env despite different gp41CTs, suggest that the number of Env spikes at the surface of HIV virions is constrained, despite the cost in fitness, most likely to evade the humoral immune response. This is also consistent with the observation that presentation by MDDCs did not increase viral replication capacity (Fig 3). DCs play a crucial role in the establishment of HIV infection and in the expansion of HIV infection through cell-to-cell transmission to T cells thereby enabling persistence of infection. Mature MDDCs do not support direct viral infection but rather mediate trans-infection. Because these cells express high levels of C-type lectins, particularly DC-SIGN and ICAM-1, whereby they bind virus [112-119], higher Env levels may not be useful in the context of presentation by cells that express very high levels of these molecules and very efficiently transmit virions.

In our approach the p55Gag polyprotein is NL3.4-derived and constant while Env or the Env ectodomain is derived from patients. Therefore it could not be excluded *a priori* that polymorphisms within the gp41CT may have been compensated for by mutations within MA that would restore viral replication capacity. Nonetheless, all residues within MA known to be involved in the MA/Env interaction [40, 47, 52, 53, 55, 70, 74, 78-83] were highly conserved across subtypes B and C [120] as were residues involved in p55Gag trafficking via adaptor proteins, such as Y₁₃₂ and V₁₃₅ at the MA-CA junction [84-87]. Serine 9 was replaced by Arg in all subtype C strains. This residue may potentially play a role in the interaction with Env and Env incorporation into virions, and would warrant further investigations. Of note, Arg was found in all subtype C strains, and did not correlate with poor

replication capacity or with the presence of subtype C polymorphisms within the gp41CT. Furthermore, no difference in viral production was recorded in MDMs, and viral production by transfection of HEK293T generated comparable amounts of recombinant viruses, as assessed by p24 measurement in cell supernatants (data not shown), arguing against a bias in the use of subtype C gp41CTs with a subtype B MA. Lastly, for some Envs (e.g. 0148, 0236, 0209), only wt Env from the Env recombinant could be detected by ELISA, but not the chimeric EnvEC from the EnvEC recombinant. Because the gp41CT modulates the conformation of the gp41 ectodomain [39, 81] and of gp120 [121] as well as gp120 stability [39], the chimeric Env may adopt a conformation that interferes with binding of the anti-gp120 antibody used. This is however unlikely, as we used polyclonal anti-gp120 antibodies described to bind subtype B and C Envs to minimize this possibility, and as Env conformational changes would be expected to affect their infectivity, whereas we found that chimeric subtype C Envs had higher replication capacity than their wt counterpart.

Mutational analysis revealed that subtype C polymorphisms in the gp41CT seemed to be functionally linked, as the triple mutant Envs (i.e. Envs 0266 and 1671 containing the subtype B-like LLL₇₇₆ + LL₈₅₆ and no 7AA insertion) partly recovered replication capacity compared to each single mutant Env (Fig 7B and 7C). For Env 1671, reverting one or both polymorphisms within LLP-2 restored replication capacity of the 1671^{LL856} mutant to near wt levels (Fig 7B). For Env 0266, the 0266^{-7AA+LL856} and 0266^{LLL776+LL856} double mutants had some, although low, replication capacity (~20%), and the 0266^{7AA+LLL776+LL856} triple mutant reached ~40% replication capacity (Fig 7C). Subtype C polymorphisms inserted into NLAD8 also affected replication capacity to some extent when simultaneously present (Fig 7A). Inserting subtype C polymorphisms into NLAD8 had a lesser impact than reverting the same residues in subtype C Envs, indicating that other determinants must be involved, although we could not identify them. The FIL₇₇₆ motif and the 7 AA insertion lie close to each other in LLP-2, but the C-terminal LL₈₅₆ lies at the C-terminus of LLP-1. A cumulative effect of mutations may be needed to preserve the overall structure of LLP-2/3 and their biophysical properties, as suggested previously [42]. Truncation studies have reported that removing LLP-1 and LLP-3 (59 to 90 AA) strongly decreases Env incorporation into virions whereas removing the three LLP α -helices does not affect Env incorporation [122, 123] suggesting that LLP-2 does not adopt an appropriate conformation in the absence of LLP-1 and fails to accommodate the p55Gag precursor. In the case of subtype C

polymorphisms, lower replication capacity may reflect gp41CTs that less efficiently interact with MA and/or with a cellular factor favoring the p55Gag/Env interaction.

The C-terminal LQ₈₅₆ was indispensable to replication capacity of Envs 1671 and 0266 (Fig 7B and 7C). It is also noteworthy that two subtype C Envs with strongly impaired replicative capacity (Envs 0148 and 0097) compared to their isogenic EnvEC recombinant harbored LL₈₅₆ instead of the LQ₈₅₆, further underscoring the chief role of this polymorphism in replication of subtype C Envs. Dileucine motifs play important roles in protein internalization and intracellular trafficking and similar signals in cellular proteins or in the glycoproteins of other viruses (e.g. alpha herpes viruses) affect endocytosis as well as intracellular trafficking in endosomes and the TGN, and can determine basolateral versus apical sorting (reviewed in [12, 124]). In the case of HIV-1 NL4.4, the C-terminal LL₈₅₆ has been shown to partake in Env PM expression and incorporation into virions by at least two mechanisms, i.e. (i) regulating Env internalization in conjunction with the membrane-proximal Y₇₁₂SPL motif and the correct post-Golgi routing of Env via AP-1 and AP-2 [65, 125], and (ii) enhancing Env accumulation at the PM and incorporation into virions in a Nef-dependent manner [126]. In agreement with these studies, inserting LQ₈₅₆ into NLAD8 slightly decreased replication capacity (Fig 7A). However, LQ₈₅₆ did not affect Env incorporation into virions (Fig 7D and 7E), perhaps reflecting modulations of Env intracellular trafficking by the naturally-occurring LQ₈₅₆ polymorphism versus a deletion of the LL₈₅₆ [126]. It is possible that the overall level of Env expression and its intracellular localization/interaction with p55Gag in the presence of the LQ₈₅₆ motif may result from a balance between Env levels at the PM when LL₈₅₆ is disrupted and Nef-dependent modulations of Env trafficking. Modulating Env intracellular trafficking or localization may play a compensatory by ultimately favoring the p55Gag/Env interaction through yet unknown mechanisms and would require further investigations.

Conclusions

In conclusion, polymorphisms in the gp41CT contribute to the lower replication capacity of subtype C Envs in CD4⁺ T cells but not in macrophages. This cell type-related phenotype was not linked to changes in Env intracellular localization or in Env incorporation into virions but was associated with a defect in gp41CT-mediated p55Gag polarized clustering in CD4⁺ T cells. The underlying mechanisms remain speculative, but the observations that the Y₇₁₂SPL motif responsible for Env polarization at the

basolateral membrane was intact, that polymorphisms within LLP-2/3 and LLP-1 are linked, and that this phenotype was only observed in activated CD4⁺ T cells are strongly indicative that the p55Gag/gp41CT involves a third factor present in activated CD4⁺ T cells but not expressed in MDMs. We propose a model whereby a direct p55Gag/gp41CT interaction provides “basal” levels of p55Gag/gp41CT interaction, sufficient to ensure Env incorporation into virions and viral propagation. In activated CD4⁺ T cells, p55Gag recruitment at the VS is favored by a third party, leading to the high viral production typical of activated CD4⁺ T cells. Polymorphisms changing the conformation of may contribute to the capacity of the viral gp41CT to induce p55Gag clustering, thereby favoring viral replication. Understanding how these polymorphisms interplay with cellular factors in CD4⁺ T cells may enlarge therapeutic possibilities.

Materials and Methods

Cell lines: HEK293T cells (ATCC through the NIH AIDS Research and Reference Reagent Program) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 1% L-Glutamine, 50 µg/mL Penicillin and 50 µg/mL Streptomycin (all from Invitrogen, Merelbeke, Belgium).

Isolation and culture of primary cells: Human PBMCs were isolated from buffy coats obtained from healthy volunteer HIV-1 seronegative donors (Red Cross Luxembourg) by Lymphoprep density gradient centrifugation (Axis-Shield, Oslo, Norway). To isolate CD4⁺ T lymphocytes, PBMCs were incubated in RPMI 1640, 1% Glutamine, 50 µg/mL Penicillin and 50µg/mL Streptomycin (CD4 medium) supplemented with 2% FCS (all from Invitrogen, Merelbeke, Belgium) for 1 hour at 37°C and 5% CO₂ prior to negative selection using a Miltenyi kit (Miltenyi Biotec, Leiden, Netherlands). Isolated CD4⁺ T cells were washed and incubated at a density of 10⁶ cells/ml in CD4 medium supplemented with 10% FCS and 5 µg/ml PHA-P (Sigma, Borneim, Belgium) for 48 hours, then incubated for 24 hours with interleukin 2 (IL-2) at 10 U/ml (Roche, Mannheim, Germany) prior to infection.

To obtain MDMs, monocytes were isolated from PBMCs by adherence to plastic as described in [127]. Briefly, PBMCs were incubated for 1 hour at 37°C in MDM medium (RPMI 1640, 1% Glutamine, 50 µg/mL Penicillin and 50 µg/mL Streptomycin, 10 mM HEPES, 1% MEM vitamins, non-essential amino acids (AA), 50 µM β-mercaptoethanol, 1% sodium pyruvate) (all from Invitrogen, Merelbeke, Belgium) supplemented with 2% human AB serum (Sigma, Bornem, Belgium). After extensive washing, adherent monocytes were incubated overnight and harvested the following morning, then seeded in 96-well plates ($3 \cdot 10^5$ cells/well) and allowed to differentiate into macrophages for 7 days in MDM medium supplemented with 15% human AB serum.

To differentiate monocytes into monocyte-derived dendritic cells (MDDCs), monocytes were seeded in 75 cm² flasks with CD4 medium containing 2% FCS for one hour at 37°C, washed extensively with PBS and cultured for 5 days in CD4 medium supplemented with 10% FCS and 20 ng/ml GM-CSF and 20 ng/ml IL-4. After 5 days, immature MDDCs were matured into unpolarized type 0 MDDCs with 50 ng/ml TNF-α and 10 ng/ml IL-1β for a further 48 hours. Cells were harvested and seeded (10^5 cells/well of a 96-well plate) for loading with recombinant viruses.

Viral Samples: Plasma samples from 22 treatment-naive patients infected with HIV-1 subtypes B (10) and subtype C (12) were included in the study. Ethical approval was obtained from the Comité National d'Ethique pour la Recherche in Luxembourg. Mean plasma viral load (VL) (Abbott m2000 RealTime HIV-1 assay) and CD4 counts were comparable for patients infected with either subtype: mean VL for subtype B was 34181 RNA copies/ml [range: 635-124657] and mean VL for subtype C was 38223 RNA copies/ml [range: 1759->500000]; mean CD4 count was 386 cell/mm³ [range: 110-610] for subtype B-infected patients and 368 [range: 100-760] for subtype C-infected patients. HIV-1 subtype was assigned from HIV-1 PR-RT and Env sequences (V3-loop and gp41CT) using COMET (www.comet.retrovirology.lu) and the REGA HIV subtyping tool [128]. A phylogenetic tree (GTR+G model) with 1000 bootstrap values was inferred for the PR-RT and Env gp41CT sequences with RAxML version 7.6.0. Reference strain G.BE.96.DRCBL.AF084936 was used as the outgroup for the tree. Phylogenetic analysis confirmed the samples were unrelated (data not shown). Coreceptor usage was determined as described in [129]. Subtype B samples included 7 strictly R5, 1 R5/X4 and 2 strictly X4 strains; 10 subtype C strains were strictly R5 and 2 were R5/X4.

Env amplification: One ml of plasma was centrifuged at 24 000g for 1 hour at 4°C and viral RNA was extracted from the pellet using the Qiagen Viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral cDNA was synthesized in a one-step RT-PCR reaction using forward primer KVL008 and reverse primer KVL009 [130] as described in [131]. A nested PCR was performed using 2 µl of the amplified cDNA. To amplify the sequence spanning the Env ectodomain (gp140) forward primer rec.envHXB2_Fp (5'-TAGGCATYTCCTATGGCAGGAA'-3') and reverse primer rec.HR1-2_RP (5'-CTCTCTCTCCACCTTCTTCTTC-3') were used as described in [132]. To amplify the full Env (gp160) we used the same forward primer (rec.envHXB2_Fp) and subtype-dependent reverse primers: Rp-fullEnv-B (5'-TCGTCTCATTCTTTCCCTTAC-3') for subtype B samples and Rp-fullEnv-C (5'-TCMTCTYATTCTTTCYCTTAC-3') for subtype C samples. Nested PCR reactions were performed in 50 µl mix containing 2 mM MgSO₄, 0.2 mM of each dNTP, 20 µM of each primer, 2.5 U Platinum *Taq* High Fidelity DNA. The amplification conditions were: initial denaturation cycle of 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec and extension at 68°C for 3 min, and a final 10 min extension step at 68°C. Amplification was verified by agarose gel electrophoresis. To avoid PCR selection, five independent amplifications were performed in parallel for each sample and were pooled for recombinant virus production and for DNA sequencing. Two reference clones NL4.3 (X4 tropic) [133] and NLAD8 (R5 tropic) [82] were included as controls in all our experiments.

Sequencing of gp41CT and MA: The V3-loop and gp41CT from patients were sequenced from the same amplicons used to produce Env-recombinant viruses to avoid PCR-selection biases. Sequencing was performed using BigDye Terminator v3.1 dye on a Applied Biosystems 3500 xL DX genetic analyzer (Applied Biosystems Europe BV, Belgium). Sense primers 6951 and 6990 and reverse primer 7336 [134] were used to sequence the V3-loop; sense primer AV326 and reverse primer AV331 [130] were used to sequence the gp41CT.

MA was sequenced from the same RNA extracted and used for Env amplification. A cDNA was synthesized and amplified from 10 µl RNA in a one-step PCR reaction using forward primer KVL64 and Reverse primer KVL79 [135] (20 µM each) and 2 µl Superscript III One Step RT-PCR and Platinum High Fidelity *Taq* (Invitrogen) as described in [135] and 2 µl of cDNA were further amplified using Forward primer KVL66 and Reverse primer KVL80 [135] as described in [135]. Amplicon size

and quality was verified by agarose gel electrophoresis and sequenced using primers KVL066, KVL080, KVL081 and GA1 [135]. Sequences were aligned and analyzed using the CLC Bio Main Workbench 6.82 software. The consensus sequence logos were generated with WebLogo3.3.

Construction of viral backbones: pNL4.3ΔEnv (Fig 1A) and pNL4.3ΔEC (Fig 1B) were generated by deleting the Env ectodomain or the full Env from pNL4.3 respectively and replacing it by a *AfeI* restriction site for linearization. To construct NL4.3ΔEC, the *Sall-XhoI* fragment from pNL4.3ΔEnv.EC.Luc [132] was cloned into pNL4.3. pNL4.3ΔEnv.EC.Luc [132] is deleted of the Env ectodomain (gp140 and contains a *AfeI* restriction site for linearization. The *Sall-BamHI* fragment containing the remaining cytoplasmic tail of gp41 was then cloned back into the *Sall-BamHI*-digested plasmid pNL4.3. To construct pNL4.3ΔEnv. The *EcoRI-XhoI* fragment from pNL4.3 was subcloned into pBluescript KS+ (Stratagene, Amsterdam, Netherlands), the entire Envelope (gp160) was deleted from pBluescript.Env through inverse PCR using phosphorylated primers Rp_del.full.Env: 5'-GCTIGTCTTCTGCTCTTTCTATTAG-3' and Fp_del.full.Env: 5'-GCTATGGGTGGCAAGTGGTC-3' designed to introduce a *AfeI* restriction site (underlined), *DpnI* digested and ligated to create pBluescriptΔEnv.AfeI. The *EcoRI-XhoI* fragment from pBluescriptΔEnv.AfeI was transferred back to pNL4.3, generating pNL4.3ΔEnv. All constructs were sequenced to verify correct cloning.

Production of Env and EnvEC Recombinant Viruses: Recombinant viral particles were produced by cotransfection of 8 µg of *AfeI*-linearized pNL4.3ΔEnv or pNL4.3ΔEC backbones (Westburg, Leusden, Netherlands) and patient-derived Env PCR amplicons (10 µl of EnvEC amplicon and 30 µl full Env amplicon) using Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) according to the manufacturer's recommendations. Cell-free culture supernatants were collected 48 hours post transfection, clarified by centrifugation and stored at -80°C until use. Amplicons produced from pNL4.3 (X4) and pNLAD8 (R5) plasmids were used as positive controls and viral-like particles lacking an envelope produced by transfecting solely the linearized backbone was used to assess background noise. Viral production was determined by quantifying p24 capsid protein using a p24 ELISA test (Innogenetics, Belgium).

Infections: PHA-P-activated CD4⁺ T cells (10⁵ cells/ well of U-shaped 96-well plates) or differentiated MDMs (3x10⁵ cells/well of a 96-well plate) maintained in MDM medium supplemented with 20% FCS were infected with normalized amounts of Env and EC- recombinant virus pairs (10 ng/ml equivalent p24) by spinoculation (1200g for 2 hours at 25°C). Because it has been shown that the impact of gp41CT truncations in primary CD4⁺ T cells and in non-permissive T cells lines can be overrode by high viral gene expression such as those reached at saturating viral inputs [71], we first tested serially diluted viral inputs that reproducibly infected primary CD4⁺ T cells and MDMs without saturating the system, and found 10 ng/ml equivalent p24 to meet these criteria (data not shown). After spinoculation, CD4⁺ T cells were incubated for 1 hour at 37°C and the medium was replaced by fresh IL-2-containing medium. MDMs were incubated for 2 hours at 37°C and medium was replaced by fresh MDM medium containing 10% FCS. Viral supernatants were collected at days 3, 5, 7 and 9-10 for CD4⁺ T cells and at days 4, 7 and 10 for MDMs and stored at -80°C. Viral replication was assayed by measuring p24 in culture supernatants by ELISA (Innogenetics, Belgium). All infections were performed in triplicate or quadruplicate wells. For some experiments, MDM infections were repeated in the presence of 2.5 mM deoxynucleosides (Sigma, Borneim, Belgium) added to cells 12 hours prior to infection with recombinant viruses. To exclude any intrinsic bias due to the recombination step, NL4.3 and AD8 Env- and EnvEC-recombinant virus pairs were included in each experiment to verify they had comparable replication levels, as their Envs, and particularly the gp41CT (NL4.3-derived) is identical regardless of the backbone and Env amplicon used.

For presentation of virions by DCs, 10⁵ MDDCs were seeded per well of a 96-well plate, loaded with 10 ng/ml equivalent p24 and spinoculated for 2 hours at 25°C at 1200g, cells were cultured for 1 hour at 37°C, washed, then 10⁵ CD4⁺ T cells were added to the MDDC culture. Medium was replaced every 2-3 days, and p24 in culture supernatants was monitored at days 5 and 7.

Site-directed-mutagenesis: To introduce subtype C polymorphisms into pNLAD8 Env, the *EcoRI*-*XhoI* fragment of pNLAD8 was subcloned into pBluescript KS(+). To insert the seven AA into pNLAD8, a synthetic gene spanning the *BamHI*-*XhoI* fragment of pNLAD8 and containing the 7 amino acid sequence insert SSLRGLQ was generated by Genscript and cloned into pBluescript-NLAD8Env generating pBluescript-NLAD8^{7AA}. The following sets of primers were designed to introduce the FIL₇₇₆ motif: Fp_FIL4HXB2 (5'-GAGACTTCATCTTGATTGTAACG-3') and Rp_FIL4HXB2 (5'-

CGTTACAATCAAGATGAAGTCTC-3'), the LQ₈₅₆ motif: Fp_LQ4HXB2 (5'-GGATTTTGCAATAAGATGGGTG-3') and Rp_LQ4HXB2 (5'-CACCCATCTTATTGCAAAATCC-3') into pBluescript-NLAD8Env and pBluescript-NLAD8^{-7AA} using the QuikChange II *Site-Directed Mutagenesis* Kit (Agilent Technologies, Netherlands). The following mutant NLAD8 Envs were generated: NLAD8Env^{FIL}, NLAD8Env^{LQ}, NLAD8Env^{FIL+LQ}, NLAD8Env^{+7AA}, NLAD8Env^{+7AA+FIL}, NLAD8Env^{+7AA+LQ}, NLAD8Env^{+7AA+FIL+LQ}.

To revert polymorphisms within subtype C Envs, two Envs (0266 and 1671) containing all the subtype-C specific polymorphisms associated respectively with very poor (0266) and with intermediate (1671) viral replication were selected. PCR amplicons spanning the full Env (gp160) were TA-cloned into the pGEM-T-easy vector (Promega) and site-directed mutagenesis was performed using the following primers: Fp_LL_gp41end (5'-GAAGCAGCTTTGCTATAAAATGG-3') and Rp_LL_gp41end(5'-CCATTTTATAGCAAAGCTGCTTC-3') to revert the C-terminal LQ₈₅₆ motif to LL₈₅₆ in both Envs; for reversion of FIL₇₇₆ to LLL₇₇₆, virus specific primer pairs were used to preserve flanking sequences: for Env 0266 primers Fp_LLLIso (5'-ATTGAGAGACTTGTTATTGATTG-3') and Rp_LLLIso (5'-CAATCAATAACAAGTCTCTCAAT-3'), and for Env 1671 primers: Fp_LLLValine (5'-ATTGAGAGACTTGTTATTGGTG-3') and Rp_LLLValine (5'-CACCAATAACAAGTCTCTCAAT-3'). The 7 amino acid insertion was removed by inverse PCR using phosphorylated primers: Elim7AAreverse (5'-GTGGAAGTCTGGGACGCAG-3') and Elim7AAforward (5'-GGGGTGGGAAGCCCTTAAATATCTG -3'). The following mutants were generated for Envs 0266 and 1671: 0266/1671Env^{LLL}, 0266/1671Env^{LL856}, 0266/1671Env^{LLL+LL856}, 0266/1671Env^{-7AA}, 0266/1671Env^{-7AA+LLL}, 0266/1671Env^{-7AA+LL856}, 0266/1671Env^{-7AA+LLL+LL856}.

Mutagenesis was verified by sequencing for all mutants. Env mutants expressed in pBluescript were used to generate inner full Env PCR amplicons and Env recombinant viruses were produced by cotransfection with pNL4.3ΔEnv as above, and compared to the wild type (wt) Env recombinants.

Env incorporation into viral particles: Supernatants from CD4+ T cells infected with Env and EnvEC recombinant viruses were purified on a sucrose cushion composed of 60%, 50%, 40%, 30%, 20% and 10% sucrose using a Beckman Optima MAX Ultracentrifuge at 50 000 rpm for 16 hours at 4°C. The fractions containing purified virions were harvested and concentrated at 25 000g for 16 hours at 4°C, then lysed with Laemmli buffer supplemented with 2-β-mercaptoethanol at a 1:1 ratio

and the mixture was heated at 95°C for 10 min prior to loading on 12% SDS-PAGE gel (Bio-Rad, Belgium). For immunoblotting, proteins were transferred to nitrocellulose membranes, incubated overnight at 4°C in 15 ml PBS containing 0.05% Tween 20 and primary antibodies 13.3 µg/ml goat polyclonal anti-HIV-1 gp120 and 0.4 µg/ml mouse anti-HIV-1 p24 monoclonal antibody 39/6.14 (Abcam, Cambridge UK). Membranes were then washed with PBS containing 0.05% Tween 20, incubated for one hour with 0.05 µg/ml donkey anti-goat and 1.25µg/ml rabbit anti-mouse IgG conjugated to HRP (Sigma-Belgium) and the bands developed with Opti-4CN substrate (Bio-Rad, Belgium) according to manufacturer's instructions.

Gp120 at the surface of virions produced from infected CD4⁺ T cells was quantified using a gp120 ELISA adapted from [62, 136]. Briefly, infected culture supernatants were pooled and virions were lysed in TNE buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5 mM EDTA) supplemented with 1% Triton X-100 and protease inhibitors and p24 was quantified by ELISA (Innogenetics). 150 µl of viral culture supernatant and 100 µl of TBS supplemented with 1% Nonidet P-40 were incubated in 96-well microtiter Nunc plates (Thermo Electron Corporation) previously coated overnight with 5 µg/ml anti-gp120 antibody D7324 (Aalto). After a 2-hour gp120 capture step at room temperature, wells were washed twice with TBS and incubated for 1 hour at room temperature with 100 µl (1 µg/ml) of human anti-gp120 antibody (2G12, Polynum) diluted in TMT/SS buffer (TBS containing 4% skimmed milk, 0.5% Tween20 and 20% sheep serum) and for one hour with 100 µl peroxidase-conjugated anti-human secondary F(ab)₂ antibody (Invitrogen) (1/2000 in TMT/SS buffer). The gp120/p24 ratio was calculated.

Confocal Microscopy: For CD4⁺ T cell staining, quadruplicate wells infected with Env and EnvEC recombinants as described above were washed at day 7, pooled and seeded in Nunc Lab-Teck chambers previously coated with 100 µg/ml poly-L-lysine for 2 hours at 37°C. For MDM staining, 2.5x10⁵ monocytes/chamber were plated in Nunc Lab-Teck 8-chamber microscope slides (VWR, Leuven, Belgium), allowed to differentiate into MDMs for 7 days, and infected with Env and EnvEC recombinant viruses (10 ng/ml equivalent p24). For staining, cells were washed and fixed with cold absolut ethanol for 10 min, and incubated with goat polyclonal anti-HIV-1 gp120 and mouse anti-MA clone 17-1 (Abcam) in blocking buffer (PBS containing 2% BSA and 5% human serum) for 1 hour, washed with PBS three times and subsequently incubated with donkey anti-goat IgG then goat anti-

mouse IgG secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 568 (all from Invitrogen). Confocal microscopy images were captured with a Zeiss LSM510 META confocal laser scanning microscope (Jena, Germany) equipped with a 40x Plan-NeoFluar oil immersion objective (numerical aperture 1.3). Analysis was performed with Huygens Essential image processing software package.

Acknowledgements

The authors are deeply grateful to the patients who participated in this study, to René Brons for support and assistance with cell staining and to Andy Chevigné and Gilbert Faure for fruitful discussions.

Funding

This work was supported by the Luxembourg Ministry of Research and Education Grant #MCESR LRTV20100604. ESS and MM were supported by PhD scholarships from the Fond National de la Recherche (AFR grants #PHD-09-115 and #PHD08-074 respectively).

Authors' contribution

ESS and MM constructed the viral tools, performed infections of primary cells. ESS and AR generated Env mutants and revertants, as well as site-directed mutagenesis. MM, ESS, CM and purified viral particles, MM and CM performed WB and ESS and ML gp120 ELISA. Confocal microscopy was performed by ML and sequencing (samples + mutants) by JYS. DPB conceived and designed the study. CSD and JCS ensured scientific guidance and contributed patient samples. ESS and DPB analyzed the data and drafted the manuscript.

Ethical clearance

The present study was approved by the Comité National d'Ethique in Luxembourg.

Transparency declaration

The authors declare no conflict of interest.

References

1. Blanco J, Bosch B, Fernandez-Figueras MT, Barretina J, Clotet B, Este JA: **High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells.** *J Biol Chem* 2004, **279**:51305-51314.
2. Chen P, Hubner W, Spinelli MA, Chen BK: **Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses.** *J Virol* 2007, **81**:12582-12595.
3. Hubner W, McNerney GP, Chen P, Dale BM, Gordon RE, Chuang FY, Li XD, Asmuth DM, Huser T, Chen BK: **Quantitative 3D video microscopy of HIV transfer across T cell virological synapses.** *Science* 2009, **323**:1743-1747.
4. Ruggiero E, Bona R, Muratori C, Federico M: **Virological consequences of early events following cell-cell contact between human immunodeficiency virus type 1-infected and uninfected CD4+ cells.** *J Virol* 2008, **82**:7773-7789.
5. Sourisseau M, Sol-Foulon N, Porrot F, Blanchet F, Schwartz O: **Inefficient human immunodeficiency virus replication in mobile lymphocytes.** *J Virol* 2007, **81**:1000-1012.
6. Sowinski S, Jolly C, Berninghausen O, Purbhoo MA, Chauveau A, Kohler K, Oddos S, Eissmann P, Brodsky FM, Hopkins C, et al: **Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission.** *Nat Cell Biol* 2008, **10**:211-219.
7. Haller C, Fackler OT: **HIV-1 at the immunological and T-lymphocytic virological synapse.** *Biol Chem* 2008, **389**:1253-1260.
8. Jolly C, Sattentau QJ: **Retroviral spread by induction of virological synapses.** *Traffic* 2004, **5**:643-650.
9. Sattentau QJ: **Cell-to-Cell Spread of Retroviruses.** *Viruses* 2010, **2**:1306-1321.
10. Carr JM, Hocking H, Li P, Burrell CJ: **Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood lymphocytes.** *Virology* 1999, **265**:319-329.
11. Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, Martin MA: **Quantitation of human immunodeficiency virus type 1 infection kinetics.** *J Virol* 1993, **67**:2182-2190.
12. Johnson DC, Huber MT: **Directed egress of animal viruses promotes cell-to-cell spread.** *J Virol* 2002, **76**:1-8.
13. Rudnicka D, Feldmann J, Porrot F, Wietgreffe S, Guadagnini S, Prevost MC, Estaquier J, Haase AT, Sol-Foulon N, Schwartz O: **Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses.** *J Virol* 2009, **83**:6234-6246.
14. Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks E, Parks W, Josephs S, Gallo R, et al: **Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS.** *Cell* 1986, **45**:637-648.
15. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: **CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.** *Science* 1996, **272**:1955-1958.
16. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, et al: **Identification of a major co-receptor for primary isolates of HIV-1.** *Nature* 1996, **381**:661-666.
17. Feng Y, Broder CC, Kennedy PE, Berger EA: **HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.** *Science* 1996, **272**:872-877.
18. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA: **HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.** *Nature* 1996, **381**:667-673.

19. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: **CCR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.** *Science* 1996, **272**:1955-1958.
20. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Marzio PD, Marmon S, Sutton RE, Hill CM, et al: **Identification of a major co-receptor for primary isolates of HIV-1.** *Nature* 1996, **381**:661-666.
21. Feng Y, Broder CC, Kennedy PE, Berger EA: **HIV-1 entry cofactor : functional cDNA cloning of a seven-transmembrane G protein-coupled receptor.** *Science* 1996, **272**:872-877.
22. Dragic T: **HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.** *Nature* 1996, **381**:667-673.
23. Zwick MB, Saphire EO, Burton DR: **gp41: HIV's shy protein.** *Nat Med* 2004, **10**:133-134.
24. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA: **Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.** *Nature* 1998, **393**:648-659.
25. Hunter E, Swanstrom R: **Retrovirus envelope glycoproteins.** *Curr Top Microbiol Immunol* 1990, **157**:187-253.
26. Steckbeck JD, Craig JK, Barnes CO, Montelaro RC: **Highly conserved structural properties of the C-terminal tail of HIV-1 gp41 protein despite substantial sequence variation among diverse clades: implications for functions in viral replication.** *J Biol Chem* 2011, **286**:27156-27166.
27. Buratti E, McLain L, Tisminetzky S, Cleveland SM, Dimmock NJ, Baralle FE: **The neutralizing antibody response against a conserved region of human immunodeficiency virus type 1 gp41 (amino acid residues 731-752) is uniquely directed against a conformational epitope.** *J Gen Virol* 1998, **79 (Pt 11)**:2709-2716.
28. Reading SA, Heap CJ, Dimmock NJ: **A novel monoclonal antibody specific to the C-terminal tail of the gp41 envelope transmembrane protein of human immunodeficiency virus type 1 that preferentially neutralizes virus after it has attached to the target cell and inhibits the production of infectious progeny.** *Virology* 2003, **315**:362-372.
29. Heap CJ, Reading SA, Dimmock NJ: **An antibody specific for the C-terminal tail of the gp41 transmembrane protein of human immunodeficiency virus type 1 mediates post-attachment neutralization, probably through inhibition of virus-cell fusion.** *J Gen Virol* 2005, **86**:1499-1507.
30. Cheung L, McLain L, Hollier MJ, Reading SA, Dimmock NJ: **Part of the C-terminal tail of the envelope gp41 transmembrane glycoprotein of human immunodeficiency virus type 1 is exposed on the surface of infected cells and is involved in virus-mediated cell fusion.** *J Gen Virol* 2005, **86**:131-138.
31. Miller MA, Garry RF, Jaynes JM, Montelaro RC: **A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides.** *AIDS Res Hum Retroviruses* 1991, **7**:511-519.
32. Checkley MA, Luttge BG, Freed EO: **HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation.** *J Mol Biol* 2011, **410**:582-608.
33. Postler TS, Desrosiers RC: **The Tale of the Long Tail: the Cytoplasmic Domain of HIV-1 gp41.** *J Virol* 2012.
34. Steckbeck JD, Kuhlmann AS, Montelaro RC: **C-terminal tail of human immunodeficiency virus gp41: functionally rich and structurally enigmatic.** *J Gen Virol* 2012.
35. Santos da Silva E, Mulinge M, Perez Bercoff D: **The frantic play of the concealed HIV envelope cytoplasmic tail.** *Retrovirology* 2013, **10**:54.
36. Owens RJ, Dubay JW, Hunter E, Compans RW: **Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells.** *Proc Natl Acad Sci U S A* 1991, **88**:3987-3991.

37. Lodge R, Gottlinger H, Gabuzda D, Cohen EA, Lemay G: **The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells.** *J Virol* 1994, **68**:4857-4861.
38. Lodge R, Lalonde JP, Lemay G, Cohen EA: **The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells.** *EMBO J* 1997, **16**:695-705.
39. Edwards TG, Wyss S, Reeves JD, Zolla-Pazner S, Hoxie JA, Doms RW, Baribaud F: **Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein.** *J Virol* 2002, **76**:2683-2691.
40. Kalia V, Sarkar S, Gupta P, Montelaro RC: **Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation.** *J Virol* 2003, **77**:3634-3646.
41. Wyss S, Dimitrov AS, Baribaud F, Edwards TG, Blumenthal R, Hoxie JA: **Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail.** *J Virol* 2005, **79**:12231-12241.
42. Bhakta SJ, Shang L, Prince JL, Claiborne DT, Hunter E: **Mutagenesis of tyrosine and di-leucine motifs in the HIV-1 envelope cytoplasmic domain results in a loss of Env-mediated fusion and infectivity.** *Retrovirology* 2011, **8**:37.
43. Emerson V, Haller C, Pfeiffer T, Fackler OT, Bosch V: **Role of the C-terminal domain of the HIV-1 glycoprotein in cell-to-cell viral transmission between T lymphocytes.** *Retrovirology* 2010, **7**:43.
44. Lee SF, Wang CT, Liang JY, Hong SL, Huang CC, Chen SS: **Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41.** *J Biol Chem* 2000, **275**:15809-15819.
45. Zhu Y, Lu L, Chao L, Chen YH: **Important changes in biochemical properties and function of mutated LLP12 domain of HIV-1 gp41.** *Chem Biol Drug Des* 2007, **70**:311-318.
46. Chojnacki J, Staudt T, Glass B, Bingen P, Engelhardt J, Anders M, Schneider J, Muller B, Hell SW, Krausslich HG: **Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy.** *Science* 2012, **338**:524-528.
47. Muranyi W, Malkusch S, Muller B, Heilemann M, Krausslich HG: **Super-resolution microscopy reveals specific recruitment of HIV-1 envelope proteins to viral assembly sites dependent on the envelope C-terminal tail.** *PLoS Pathog* 2013, **9**:e1003198.
48. Roy NH, Chan J, Lambele M, Thali M: **Clustering and Mobility of HIV-1 Env at Viral Assembly Sites Predict Its Propensity To Induce Cell-Cell Fusion.** *J Virol* 2013, **87**:7516-7525.
49. Dubay JW, Roberts SJ, Hahn BH, Hunter E: **Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity.** *J Virol* 1992, **66**:6616-6625.
50. Yu X, Yuan X, McLane MF, Lee TH, Essex M: **Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions.** *J Virol* 1993, **67**:213-221.
51. Cosson P: **Direct interaction between the envelope and matrix proteins of HIV-1.** *EMBO J* 1996, **15**:5783-5788.
52. Lee YM, Tang XB, Cimasky LM, Hildreth JE, Yu XF: **Mutations in the matrix protein of human immunodeficiency virus type 1 inhibit surface expression and virion incorporation of viral envelope glycoproteins in CD4+ T lymphocytes.** *J Virol* 1997, **71**:1443-1452.
53. Murakami T, Freed EO: **The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions.** *Proc Natl Acad Sci U S A* 2000, **97**:343-348.

54. Murakami T, Freed EO: **Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail.** *J Virol* 2000, **74**:3548-3554.
55. Piller SC, Dubay JW, Derdeyn CA, Hunter E: **Mutational analysis of conserved domains within the cytoplasmic tail of gp41 from human immunodeficiency virus type 1: effects on glycoprotein incorporation and infectivity.** *J Virol* 2000, **74**:11717-11723.
56. Ohno H: **Interaction of tyrosine-based sorting signals with clathrin-associated proteins.** *Science* 1995, **269**:1872-1875.
57. Ohno H, Aguilar RC, Fournier MC, Hennecke S, Cosson P, Bonifacino JS: **Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family.** *Virology* 1997, **238**:305-315.
58. Ohno H, Fournier MC, Poy G, Bonifacino JS: **Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains.** *J Biol Chem* 1996, **271**:29009-29015.
59. Dong X, Li H, Derdowski A, Ding L, Burnett A, Chen X, Peters TR, Dermody TS, Woodruff E, Wang JJ, Spearman P: **AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly.** *Cell* 2005, **120**:663-674.
60. Blot G, Janvier K, Le Panse S, Benarous R, Berlioz-Torrent C: **Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity.** *J Virol* 2003, **77**:6931-6945.
61. Murray JL, Mavrikakis M, McDonald NJ, Yilla M, Sheng J, Bellini WJ, Zhao L, Le Doux JM, Shaw MW, Luo CC, et al: **Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses, and measles virus.** *J Virol* 2005, **79**:11742-11751.
62. Lambele M, Labrosse B, Roch E, Moreau A, Verrier B, Barin F, Roingeard P, Mammano F, Brand D: **Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of envelope glycoproteins and viral assembly.** *J Virol* 2007, **81**:125-140.
63. Lopez-Verges S, Camus G, Blot G, Beauvoir R, Benarous R, Berlioz-Torrent C: **Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions.** *Proc Natl Acad Sci U S A* 2006, **103**:14947-14952.
64. Bauby H, Lopez-Verges S, Hoeffel G, Delcroix-Genete D, Janvier K, Mammano F, Hosmalin A, Berlioz-Torrent C: **TIP47 is required for the production of infectious HIV-1 particles from primary macrophages.** *Traffic* 2010, **11**:455-467.
65. Wyss S, Berlioz-Torrent C, Boge M, Blot G, Honing S, Benarous R, Thali M: **The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter].** *J Virol* 2001, **75**:2982-2992.
66. Byland R, Vance PJ, Hoxie JA, Marsh M: **A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein.** *Mol Biol Cell* 2007, **18**:414-425.
67. Postler TS, Desrosiers RC: **The cytoplasmic domain of the HIV-1 glycoprotein gp41 induces NF-kappaB activation through TGF-beta-activated kinase 1.** *Cell Host Microbe* 2012, **11**:181-193.
68. Wilk T, Pfeiffer T, Bosch V: **Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product.** *Virology* 1992, **189**:167-177.
69. Akari H, Fukumori T, Adachi A: **Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions.** *J Virol* 2000, **74**:4891-4893.

70. Dubay JW, Roberts SJ, Hahn BH, Hunter E: **Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity.** *J Virol* 1992, **66**:6616-6625.
71. Emerson V, Haller C, Pfeiffer T, Fackler O, Bosch V: **Role of the C-terminal domain of the HIV-1 glycoprotein in cell-to-cell viral transmission between T lymphocytes.** *Retrovirology* 2010, **7**:43.
72. Murakami T, Freed E: **The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions.** *Proc Natl Acad Sci USA* 2000, **97**:343 - 348.
73. Iwatani Y, Ueno T, Nishimura A, Zhang X, Hattori T, Ishimoto A, Ito M, Sakai H: **Modification of virus infectivity by cytoplasmic tail of HIV-1 TM protein.** *Virus Res* 2001, **74**:75-87.
74. Yu X, Yuan X, McLane M, Lee T, Essex M: **Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions.** *J Virol* 1993, **67**:213 - 221.
75. Salemi M, de Oliveira T, Soares MA, Pybus O, Dumans AT, Vandamme AM, Tanuri A, Cassol S, Fitch WM: **Different epidemic potentials of the HIV-1B and C subtypes.** *J Mol Evol* 2005, **60**:598-605.
76. Arts EJ: **Infection with subtype C HIV-1 of lower replicative fitness as compared to subtypes A and D leads to slower disease progression in Zimbabwean and Ugandan women.** 2006.
77. Pollakis G: **Phenotypic and genotypic comparisons of CCR5- and CXCR4-tropic human immunodeficiency virus type 1 biological clones isolated from subtype C-infected individuals.** *J Virol* 2004, **78**:2841-2852.
78. Murakami T, Freed E: **Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail.** *J Virol* 2000, **74**:3548 - 3554.
79. Monel B, Beaumont E, Vendrame D, Schwartz O, Brand D, Mammano F: **HIV cell-to-cell transmission requires the production of infectious virus particles and does not proceed through env-mediated fusion pores.** *J Virol* 2012, **86**:3924-3933.
80. Freed E, Martin M: **Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions.** *J Virol* 1996, **70**:341 - 351.
81. Durham ND, Yewdall AW, Chen P, Lee R, Zony C, Robinson JE, Chen BK: **Neutralization Resistance of Virological Synapse-Mediated HIV-1 Infection Is Regulated by the gp41 Cytoplasmic Tail.** *Journal of virology* 2012, **86**:7484-7495.
82. Freed EO, Englund G, Martin MA: **Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection.** *J Virol* 1995, **69**:3949-3954.
83. Cosson P: **Direct interaction between the envelope and matrix proteins of HIV-1.** *EMBO J* 1996, **15**:5783-5788.
84. Batonick M, Favre M, Boge M, Spearman P, Honing S, Thali M: **Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2.** *Virology* 2005, **342**:190-200.
85. Berlioz-Torrent C, Shacklett BL, Erdtmann L, Delamarre L, Bouchaert I, Sonigo P, Dokhelar MC, Benarous R: **Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins.** *J Virol* 1999, **73**:1350-1361.
86. Camus G, Segura-Morales C, Molle D, Lopez-Verges S, Begon-Pescia C, Cazevielle C, Schu P, Bertrand E, Berlioz-Torrent C, Basyuk E: **The clathrin adaptor complex AP-1 binds HIV-1 and MLV Gag and facilitates their budding.** *Mol Biol Cell* 2007, **18**:3193-3203.
87. Liu L, Sutton J, Woodruff E, Villalta F, Spearman P, Dong X: **Defective HIV-1 particle assembly in AP-3-deficient cells derived from patients with Hermansky-Pudlak syndrome type 2.** *J Virol* 2012, **86**:11242-11253.

88. Rangel HR, Weber J, Chakraborty B, Gutierrez A, Marotta ML, Mirza M, Kiser P, Martinez MA, Este JA, Quinones-Mateu ME: **Role of the human immunodeficiency virus type 1 envelope gene in viral fitness.** *J Virol* 2003, **77**:9069-9073.
89. Jacobs GB, Bock S, Schuch A, Moschall R, Schrom EM, Zahn J, Reuter C, Preiser W, Rethwilm A, Engelbrecht S, et al: **Construction of a high titer infectious HIV-1 subtype C proviral clone from South Africa.** *Viruses* 2012, **4**:1830-1843.
90. Baldauf HM, Pan X, Erikson E, Schmidt S, Daddacha W, Burggraf M, Schenkova K, Ambiel I, Wabnitz G, Gramberg T, et al: **SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells.** *Nat Med* 2012.
91. Lahouassa H, Daddacha W, Hofmann H, Ayinde D, Logue EC, Dragin L, Bloch N, Maudet C, Bertrand M, Gramberg T, et al: **SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates.** *Nat Immunol* 2012, **13**:223-228.
92. Gavegnano C, Kennedy EM, Kim B, Schinazi RF: **The Impact of Macrophage Nucleotide Pools on HIV-1 Reverse Transcription, Viral Replication, and the Development of Novel Antiviral Agents.** *Mol Biol Int* 2012, **2012**:625983.
93. Rowell JF, Stanhope PE, Siliciano RF: **Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC.** *J Immunol* 1995, **155**:473-488.
94. Bultmann A, Muranyi W, Seed B, Haas J: **Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein that inhibit cell surface expression.** *J Virol* 2001, **75**:5263-5276.
95. Blot G, Janvier K, Le Panse S, Benarous R, Berlioz-Torrent C: **Targeting of the Human Immunodeficiency Virus Type 1 Envelope to the trans-Golgi Network through Binding to TIP47 Is Required for Env Incorporation into Virions and Infectivity.** *Journal of Virology* 2003, **77**:6931-6945.
96. Murray JL: **Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses, and measles virus.** *J Virol* 2005, **79**:11742-11751.
97. Lopez-Vergès S, Camus G, Blot G, Beauvoir R, Benarous R, Berlioz-Torrent C: **Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions.** *Proceedings of the National Academy of Sciences* 2006, **103**:14947-14952.
98. Bauby H, Lopez-Vergès S, Hoeffel G, Delcroix-Genête D, Janvier K, Mammano F, Hosmalin A, Berlioz-Torrent C: **TIP47 is Required for the Production of Infectious HIV-1 Particles from Primary Macrophages.** *Traffic* 2010, **11**:455-467.
99. Deschambeault J, Lalonde JP, Cervantes-Acosta G, Lodge R, Cohen EA, Lemay G: **Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission.** *J Virol* 1999, **73**:5010-5017.
100. Gousset K, Ablan SD, Coren LV, Ono A, Soheilian F, Nagashima K, Ott DE, Freed EO: **Real-time visualization of HIV-1 GAG trafficking in infected macrophages.** *PLoS Pathog* 2008, **4**:e1000015.
101. Sharova N, Swingler C, Sharkey M, Stevenson M: **Macrophages archive HIV-1 virions for dissemination in trans.** *EMBO J* 2005, **24**:2481-2489.
102. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M: **In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53.** *J Cell Biol* 2007, **177**:329-341.
103. Welsch S, Keppler OT, Habermann A, Allespach I, Krijnse-Locker J, Krausslich HG: **HIV-1 buds predominantly at the plasma membrane of primary human macrophages.** *PLoS Pathog* 2007, **3**:e36.
104. Koppensteiner H, Banning C, Schneider C, Hohenberg H, Schindler M: **Macrophage internal HIV-1 is protected from neutralizing antibodies.** *J Virol* 2012, **86**:2826-2836.

105. Murakami T: **Retroviral env glycoprotein trafficking and incorporation into virions.** *Mol Biol Int* 2012, **2012**:682850.
106. Ghanam RH, Samal AB, Fernandez TF, Saad JS: **Role of the HIV-1 Matrix Protein in Gag Intracellular Trafficking and Targeting to the Plasma Membrane for Virus Assembly.** *Front Microbiol* 2012, **3**:55.
107. Qi M, Williams JA, Chu H, Chen X, Wang JJ, Ding L, Akhirome E, Wen X, Lapierre LA, Goldenring JR, Spearman P: **Rab11-FIP1C and Rab14 direct plasma membrane sorting and particle incorporation of the HIV-1 envelope glycoprotein complex.** *PLoS Pathog* 2013, **9**:e1003278.
108. Nabel G, Baltimore D: **An inducible transcription factor activates expression of human immunodeficiency virus in T cells.** *Nature* 1987, **326**:711-713.
109. !!! INVALID CITATION !!!
110. Postler T, Desrosiers R: **The Cytoplasmic Domain of the HIV-1 Glycoprotein gp41 Induces NF- κ B Activation through TGF- β -Activated Kinase 1.** *Cell Host & Microbe* 2012, **11**:181-193.
111. Pace MJ, Graf EH, Agosto LM, Mexas AM, Male F, Brady T, Bushman FD, O'Doherty U: **Directly infected resting CD4+T cells can produce HIV Gag without spreading infection in a model of HIV latency.** *PLoS Pathog* 2012, **8**:e1002818.
112. Sanders RW, de Jong EC, Baldwin CE, Schuitemaker JH, Kapsenberg ML, Berkhout B: **Differential transmission of human immunodeficiency virus type 1 by distinct subsets of effector dendritic cells.** *J Virol* 2002, **76**:7812-7821.
113. Patterson S, Rae A, Hockey N, Gilmour J, Gotch F: **Plasmacytoid dendritic cells are highly susceptible to human immunodeficiency virus type 1 infection and release infectious virus.** *J Virol* 2001, **75**:6710-6713.
114. Turville SG, Arthos J, Donald KM, Lynch G, Naif H, Clark G, Hart D, Cunningham AL: **HIV gp120 receptors on human dendritic cells.** *Blood* 2001, **98**:2482-2488.
115. Turville SG, Cameron PU, Arthos J, MacDonald K, Clark G, Hart D, Cunningham AL: **Bitter-sweet symphony: defining the role of dendritic cell gp120 receptors in HIV infection.** *J Clin Virol* 2001, **22**:229-239.
116. Soilleux EJ, Coleman N: **Langerhans cells and the cells of Langerhans cell histiocytosis do not express DC-SIGN.** *Blood* 2001, **98**:1987-1988.
117. Turville SG, Cameron PU, Handley A, Lin G, Pohlmann S, Doms RW, Cunningham AL: **Diversity of receptors binding HIV on dendritic cell subsets.** *Nat Immunol* 2002, **3**:975-983.
118. Granelli-Piperno A, Pritsker A, Pack M, Shimeliovich I, Arrighi JF, Park CG, Trumppfeller C, Piguat V, Moran TM, Steinman RM: **Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction.** *J Immunol* 2005, **175**:4265-4273.
119. Wu L, KewalRamani VN: **Dendritic-cell interactions with HIV: infection and viral dissemination.** *Nat Rev Immunol* 2006, **6**:859-868.
120. Leitner T: **HIV Sequence Compendium 2003.** Nature Publishing Group; 2003.
121. Davis MR, Jiang J, Zhou J, Freed EO, Aiken C: **A mutation in the human immunodeficiency virus type 1 Gag protein destabilizes the interaction of the envelope protein subunits gp120 and gp41.** *J Virol* 2006, **80**:2405-2417.
122. Mammano F, Kondo E, Sodroski J, Bukovsky A, Gottlinger HG: **Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains.** *J Virol* 1995, **69**:3824-3830.
123. Freed EO, Martin MA: **Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions.** *J Virol* 1996, **70**:341-351.
124. Dingwell KS, Johnson DC: **The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions.** *J Virol* 1998, **72**:8933-8942.

125. Byland R, Vance PJ, Hoxie JA, Marsh M: **A Conserved Dileucine Motif Mediates Clathrin and AP-2-dependent Endocytosis of the HIV-1 Envelope Protein.** *Molecular Biology of the Cell* 2007, **18**:414-425.
126. Schiavoni I, Trapp S, Santarcangelo AC, Piacentini V, Pugliese K, Baur A, Federico M: **HIV-1 Nef enhances both membrane expression and virion incorporation of Env products. A model for the Nef-dependent increase of HIV-1 infectivity.** *J Biol Chem* 2004, **279**:22996-23006.
127. Perez-Bercoff D, Wurtzer S, Compain S, Benech H, Clavel F: **Human immunodeficiency virus type 1: resistance to nucleoside analogues and replicative capacity in primary human macrophages.** *J Virol* 2007, **81**:4540-4550.
128. de Oliveira T, Deforche K, Cassol S, Salminen M, Paraskevis D, Seebregts C, Snoeck J, van Rensburg EJ, Wensing AMJ, van de Vijver DA, et al: **An automated genotyping system for analysis of HIV-1 and other microbial sequences.** *Bioinformatics* 2005, **21**:3797-3800.
129. Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, Verhofstede C, Lie Y, Seguin-Devaux C, Schmit JC, Bercoff DP: **Correction: HIV-1 Tropism Determination Using a Phenotypic Env Recombinant Viral Assay Highlights Overestimation of CXCR4-Usage by Genotypic Prediction Algorithms for CRRF01_AE and CRF02_AG.** *PLoS One* 2013, **8**.
130. Van Laethem K, Schrooten Y, Lemey P, Wijngaerden EV, Wit SD, Ranst MV, Vandamme A-M: **A genotypic resistance assay for the detection of drug resistance in the human immunodeficiency virus type 1 envelope gene.** *Journal of Virological Methods* 2005, **123**:25-34.
131. Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, Verhofstede C, Lie Y, Seguin-Devaux C, Schmit JC, Bercoff DP: **HIV-1 Tropism Determination Using a Phenotypic Env Recombinant Viral Assay Highlights Overestimation of CXCR4-Usage by Genotypic Prediction Algorithms for CRRF01_AE and CRF02_AG.** *PLoS One* 2013, **8**:e60566.
132. Baatz F, Nijhuis M, Lemaire M, Riedijk M, Wensing AMJ, Servais J-Y, van Ham PM, Hoepelman AIM, Koopmans PP, Sprenger HG, et al: **Impact of the HIV-1 Genetic Context outside HR1/HR2 on Resistance to the Fusion Inhibitor Enfuvirtide and Viral Infectivity in Clinical Isolates.** *PLoS ONE* 2011, **6**:e21535.
133. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA: **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** *J Virol* 1986, **59**:284-291.
134. Verhofstede C, Brudney D, Reynaerts J, Vaira D, Franssen K, De Bel A, Seguin-Devaux C, De Wit S, Vandekerckhove L, Geretti AM: **Concordance between HIV-1 genotypic coreceptor tropism predictions based on plasma RNA and proviral DNA.** *HIV Medicine* 2011, **12**:544-552.
135. Van Laethem K, Schrooten Y, Dedecker S, Van Heeswijck L, Deforche K, Van Wijngaerden E, Van Ranst M, Vandamme AM: **A genotypic assay for the amplification and sequencing of gag and protease from diverse human immunodeficiency virus type 1 group M subtypes.** *J Virol Methods* 2006, **132**:181-186.
136. Beaumont E, Vendrame D, Verrier B, Roch E, Biron F, Barin F, Mammano F, Brand D: **Matrix and envelope coevolution revealed in a patient monitored since primary infection with human immunodeficiency virus type 1.** *J Virol* 2009, **83**:9875-9889.

Figure Legends

Figure 1: Molecular constructs pNL4.3ΔEC and pNL4.3Δenv. The molecular constructs were based on the pNL4.3 infectious clone deleted of the Env gp120+gp41 ectodomain (gp140, ΔEnvEC) (A) or of the entire Env (gp160, ΔEnv) (B) by inverse PCR. Infectious viral particles expressing patient-derived Env or EnvEC sequences are generated by co-transfecting HEK293 cells with the linearized pNL4.3ΔEnvEC or pNL4.3ΔEnv backbones and a PCR amplicon spanning the target region (gp140 or gp160 respectively).

Figure 2. Replication of Env and EnvEC recombinant virus pairs in CD4+ T cells (A and B) and in MDMs (C and D). 10^5 CD4+ T cells (A) or 3×10^5 MDMs (C) were infected with 10 ng/ml equivalent p24 of Env or EnvEC recombinant viruses. Viral infection was monitored by measuring p24 in culture supernatants at days 3, 5, 7 and 10. Infections at day 5 for CD4+ T cells and at day 7 for MDMs are reported here. Each dot represents one infection experiment performed with cells from a different donor. All infections were performed in triplicate wells. Statistically different means are indicated. (B and D): The same results are represented for each individual Env, as the ratio of p24 in supernatants from cells infected with Env recombinant viruses/p24 in the supernatants of cells infected with the corresponding EnvEC recombinant virus, in CD4+ T cells (B) and MDMs (D). A positive ratio indicates that the wt recombinant replicates better than its chimeric counterpart containing the isogenic gp140 and the gp41CT of NL4.3 and a negative ratio implies the recombinant virus containing the chimeric Env replicates better than the wt Env. All infections were performed in triplicate or quadruplicate wells. Because of inter-donor variability, results are presented as individual experiments performed with CD4+ T cells and MDMs from independent donors.

Figure 3. Transmission of subtype C Env and EnvEC recombinant virus pairs by mature MDDCs to CD4+ T cells. 10^5 MDDCs were loaded with subtype C Env and EnvEC recombinant viruses, then incubated with 10^5 CD4+ T cells. Viral infection was monitored by measuring p24 in culture supernatants at days 5 and 7. Infections at day 5 are presented. Each dot represents one

infection experiment performed with cells from a different donor. All infections were performed in triplicate wells. Statistically different means are indicated.

Figure 4. Intracellular localization of p55Gag and Env in CD4+ T cells and in MDMs. A and C.

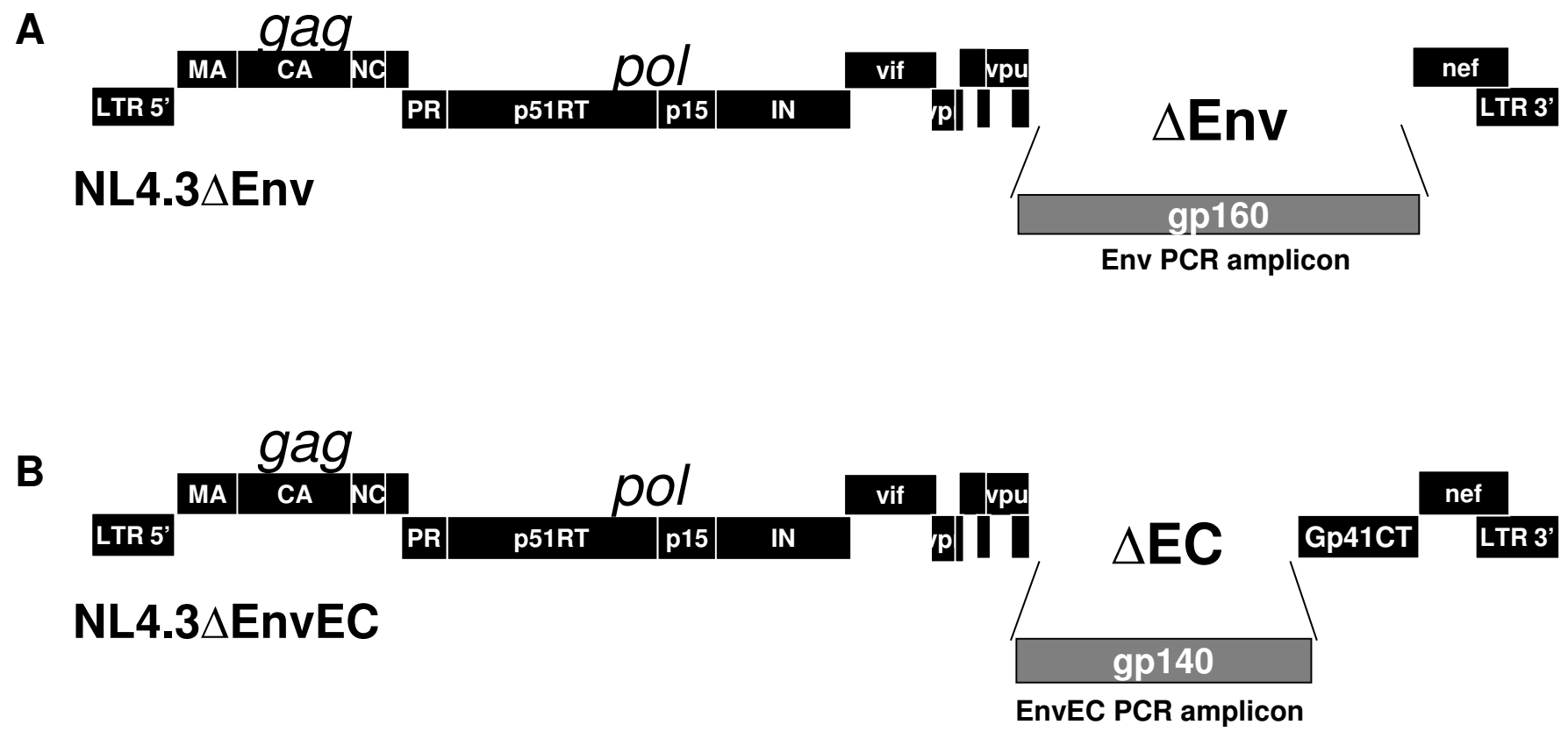
CD4+ T cells (10^5 cells per well of a 96-well plate) were infected with 10 ng/ml equivalent p24 of subtype B (A) or subtype C (C) Env and EnvEC recombinant virus pairs by spinoculation. At day 5 post-infection, cells were pooled and seeded on poly-L-lysine coated Lab-Teck coverslips. Cells were permeabilized and stained with antibodies against gp120 and p17. Intracellular localization of gp120 (green) and p55Gag (MA, red) in CD4+ T cells infected with subtype B Env and EnvEC recombinant virus pairs, or with subtype C Env and EnvEC recombinant virus pairs. **B and D.** MDMs (2.5×10^5 cells/chamber of a Lab-Teck coverslip) were infected with 10 ng/ml equivalent p24 Env or EnvEC recombinant viruses. Intracellular localization of gp120 (red) and p17 (green) in MDMs infected with subtype B (B) and subtype C (D) Env and EnvEC recombinant viruses. Images were captured with a Zeiss LSM510 META confocal laser scanning microscope (Jena, Germany) equipped with a 40x Plan-NeoFluar oil immersion objective (numerical aperture 1.3).

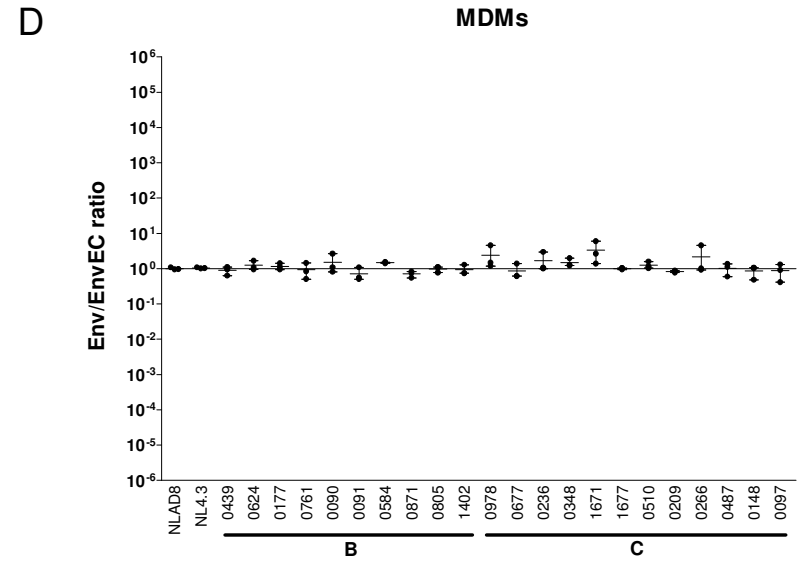
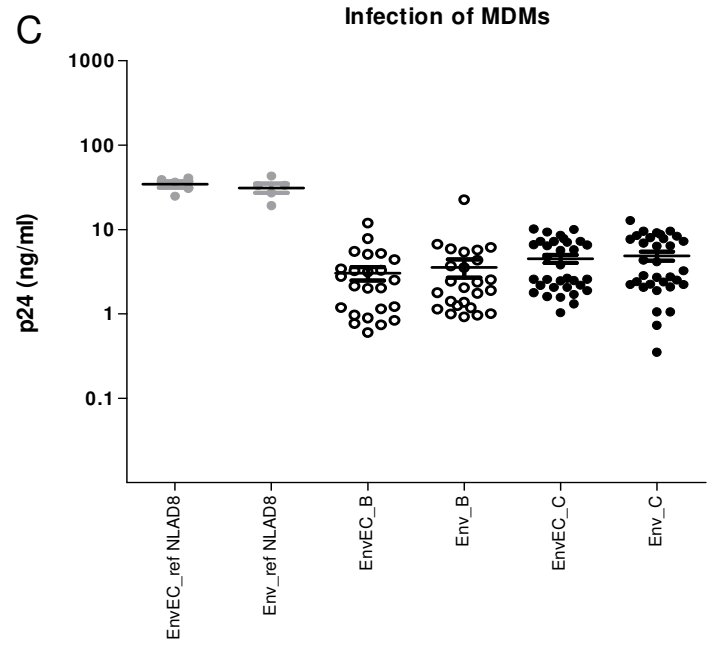
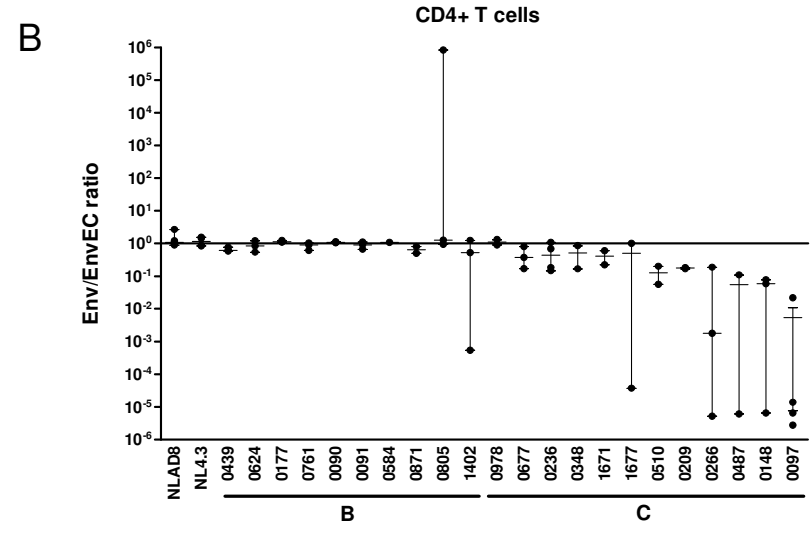
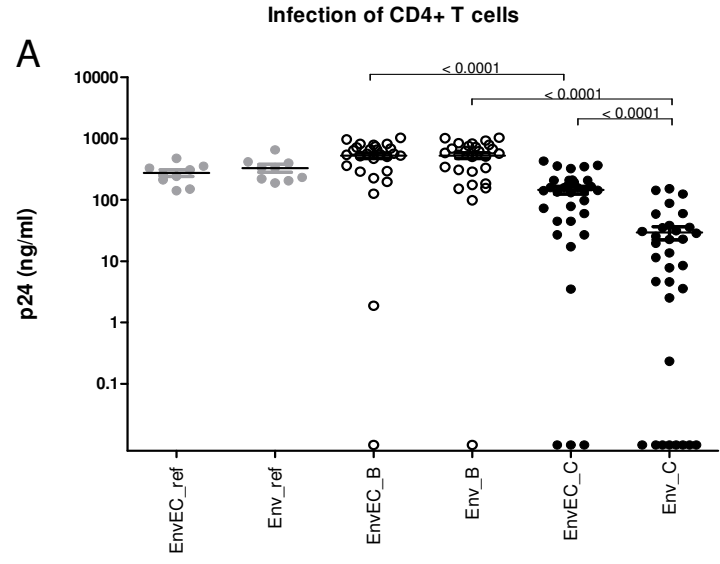
Figure 5. Env incorporation into virions. A. Virions produced from CD4+ T cells were purified by ultracentrifugation on a sucrose cushion and concentrated, then immunoblotted with mouse monoclonal antibody against p24 and goat polyclonal antibody against gp120. **B.** The gp120:p24 ratio in virions produced by CD4+ T cells was evaluated by ELISA.

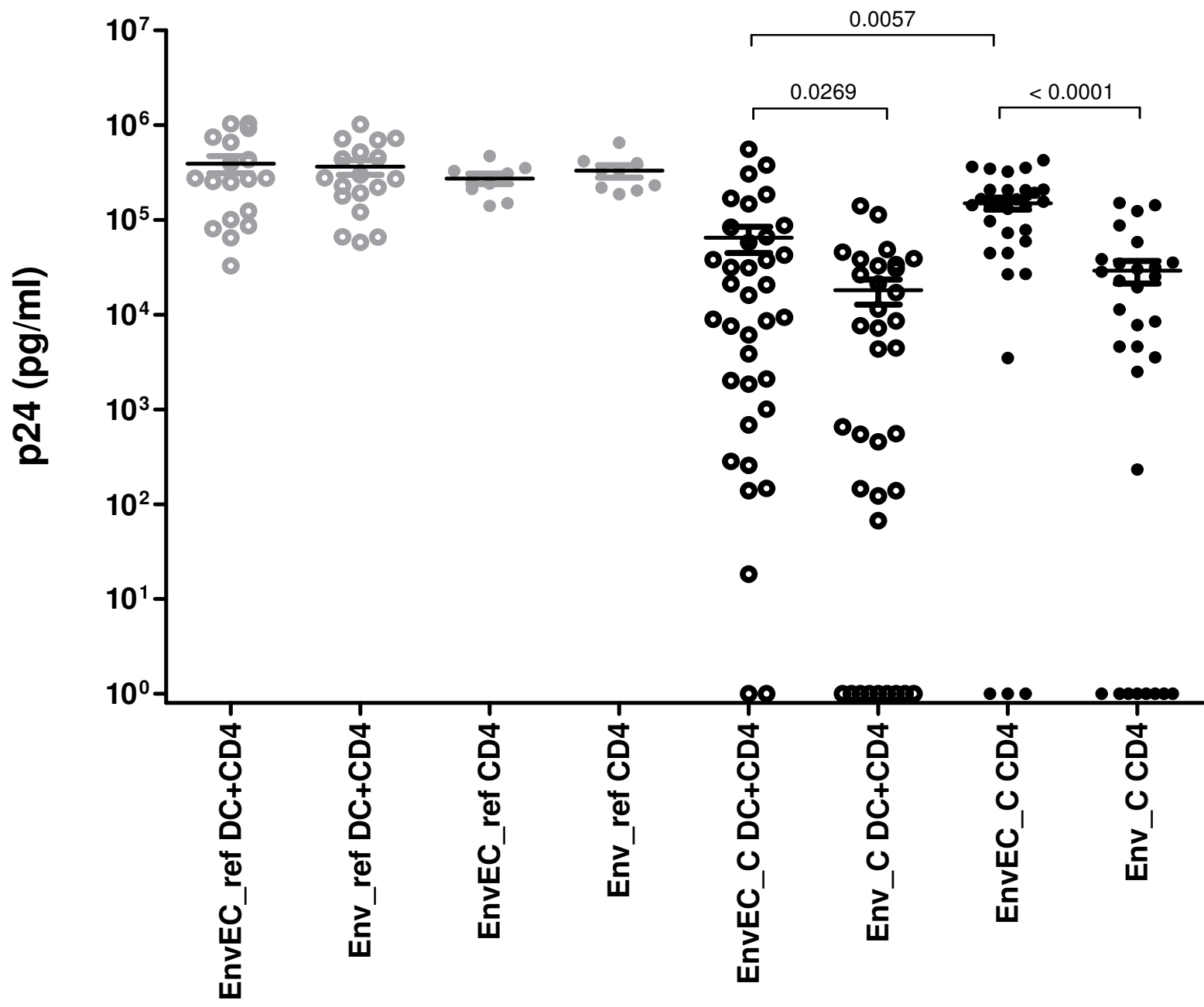
Fig 6. gp41CT sequence analysis. A sequence alignment of subtype C strain MA against the NL4.3 reference and consensus logos. Residues described to partake in the interaction of MA with Env and in Env incorporation into virions are L₈, S₉ [47, 79], L₁₂, L₃₀, V₃₄ [78, 80], K₃₂ [52], L₄₉ [81], the basic domain of MA (AA 17-21) [82], and basic residues 17-21 [40, 52, 53, 55, 70, 74, 83]. AA involved in p55Gag trafficking via adaptor proteins (AP-1, 2 and 3), include Y₁₃₂ and V₁₃₅ at the MA-CA junction [84-87]. **B. Sequence alignment of subtype B and subtype C gp41CT sequences against the NL4.3 reference.** The Y₇₁₂SPL, Y₈₀₂W₈₀₃, P_{T/R}RIR and tri-leucine LLL₇₇₆, di-leucine motifs

LL₈₀₀ and C-terminal LL₈₅₆ motifs are boxed. The locations are assigned based on the NL4.3 reference sequence. Sequence alignments were performed by CLC Main Workbench 6.8.2 Software (Aarhus, Denmark). **C. Consensus sequence logos of aligned gp41CT regions generated with WebLogo3.3.** (i) Consensus logo of subtype B (ii) Consensus logo of subtype C with mildly decreased or unaffected replication capacity, (iii) Consensus logo of subtype C gp41CT with impaired replication. The location of the LLP α -helices and sequences that were mutated are boxed (orange) and indicated by arrows. The locations are based on NL4.3. The Polymorphisms differing between subtypes B and C are boxed (green).

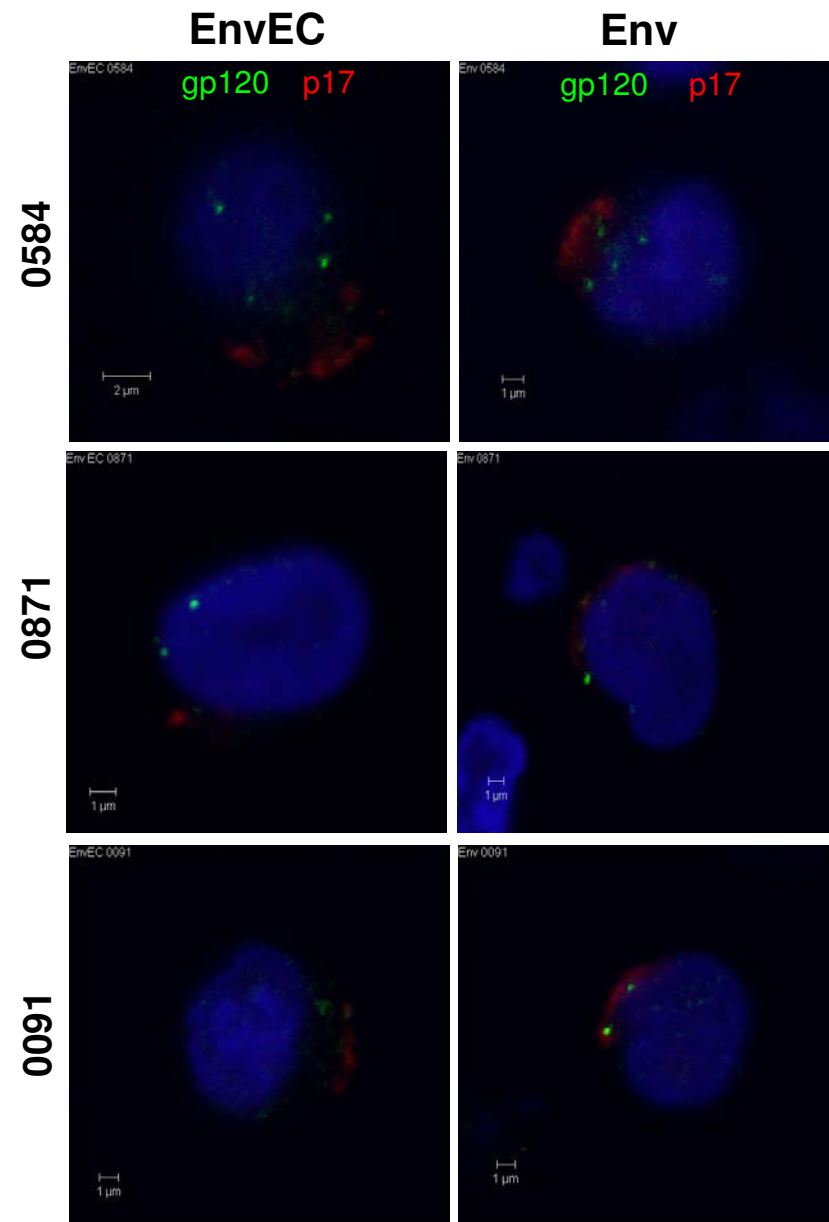
Figure 7. Replication capacity in CD4⁺ T cells and Env incorporation into virions of NLAD8^{mut}, 1671^{mut} and 0266^{mut} recombinant viruses. CD4⁺ T cells (10⁵ cells per well) were infected with the following NLAD8 mutant Env recombinant viruses: NLAD8Env^{FIL}, NLAD8Env^{LQ}, NLAD8Env^{FIL+LQ}, NLAD8Env^{+7AA}, NLAD8Env^{7AA+FIL}, NLAD8Env^{+7AA+LQ}, NLAD8Env^{+7AA+FIL+LQ} (**A**) or with the following subtype C Env recombinant viruses (**B and C**): 1671/0266Env^{LLL}, 1671/0266Env^{LL856}, 1671/0266Env^{LLL+LL856}, 1671/0266Env^{-7AA}, 1671/0266Env^{-7AA+LLL}, 1671/0266Env^{-7AA+LL856}, 1671/0266Env^{-7AA+LLL+LL856}. Viral replication (p24) was measured in the supernatant at days 3, 5, 7 and 11 post-infection, and infection at day 5 is reported here, as a percentage of wt infection. Infections were performed in triplicate wells. Individual experiments performed with CD4⁺ T cells from different donors are reported to account for inter-donor variability. **D and E.** NLAD8^{mut} (D) and 1671^{mut} (E) virions produced from CD4⁺ T cells were purified by ultracentrifugation on a sucrose cushion and concentrated, then immunoblotted with mouse monoclonal antibody against p24 and goat polyclonal antibody against gp120. Infection with the 1671^{LL856} mutant was undetectable in supernatants from CD4⁺ T cell cultures, and accordingly, p24 and gp120 could not be detected by WB. Infection with all 0266^{mut} recombinant viruses was undetectable.





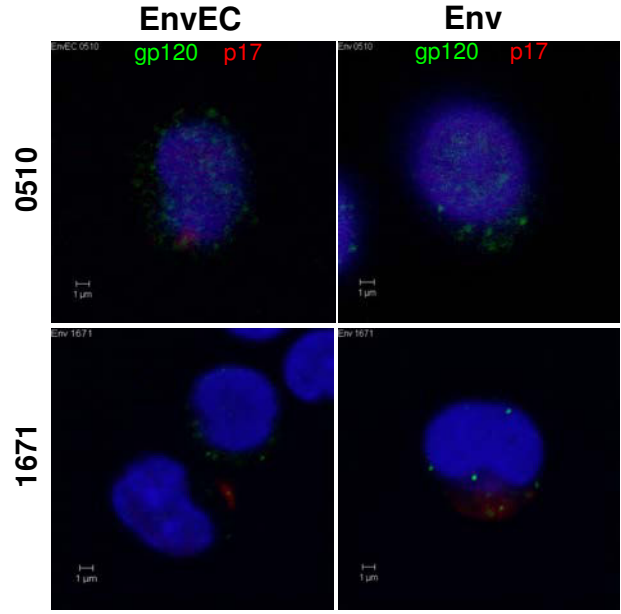


A Subtype B – CD4+ T cells



B Subtype C – CD4+ T cells

Intermediate Replication Capacity



Poor Replication Capacity

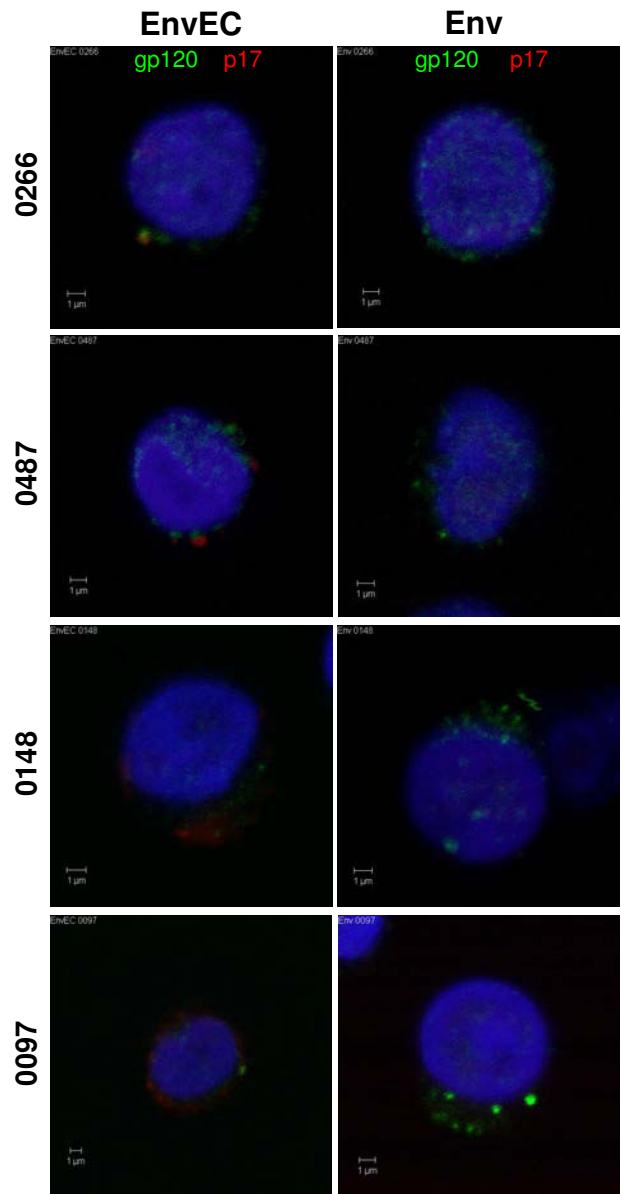


Figure 4 - continued

C Subtype B – MDMs

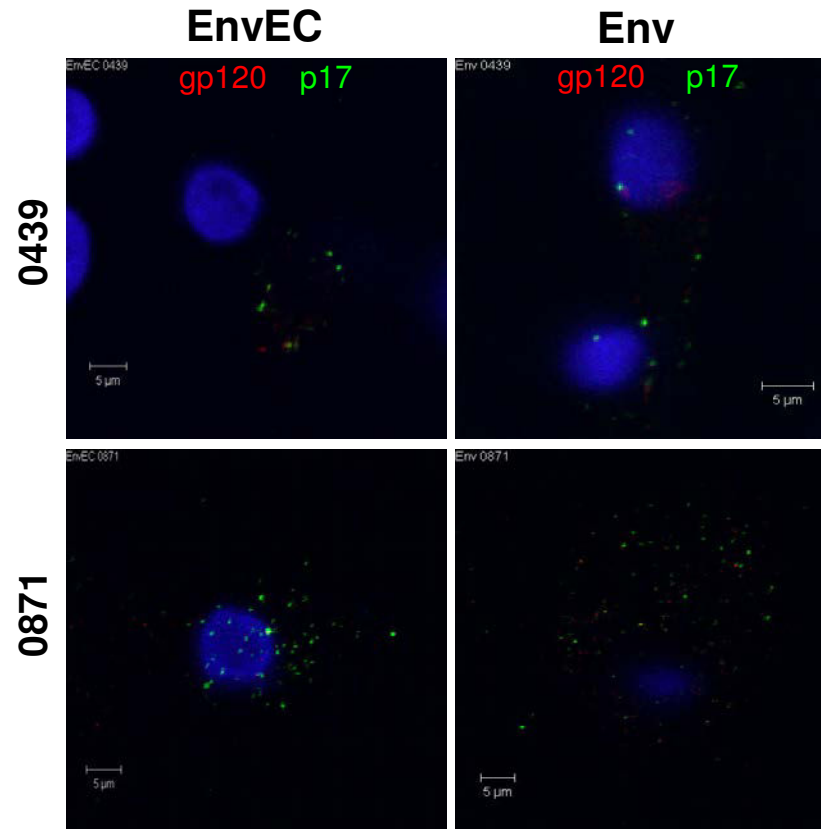
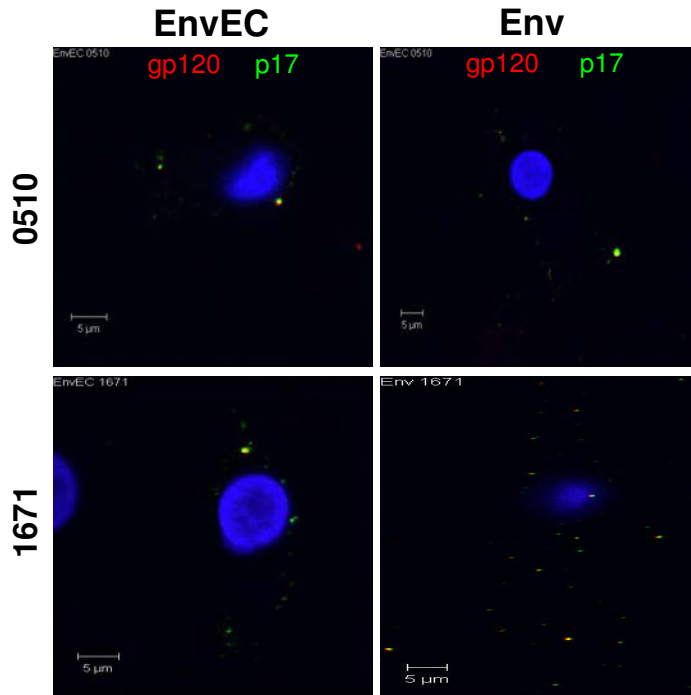


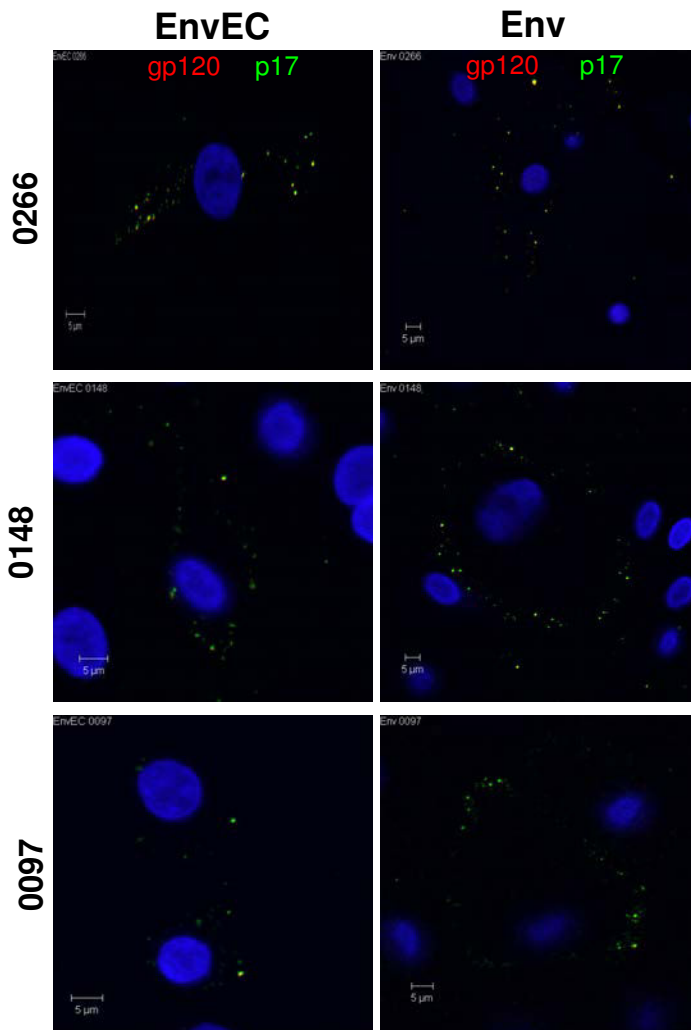
Figure 4 - continued

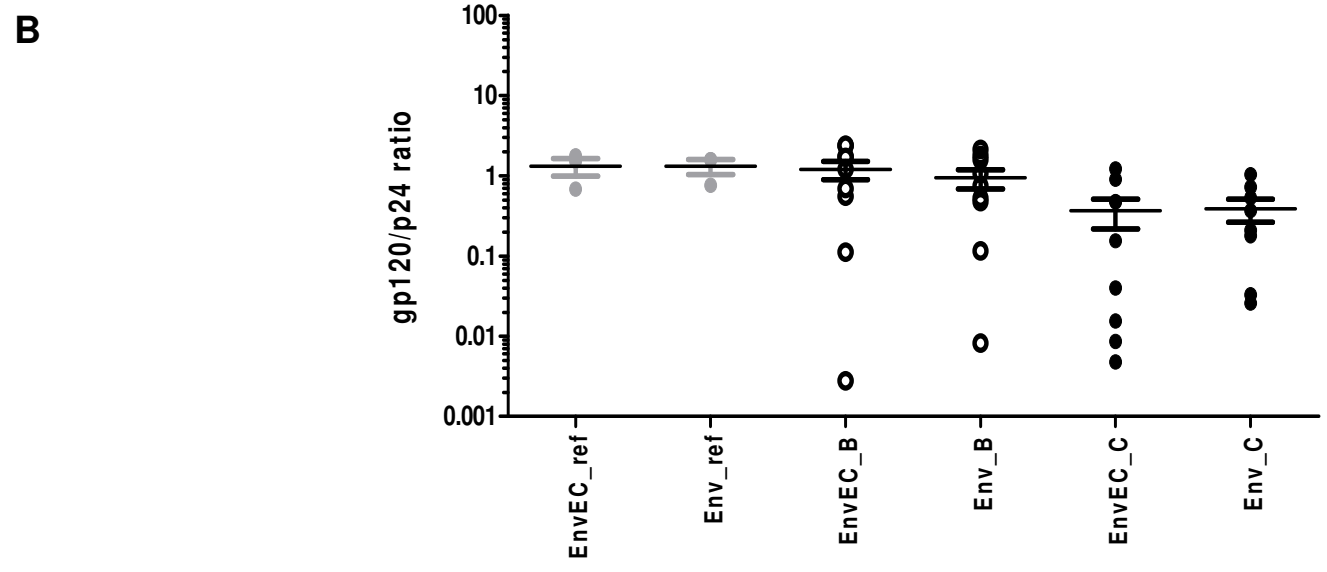
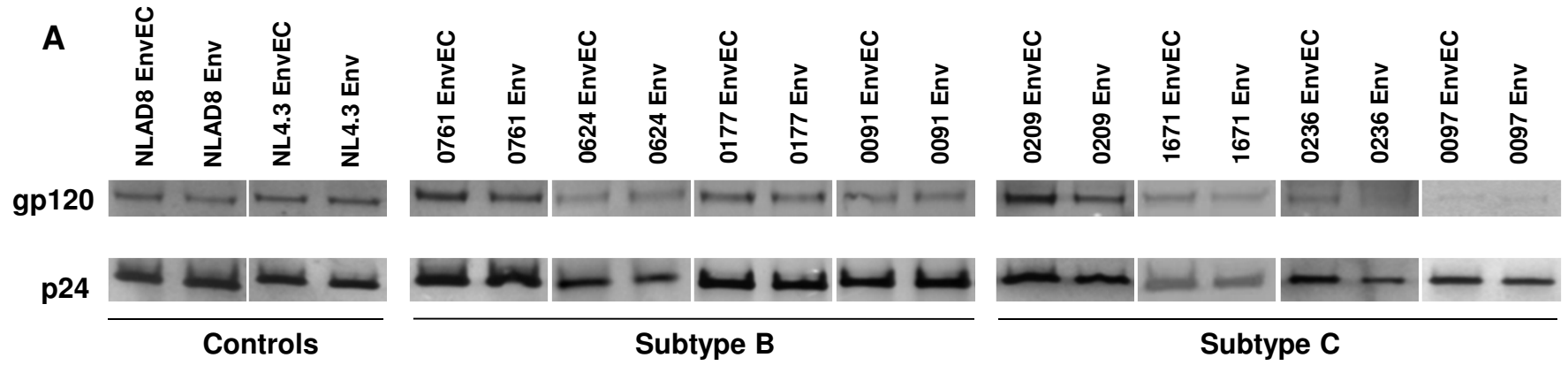
D Subtype C – MDMs

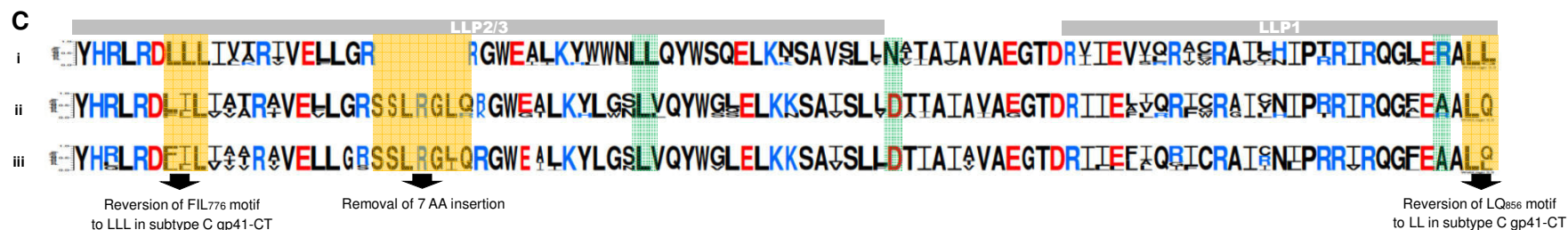
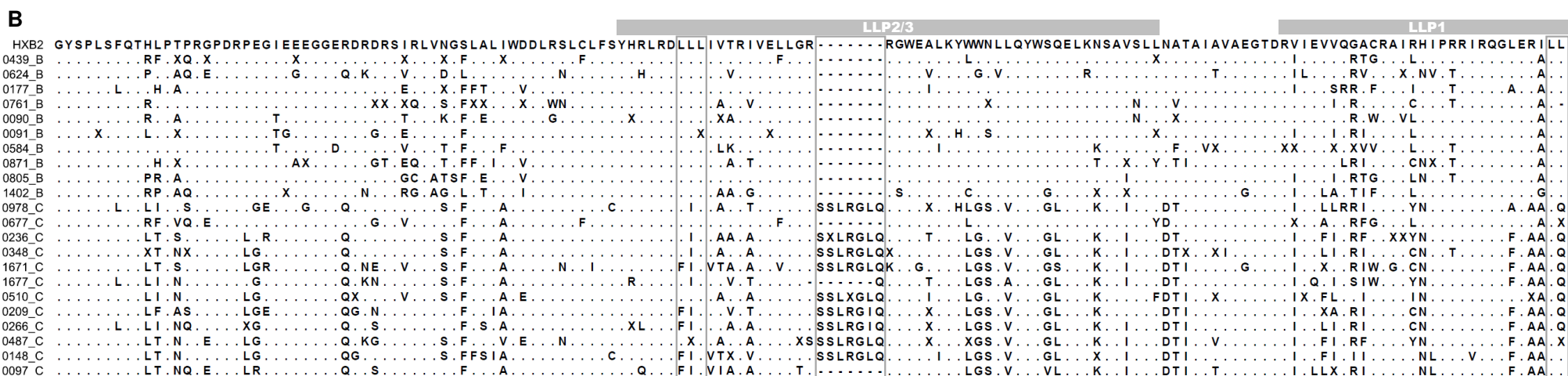
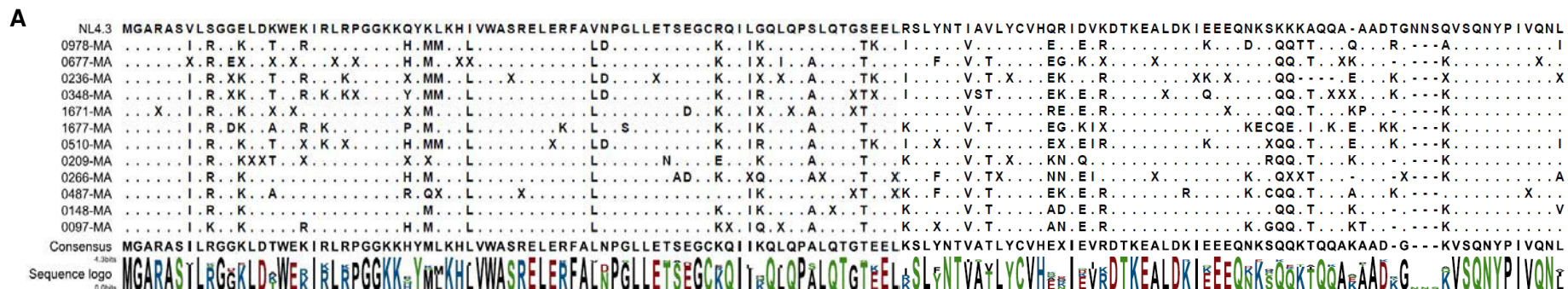
Intermediate Replication Capacity

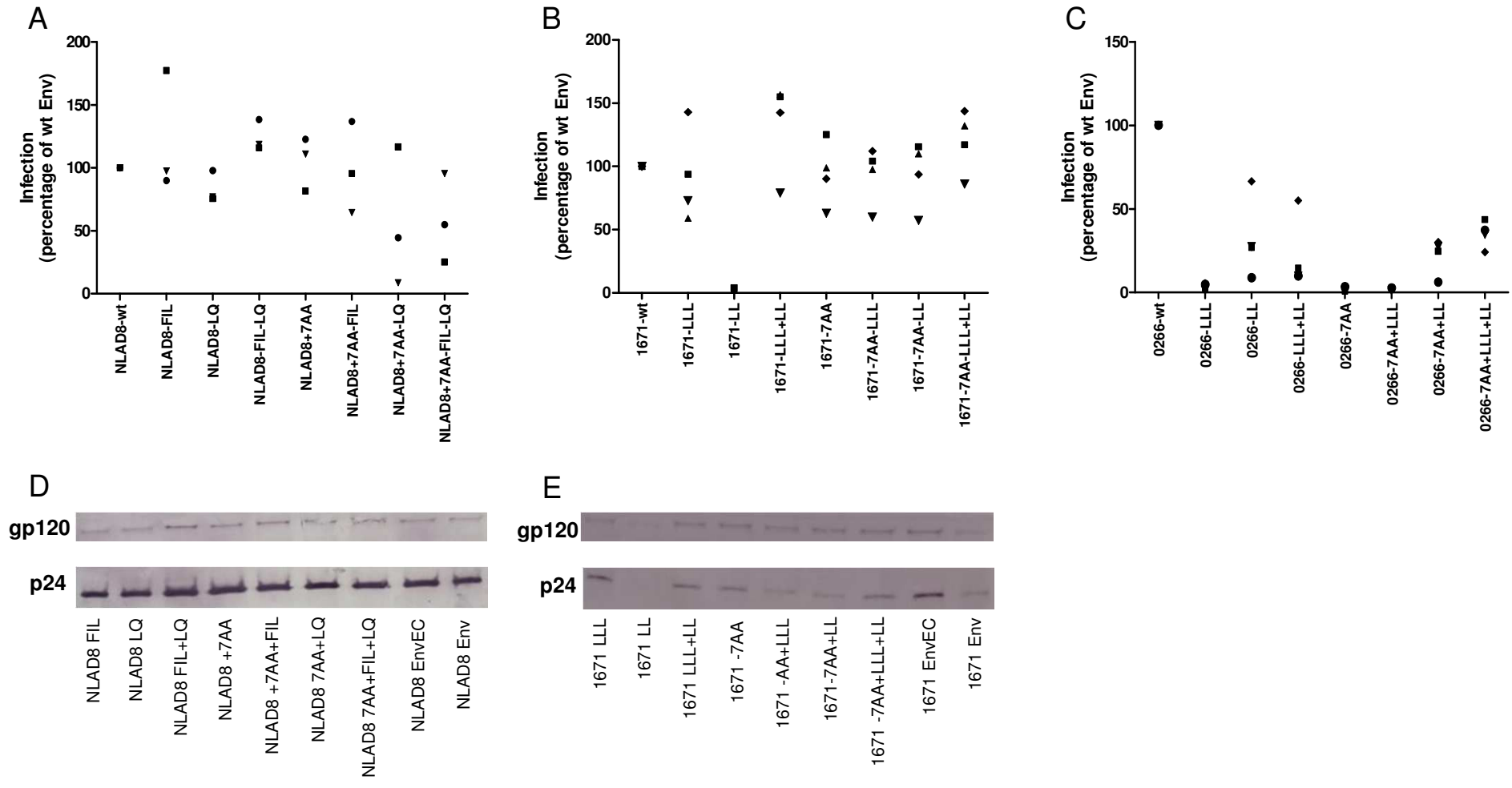


Poor Replication Capacity









DISCUSSION

Discussion

Beaucoup d'efforts ont été déployés pour décrypter les mécanismes employés par l'enveloppe virale du VIH pour permettre l'infection de cellules cibles. Néanmoins, la queue intracytoplasmique de l'enveloppe a été peu étudiée, tant au niveau de ses interactions et propriétés que des polymorphismes inhérents aux divers sous-types de VIH. La majorité des connaissances actuelles sur gp41CT proviennent d'expériences réalisées à partir du clone de référence NL4.3 ayant subi des délétions totales ou partielles de la gp41CT (363, 368, 389, 390) ou d'expériences de mutagenèse dirigée comprenant l'élimination ou la substitution de motifs ciblés intervenant dans le trafic intracellulaire (379-381, 423) et/ou la mutation de motifs dileucine (401, 403, 414) ou d'acides aminés chargés (367, 391). Seules deux équipes se sont penchées sur les polymorphismes de la gp41CT, l'une en se concentrant sur des mutations spontanées dans les motifs impliqués dans le trafic intracellulaire (424) et l'autre en étudiant la co-évolution de Env et de MA (387). Enfin, les polymorphismes rencontrés au niveau de sous-types viraux ont été décrits, cependant ces études sont restreintes à l'observation de propriétés biochimiques et structurales de peptides issus de gp41CT correspondant aux α -hélices LLP-2/3 et LLP-1 (425, 426).

Ce travail se penche sur l'impact fonctionnel de polymorphismes dans le domaine gp41CT sur l'infectivité. Nous avons adapté une stratégie permettant d'éviter le recours à des délétions d'une part, et permettant de conserver l'interaction autologue entre la gp120 et la gp41 au niveau des enveloppes primaires d'autre part. De plus, cette approche permet de préserver la variabilité des quasi-espèces virales en évitant l'utilisation de clones. Nous nous sommes concentrés sur les sous-types C et B. En effet, presque la moitié des infections mondiales sont causées par des infections de virus de sous-type C (28) et le sous-type B est le plus investigué car il sévit en Amérique du Nord, en Europe et en Australie. Or des études ont décrit que le sous-type C a une plus faible capacité répliquative *in vitro* (427). Nos résultats concordent partiellement avec ces études, dans la mesure où les virus exprimant une enveloppe de sous-type B (recombinants Env ou EnvEC) présentent une capacité répliquative supérieure à celle des virus comportant une enveloppe de sous-type C

dans les LT CD4+, mais pas dans les macrophages. La comparaison des capacités répliquatives des recombinants Env et EnvEC autologues de sous-types B et C (sous-type B Env = sous-type B EnvEC > sous-type C EnvEC > sous-type C Env) indique que des polymorphismes dans l'ectodomaine de Env ainsi que dans la gp41CT sont responsables de la faible réplication des recombinants de sous-type C. Des études précédentes montrent que l'enveloppe est un déterminant important de la capacité répliquative (428, 429), sans toutefois distinguer la contribution relative des différentes régions de Env.

Ce défaut de réplication ne semble toutefois pas provenir d'un défaut d'incorporation de Env dans les virions produits. En effet, non seulement la réplication des paires de virus Env et EnvEC était similaire dans les macrophages aussi bien que lors de la production de virus par transfection, mais encore, lorsqu'elles ont pu être dosées, les proportions Env:Gag étaient comparables entre paires de virus. Malheureusement, la faible quantité de virions produits par les LT CD4+ a compliqué, voire rendu impossible, le dosage exact des quantités d'Env incorporées dans les virions recombinants, surtout pour les recombinants Env de sous-type C. Des expériences d'infection de cellules TZM-bl (HeLa-CD4-CCR5 exprimant les gènes rapporteurs *luciferase* et *β -galactosidase* sous le contrôle du promoteur LTR) sont en cours. Cette approche est indirecte et ne permet pas de quantifier formellement le nombre de molécules Env incorporées dans chaque virus, mais fournit une indication sur l'infectivité des virus produits, qui est proportionnelle à la quantité d'Env incorporée. L'analyse par microscopie confocale de la localisation intracellulaire de Env et de Gag dans des LT CD4+ infectés montre que gp120 est exprimée normalement et forme des agrégats similaires que ce soit lors de l'infection par des recombinants Env ou EnvEC de sous-type B ou C, suggérant l'absence d'altérations de transcription et de transport intracellulaire. Cette observation est en accord avec l'extrême conservation des motifs de trafic intracellulaires, notamment du motif Y₇₁₂SPL qui est impliqué dans l'internalisation de l'enveloppe virale (379, 380), du motif Y₈₀₂W₈₀₃ qui intervient dans le transport rétrograde de l'enveloppe depuis l'endosome tardif vers le réseau trans-golgien (385, 404, 424), ainsi que des résidus cystéine de LLP-1 et des résidus aromatique qui interviendraient dans l'association avec les radeaux lipidiques (430, 431).

En revanche, la faible réplication virale dans les LT CD4+ était associée à une diminution ou à l'absence d'agrégats Pr55^{Gag} dans les LT CD4+ infectés par les virus

recombinants Env de sous-type C. A l'inverse, des agrégats Pr55^{Gag} étaient détectés à la membrane des LT CD4+ infectés par les virus appariés exprimant l'ectodomaine de sous-type C fusionné au domaine gp41CT de NL4.3 ou par des recombinants avec des Env de sous-type B. Ainsi, seules les infections par des recombinants gp41CT de sous-type C induisent une altération de l'expression ou de la distribution de Gag dans les LT CD4+. Certaines Env de sous-type C avaient une capacité répliquative plus de 10 fois plus faible que l'homologue EnvEC, et pour d'autres, la capacité répliquative est 4-6 fois plus faible. Or dans les LT CD4+ infectés par les Env à très faible capacité répliquative, les agrégats Pr55^{Gag} étaient invisibles, alors que pour les Env à capacité répliquative légèrement moins faible, quelques agrégats étaient visibles. Des études menées à l'aide de mutants délétés du domaine gp41CT montrent l'absence de polarisation de Pr55^{Gag} (56, 388, 432). Nos résultats suggèrent que des polymorphismes dans gp41CT peuvent moduler la capacité de Pr55^{Gag} à s'agréger et à polariser au site d'assemblage alors que des délétions de gp41CT abrogent complètement l'agrégation de Pr55^{Gag}.

Le domaine gp41CT interagit avec la protéine virale Pr55^{Gag} au niveau de MA lors de l'assemblage viral (55, 57, 64, 65, 433, 434) et cette association est indispensable à la génération de particules virales infectieuses. Or dans notre système, le domaine gp41CT de sous-type C se trouve dans un contexte génétique de sous-type B, ce qui pourrait a priori se traduire par une mauvaise affinité entre le domaine gp41CT de sous-type C et la protéine Gag de sous-type B. Or, des études (55, 57, 64, 65, 433, 434) ont été effectuées par transfection dans des cellules HEK, dans des systèmes comparables à celui utilisé ici pour produire les particules virales. Dans notre système, il n'y a pas de différence de production virale lors de la transfection de cellules HEK. De plus, une incompatibilité entre MA de sous-type B et domaine gp41CT de sous-type C aurait dû affecter la répliquaison dans les macrophages aussi bien que dans les LT CD4+, alors que ce phénotype n'est observé que dans les LT CD4+, mais ni dans les cellules productrices HEK transfectées, ni dans les macrophages. Deuxièmement, la quantification d'Env incorporée dans les virions indique que le nombre d'Env par virion est similaire entre les recombinants Env et les EnvEC de sous-type B et entre les virus Env et EnvEC de sous-type C. Bien que cette quantification n'ait pas pu être réalisée pour tous les virus recombinants à cause des productions virales trop faibles, les résultats sont congruents entre paires de virus. Troisièmement, le séquençage des MA correspondantes des souches de sous-type C n'a mis en évidence

aucune mutation aux positions connues pour interagir avec Env et qui permettent l'incorporation dans les particules virales (55, 59, 63, 69, 368, 435, 436). Le clonage de deux MA de sous-type C (d'une souche avec capacité répliquative intermédiaire et d'une souche avec faible capacité répliquative) est en cours, afin de vérifier formellement que l'utilisation d'un backbone de sous-type B avec des gp41CT de sous-type C n'introduit pas un biais intrinsèque au système. L'ensemble de ces éléments indique que les phénotypes d'infection différents ne proviendraient donc pas d'un manque d'enveloppe à la surface des virions ayant la gp41CT sous-type C, mais au faible nombre de particules virales produites par chaque LT CD4+ infecté.

Dans les MDMs, où la réplication virale est comparable quelle que soit l'enveloppe et quelle que soit l'origine du domaine gp41CT, la microscopie confocale permet de visualiser des agrégats Env aussi bien que des agrégats Gag, ainsi que plusieurs sites de colocalisation de Gag et Env. Aucune différence de distribution ou de niveau d'expression n'a pu être visualisée lors de l'infection par des virus exprimant des enveloppes de sous-type B ou C, ce qui indique que les polymorphismes du domaine gp41CT n'ont pas le même impact dans ces cellules. Ces résultats sont à première vue surprenants puisque lors d'expériences basées sur des délétions de gp41CT, les phénotypes résultants étaient similaires dans les MDMs et les LT CD4+ (368). Néanmoins, dans ces études, la délétion de gp41CT inhibe la réplication virale dans les cellules non permissives telles que les LT CD4+ et les MDMs en diminuant l'incorporation de Env dans les virions produits. Ce mécanisme diffère de celui que nous avons observé dans cette étude.

L'infection des MDMs par les virus recombinants résulte en des taux de réplication virale nettement plus faibles que ceux atteints dans les LT CD4+ (l'infection est inférieure de 2 à 3 ordres de grandeur). Ceci s'explique généralement par le fait que les MDMs possèdent un réservoir de dNTPs restreint, ainsi que des facteurs de restriction qui répriment l'infection, tels que SAMHD1 (256-260, 294). Alimenter le réservoir de dNTPs afin d'être dans des conditions plus proches des LT CD4+ (437) a effectivement augmenté la production virale dans les MDMs, mais n'a pas changé le phénotype gp41CT-induit, suggérant un mécanisme indépendant du pool de dNTPs.

Il a été montré que dans les MDMs, ou dans les cocultures MDM-LT CD4+, l'agrégation de Gag au niveau de la synapse virologique est indépendante de Env (438), alors que les LT CD4+ requièrent l'interaction Env-Gag pour la formation de synapses

virologiques (439, 440). Ces observations s'accordent avec l'homogénéité des capacités répliquatives mesurées dans les MDMs mais non dans les LT CD4+ et suggèrent que les différences de répllication virale liées à gp41CT observées dans les LT CD4+ pourraient impliquer un facteur cellulaire qui diffère entre les LT CD4+ et les MDMs. De plus amples investigations doivent être menées afin de déterminer s'il s'agit d'un facteur de restriction qui est inactivé par les gp41CT de sous-type B dans les LT CD4+, et qui serait absent des MDMs, ou à l'inverse d'un facteur cellulaire favorisant la répllication virale qui interagirait mieux avec le domaine gp41CT de sous-type B et qui serait absent dans les MDMs. Le fait qu'il n'y ait pas de différence entre les infections par le sous-type B ou C dans les MDMs et que la répllication soit bien plus faible dans ces derniers soutient plutôt la dernière hypothèse.

En regard des quatre modèles d'assemblage viral proposés (334) (6.3.4.1), il n'est pas exclu que plusieurs modèles co-existent, et dépendent du type cellulaire et/ou de son état (activation, différenciation...). Ainsi, nous proposons qu'un facteur cellulaire présent dans les LT CD4+ activés participe à l'agrégation de Pr55^{Gag} induite par le domaine gp41CT. Le domaine gp41CT de sous-type B interagirait efficacement avec ce facteur et induirait l'agrégation et la polarisation de Pr55^{Gag} à la membrane cellulaire, alors que cette interaction serait moins efficace avec le domaine gp41CT d'autres sous-types, et notamment de sous-type C. L'absence de ce facteur des MDMs expliquerait les capacités répliquatives faibles et similaires. Ceci laisse penser qu'il y a un assemblage basal qui assure un minimum d'interaction Gag/Env. Dans les macrophages, cellules présentatrices par excellence, le réseau de vésicules impliquées dans le trafic pourraient faciliter la rencontre Env/Gag. Dans les LT CD4+ activés, un facteur intermédiaire interagissant avec le domaine gp41CT et avec Gag jouerait un rôle intermédiaire ou un rôle de chaperonne favorisant l'assemblage de Gag et de Env. Ce modèle est également en accord avec la distribution de Pr55^{Gag} dans les MDMs et avec la présence de nombreux points de colocalisation Env/Gag observés dans les macrophages, alors que ceux-ci sont invisibles dans les LT CD4+. Une autre explication, non exclusive, pourrait être que les macrophages produisant peu de virus, ces signaux de colocalisation reflètent la concentration de composants viraux dans les invaginations reliées à la membrane des macrophages (297, 441, 442), ou de virions stockés dans les compartiments VCC près de la surface cellulaire (298, 443). Dans ce cas, l'ajout de dNTP exogènes pour augmenter les niveaux de répllication dans la MDMs devrait

permettre de visualiser des agrégats Pr55^{Gag} par microscopie confocale. Ces hypothèses doivent être vérifiées ultérieurement.

Plusieurs facteurs, tels que les radeaux lipidiques et la protéine TIP47 ont déjà été proposés comme jouant le rôle d'intermédiaire dans l'interaction Env-Gag (334, 385, 404). Néanmoins ces facteurs sont présents dans les macrophages. De plus, le rôle de TIP47 comme intermédiaire dans la liaison Env-Gag a été réfuté (386). Il est possible que des résultats contradictoires reflètent des différences de systèmes expérimentaux utilisés.

Il a été rapporté récemment que la transfection de souches virales résulte en de nombreux agrégats Gag et Env à la surface des lignées cellulaires transfectées (69). La proximité des protéines permettrait la formation de sites d'assemblage. L'étude estime à 50-90% le pourcentage de protéines qui forment les sites d'assemblage (69). Or la quantité et la proximité des deux protéines virales dans les cellules primaires infectées par les recombinants Env ou EnvEC est considérablement moindre. De plus, nous avons utilisé des concentrations non-saturantes de virus recombinants lors des infections afin d'éviter de masquer des effets par des niveaux très élevés de virus (370), contrairement à l'étude de Muranyi *et al.* dans laquelle le système est saturé. Ces différences pourraient expliquer les différences quantitatives que nous observons, ainsi que le faible nombre de colocalisations ou de sites d'assemblage entre Env et Gag dans les LT CD4+ même lors d'infections très productives par des Env de sous-type B. En effet, nos images sont plus proches de celles publiées par Groot *et al.* (439) dans des cellules primaires (MDMs et LT CD4+) infectés par la souche de référence BaL.

Bien que les particules recombinantes qui expriment un domaine gp41CT de sous-type C présentent une faible capacité répliquative dans les LT CD4+, il n'est pas exclu que les virus primaires de sous-type C ne possèdent dans leur génome des mutations compensatrices qui améliorent la réplication de ces virus. Ainsi, il a été récemment montré que la gp41CT intervient aussi dans l'activation du facteur de transcription NF- κ B (411). Or, les virus de sous-type C possèdent un site NF- κ B supplémentaire dans leur promoteur LTR (36), ce qui peut augmenter la transcription virale, bien que le motif Y₇₆₈XXL qui est impliqué dans l'interaction avec TAK-1 pour activer NF- κ B, soit conservé dans toutes les gp41CT incluses dans cette étude. Par ailleurs, les LT CD4+ utilisés dans cette étude étaient fortement activées (que ce soit par de la phytohémagglutinine ou par les MDDCs), alors que Postler *et al* décrivent que l'activation de la voie NF- κ B par le domaine gp41CT dans

des LT CD4+ suboptimalement activés (411). Enfin, si le défaut de réplication dans les LT CD4+ résultait d'un défaut d'activation, il est fort probable que l'expression de Env serait elle aussi diminuée. Néanmoins ces pistes doivent être explorées par des expériences de mesure d'induction de facteurs de transcription et d'induction de la transcription à partir du LTR avant de pouvoir être exclues.

Nous nous sommes également penchés sur les séquences des gp41CT de sous-type C et avons mis en évidence 3 motifs qui semblaient récurrents dans les recombinants gp41CT de sous-type C moins infectieux. Les motifs identifiés sont les suivants : (i) le motif LQ₈₅₆ situé à l'extrémité C-terminale de Env, qui est un motif dileucine dans le sous-type B, (ii) le motif FIL₇₇₆ situé dans le domaine LLP-2 et qui est un motif LLL₇₇₆ dans le sous-type B et enfin (iii) une insertion de sept acides aminées (7AA) qui est absente du sous-type B. Les deux motifs dileucine ont été investigués précédemment dans le sous-type B. Le motif dileucine LL₈₅₆ est impliqué dans le transit intracellulaire, et plus particulièrement dans l'internalisation de Env par l'intermédiaire de AP-2. Il agit conjointement au motif Y₇₁₂SPL (396, 401, 402). Le motif LLL₇₇₆ semble jouer un rôle dans la fusogénicité de Env (403). A notre connaissance, l'insertion de 7AA dans les gp41CT de sous-type C n'a encore jamais fait l'objet de recherches poussées, or cette insertion ajoute deux tours à l'hélice α au niveau des régions LLP-2/LLP-3, allongeant ces structures. De plus, la séquence SSLRGLQ des 7AA introduit un motif GxxxG que l'on retrouve dans des interactions d'hélices α transmembranaires (444). L'impact des trois motifs a été évalué par mutagenèse en les remplaçant par les motifs consensus de sous-type B au sein de deux Env sous-type C : une Env possédant une réplication intermédiaire (Env1671) et l'autre présentant une réplication extrêmement réduite (Env0266). L'effet des réversions est dépendant du contexte génétique de Env. Ainsi la réversion du polymorphisme LQ₈₅₆ abroge complètement la réplication virale de virus recombinants 1671, mais pas du recombinant 0266, pour lequel les polymorphismes dans LLP-2 (FIL₇₇₆ et l'insertion de 7AA) semblent cruciaux. Toutefois, il faut remarquer que la réversion des polymorphismes dans LLP-2 de l'Env1671 restaure la réplication virale du mutant LQ₈₅₆ \rightarrow LL₈₅₆ ; de même, l'ajout de LQ₈₅₆ \rightarrow LL₈₅₆ restaure la réplication virale des mutants Env0266 comportant une ou deux réversions dans LLP-2. Ces observations indiquent que les motifs dans LLP-2 et le motif LQ₈₅₆ dans LLP-1 sont liés. A l'inverse, l'introduction des motifs FIL₇₇₆, 7AA, et LQ₈₅₆ dans

NLAD8 ne compromet pas particulièrement la réplication des mutants dans les LT CD4+, sauf pour le double mutant NLAD8^{+7AA-LQ856} et le triple mutant NLAD8^{+7AA-FIL-LQ856}, suggérant également un effet cumulatif et lié de ces polymorphismes. Néanmoins, l'effet moindre observé sur NLAD8 indique que d'autres structures ou polymorphismes non encore identifiés peuvent être impliqués.

Une étude basée sur l'insertion progressive de mutations dans les motifs dileucine dans le domaine gp41CT conclut à un effet cumulatif des motifs qui est probablement nécessaire pour préserver la structure et les propriétés fonctionnelles de gp41CT (403). Etant donné que le motif FIL₇₇₆ et l'insertion 7AA sont situés dans LLP-2 et sont très proches, alors que le motif LQ₈₅₆ se trouve en C-terminal de LLP-1, ces résultats pointent vers une contrainte évolutive, voire une interaction entre les domaines au niveau de ces motifs et cette interaction serait déterminante pour la réplication virale. Dans le contexte de nos résultats précédents, une interprétation possible est que ces variations de séquence et de structure affectent l'interaction avec un facteur cellulaire, ce qui se répercute sur la capacité répliquative et la distribution intracellulaire de Pr55^{Gag}.

L'insertion de 7AA introduit aussi un résidu arginine supplémentaire dans l'hélice, or les résidus arginine sont hautement conservés dans la gp41CT (425), il a été proposé que ces résidus facilitent l'interaction avec la membrane phospholipidique (425) et sont impliqués dans la multimérisation de l'enveloppe (391). Une équipe a montré que la multimérisation de Env au niveau du virion assure l'infectivité virale (68, 69). Dès lors, l'ajout de deux tours d'hélice et d'un résidu Arg pourraient favoriser la capacité infectieuse des virus en augmentant la multimérisation de Env. Une autre étude a montré que le résidu phénylalanine peut favoriser des interactions d'hélices dans les régions transmembranaires grâce au motif GxxxG (445, 446). Un tel mécanisme pourrait être à l'origine de l'inefficacité des mutants simples et double-mutants LLL₇₇₆ et 7AA. Dans ce scénario, la 3^e mutation modifiant le motif dileucine restaurerait l'infection soit en restaurant la structure et fonctionnalité du domaine gp41CT par un effet cumulatif, soit par un mécanisme de compensation : en modifiant le motif dileucine, le trafic intracellulaire de Env pourrait être diminué et plus d'Env pourrait être exprimée à la membrane cellulaire. Si c'est le cas, toutefois, ceci ne se traduit pas par une augmentation de la quantité de Env incorporée dans le virion, et devrait donc impacter la fusion cellule-cellule plutôt que l'infection par des virions libres. Nous n'avons pas testé la capacité

fusiogène des Env dans ce travail à l'aide de tests de fusion parce que ces tests sont généralement basés sur l'utilisation de lignées indicatrices permissives à la délétion de gp41CT (HeLa), qui ne sont donc pas représentatives de l'infection dans les LT CD4+ primaires. L'utilisation de lignées T présentait le désavantage de ne pas exprimer CCR5, et ne pouvaient donc pas être utilisées vu que la majorité des Env incluses dans cette étude sont R5.

Le domaine gp41CT influence la conformation de l'ectodomaine de gp41 (389, 436) ainsi que de gp120 (447). Ainsi, nous nous sommes demandés si les Env de sous-type C possédaient un avantage en termes de transmission par rapport aux Env de sous-type B, et le rôle potentiel du domaine gp41CT le cas échéant. Ceci pourrait également expliquer que le sous-type C soit tellement répandu dans le monde. Néanmoins, la présentation de virus par des cellules dendritiques (MDDCs) autologues ne modifie pas le profil de capacité répliquative (EnvB = EnvEC B > EnvEC C > Env C), excluant un avantage en termes de transmission. Nous avons également constaté aussi que la transmission des recombinants EnvEC par les MDDCs était moins prononcée par rapport à l'infection par des virus recombinants EnvEC libres. Ceci peut simplement être inhérent au système, en effet, les variations de réplication semblent plus importantes dans les expériences de transmission. Il se peut aussi qu'il y ait un changement de conformation au niveau de la gp120 induit par l'interaction gp120-gp41CT (389, 436, 447) qui modifierait l'expression des glycosylations et empêcherait les récepteurs aux lectines C présents sur les MDDCs de capturer le virus et donc aussi la transmission (303).

Il est important de remarquer que l'origine des cellules primaires utilisées dans cette étude est caucasienne majoritairement. Or les infections par des virus de sous-type C sont prépondérantes dans la population africaine, on ne peut donc pas exclure que les différences que l'on observe lors de l'infection par des Env de sous-type C proviennent d'une incompatibilité entre la cellule hôte et le virus et non pas le virus même. Afin d'écartier cette hypothèse et de travailler dans un système le plus représentatif possible des interactions virus-hôte, il serait intéressant de comparer la capacité répliquative de ces paires de virus B et C dans des cellules d'origine caucasienne et africaine.

CONCLUSIONS

Conclusions

Ce travail a établi pour la première fois que des polymorphismes dans la gp41CT entravent la réplication virale dans les LT CD4+, mais pas dans les MDMs. Cette altération ne semble pas provenir de perturbations au niveau de l'expression, du trafic intracellulaire ou de l'incorporation de l'enveloppe dans les virions. La perte d'infectivité semble être associée à une altération de la distribution de la protéine virale Gag dans les LT CD4+ infectées par la gp41CT de sous-type C, qui ne s'agrège pas convenablement et n'est pas polarisée à la membrane. Etant donné que ce phénomène n'est pas observé dans les MDMs infectés ou dans les cellules productrices HEK transfectées, et que la réplication est bien plus faible dans ces derniers, un troisième intervenant, un facteur cellulaire présent dans les LT CD4+ mais pas dans les MDMS, pourrait être impliqué. Ainsi, un niveau minimal d'interaction Env/Gag serait assuré, permettant une réplication virale à bas bruit, mais cette interaction serait favorisée dans les LT CD4+ par un intermédiaire cellulaire, permettant les forts niveaux de réplication viral typiques des LT CD4+ activés. Des polymorphismes situés dans les LLP-2/3 et LLP-1 interviennent aussi dans la capacité répliquative, et le fait qu'ils sont liés suggère que le maintien de la structure de gp41CT est primordial pour la réplication virale dans les LT CD4+.

PERSPECTIVES

Perspectives

Afin d'avoir une meilleure vue d'ensemble de la localisation intracellulaire des protéines virales Gag et Env, il sera intéressant de superposer tous les plans d'une cellule infectée. Cette superposition permettra de constater et de quantifier plus finement des différences d'agrégation de Gag au niveau des Env de sous-type C.

Par ailleurs, bien que le séquençage des matrices des isolats de sous-type C inclus dans cette étude indique que les résidus impliqués dans l'interaction MA/Env sont conservés, une co-évolution entre gp41CT et MA a été décrite (387). Il est donc envisageable que les matrices autologues correspondant aux Env de sous-type C aient évolué différemment de MA de NL4-3 (ou des MA de sous-type B) et possèdent un potentiel d'agrégation supérieur dans le LT CD4+. Pour vérifier ce point, de nouvelles constructions sont en cours, dans lesquelles les matrices des virus 1671 et 0266 sont clonées, permettant de générer des virus recombinants possédant la MA autologue correspondant à l'Env. Des LT CD4+ seront infectés, et la capacité répliquative ainsi que la localisation intracellulaire de Env et de Pr55^{Gag} seront observés. Si aucune différence n'est observée entre les MA NL4.3 et les MA autologues, cela constituera un élément supplémentaire en faveur de la présence d'un facteur cellulaire spécifique interagissant avec le domaine gp41CT de Env dans les LT CD4+ activés. En parallèle, des expériences de co-immunoprécipitation de gp41CT et de Pr55^{Gag} dans des lysats de MDMs et de LT CD4+ pourraient corroborer ces points.

L'identification d'un tel facteur pourrait permettre de mieux appréhender comment contrôler la réplication virale et dévoilerait de nouvelles cibles thérapeutiques. Etant donné le faible niveau d'infection des LT CD4+ par des recombinants Env de sous-type C, des analyses de transcriptomique ne permettent pas de mettre en évidence des différences entre l'infection par du sous-type B et du sous-type C. Ainsi, une approche ciblée inhibant les diverses protéines cellulaires déjà connues comme interagissant avec la gp41CT et leur inhibition par des siRNA pourrait apporter des éléments de réponse. Une approche complémentaire basée sur des doubles co-immunoprécipitations gp41CT/facteur et MA/facteur devraient permettre d'isoler des candidats potentiels qui pourront être caractérisés par spectroscopie de masse.

Etant donné que le domaine gp41CT intervient dans l'activation de la transcription, il se pourrait que les différences observées dans LT CD4 résultent d'une diminution de la transcription, il conviendrait donc d'analyser l'état d'activation dans les cellules infectées (en comparaison du niveau d'expression à partir du LTR viral, ainsi que la quantification de NF- κ B) et de déterminer le niveau d'expression de Pr55^{Gag} dans les cellules infectées (analyser le précurseur de Gag ainsi que les protéines clivées par Western Blot dans les lysats de cellules infectées par des virus exprimant des Env de sous-type B et C).

Enfin, il serait également intéressant de compléter l'étude *in vitro* de l'impact des réversions des polymorphismes dans le domaine gp41CT de sous-type C par des modélisations de structures de gp41CT de sous-type B et sous-type C afin de vérifier ainsi l'impact des mutations sur la structure de gp41CT. Une telle approche est complémentaire des analyses fonctionnelles *in vitro*, et pourrait révéler la présence de motifs ou de domaines plus prônes à une interaction protéine-protéine, ainsi que révéler comment les polymorphismes dans LLP-2 et dans LLP-1 sont liés.

BIBLIOGRAPHIE

Bibliographie

1. **Gottlieb M, Schroff R, Schanker H, Weisman J, Fan P, Wolf R, Saxon A.** 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**:1425 - 1431.
2. **Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868-871.
3. **Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, et al.** 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500-503.
4. **Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-842.
5. **Popovic M, Sarngadharan MG, Read E, Gallo RC.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**:497-500.
6. **Sarngadharan MG, diMarzo Veronese F, Lee S, Gallo RC.** 1985. Immunological properties of HTLV-III antigens recognized by sera of patients with AIDS and AIDS-related complex and of asymptomatic carriers of HTLV-III infection. *Cancer research* **45**:4574s-4577s.
7. **UNAIDS.** 2012. 2012 UNAIDS Report on the Global AIDS Epidemic. <http://www.unaids.org/en/resources/publications/2012/name,76121,en.asp>.
8. **Rothenberg R, Woelfel M, Stoneburner R, Milberg J, Parker R, Truman B.** 1987. Survival with the acquired immunodeficiency syndrome. Experience with 5833 cases in New York City. *N Engl J Med* **317**:1297-1302.
9. **Clavel F, Guyader M, Guetard D, Salle M, Montagnier L, Alizon M.** 1986. Molecular cloning and polymorphism of the human immune deficiency virus type 2. *Nature* **324**:691-695.
10. **Guyader M, Emerman M, Sonigo P, Clavel F, Montagnier L, Alizon M.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. *Nature* **326**:662-669.
11. **Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, Hahn BH.** 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**:436-441.
12. **Peeters M, Honore C, Huet T, Bedjabaga L, Ossari S, Bussi P, Cooper RW, Delaporte E.** 1989. Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. *AIDS* **3**:625-630.
13. **Gao F, Yue L, White AT, Pappas PG, Barchue J, Hanson AP, Greene BM, Sharp PM, Shaw GM, Hahn BH.** 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. *Nature* **358**:495-499.
14. **Chen Z, Telfier P, Gettie A, Reed P, Zhang L, Ho DD, Marx PA.** 1996. Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. *J Virol* **70**:3617-3627.
15. **Sharp PM, Hahn BH.** 2011. Origins of HIV and the AIDS pandemic. *Cold Spring Harbor perspectives in medicine* **1**:a006841.
16. **Hooper E.** 2000. Search for the origin of HIV and AIDS. *Science* **289**:1140-1141.
17. **Simon F, Mauclele P, Roques P.** 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat. Med.* **4**:1032-1037.
18. **Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, et al.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**:117-122.
19. **Pathak VK, Temin HM.** 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. *Proc Natl Acad Sci U S A* **87**:6019-6023.
20. **Mansky LM, Temin HM.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* **69**:5087-5094.
21. **Ramirez BC, Simon-Lorriere E, Galetto R, Negroni M.** 2008. Implications of recombination for HIV diversity. *Virus Research* **134**:64-73.
22. **Srinivasan A, York D, Jannoun-Nasr R, Kalyanaraman S, Swan D, Benson J, Bohan C, Luciw PA, Schnoll S, Robinson RA, et al.** 1989. Generation of hybrid human immunodeficiency virus by homologous recombination. *Proc Natl Acad Sci U S A* **86**:6388-6392.
23. **Kanki PJ, Peeters M, Gueye-Ndiaye A.** 1997. Virology of HIV-1 and HIV-2: implications for Africa. *AIDS* **11 Suppl B**:S33-42.
24. **Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V.** 2001. Evolutionary and immunological implications of contemporary HIV-1 variation. *British medical bulletin* **58**:19-42.

25. **Hemelaar J, Gouws E, Ghys PD, Osmanov S.** 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* **20**:W13-23.
26. **Taylor BS, Hammer SM.** 2008. The challenge of HIV-1 subtype diversity. *N Engl J Med* **359**:1965-1966.
27. **Gao F, Robertson DL, Carruthers CD, Morrison SG, Jian B, Chen Y, Barre-Sinoussi F, Girard M, Srinivasan A, Abimiku AG, Shaw GM, Sharp PM, Hahn BH.** 1998. A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. *J Virol* **72**:5680-5698.
28. **Hemelaar J, Gouws E, Ghys PD, Osmanov S, Isolation W-UNfH, Characterisation.** 2011. Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* **25**:679-689.
29. **Butler IF, Pandrea I, Marx PA, Apetrei C.** 2007. HIV genetic diversity: biological and public health consequences. *Current HIV research* **5**:23-45.
30. **Roquebert B, Damond F, Brun-Vezinet F, Descamps D.** 2009. [HIV genetic diversity and its consequences]. *Pathologie-biologie* **57**:142-148.
31. **Arien KK, Vanham G, Arts EJ.** 2007. Is HIV-1 evolving to a less virulent form in humans? *Nature reviews. Microbiology* **5**:141-151.
32. **Frankel AD, Young JA.** 1998. HIV-1: fifteen proteins and an RNA. *Annual review of biochemistry* **67**:1-25.
33. **Cassan M, Delaunay N, Vaquero C, Rousset JP.** 1994. Translational frameshifting at the gag-pol junction of human immunodeficiency virus type 1 is not increased in infected T-lymphoid cells. *J Virol* **68**:1501-1508.
34. **Pettit SC, Moody MD, Wehbie RS, Kaplan AH, Nantermet PV, Klein CA, Swanstrom R.** 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. *J Virol* **68**:8017-8027.
35. **Ramirez de Arellano E, Soriano V, Alcamil J, Holguin A.** 2006. New findings on transcription regulation across different HIV-1 subtypes. *AIDS reviews* **8**:9-16.
36. **Jeeninga RE, Hoogenkamp M, Armand-Ugon M, de Baar M, Verhoef K, Berkhout B.** 2000. Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. *J Virol* **74**:3740-3751.
37. **van Opijnen T, Jeeninga RE, Boerlijst MC, Pollakis GP, Zetterberg V, Salminen M, Berkhout B.** 2004. Human immunodeficiency virus type 1 subtypes have a distinct long terminal repeat that determines the replication rate in a host-cell-specific manner. *J Virol* **78**:3675-3683.
38. **Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ.** 2000. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. *Nucleic acids research* **28**:663-668.
39. **Karn J.** 1999. Tackling Tat. *Journal of molecular biology* **293**:235-254.
40. **Marquet R, Isel C, Ehresmann C, Ehresmann B.** 1995. tRNAs as primer of reverse transcriptases. *Biochimie* **77**:113-124.
41. **Berkhout B, van Wamel JL.** 2000. The leader of the HIV-1 RNA genome forms a compactly folded tertiary structure. *Rna* **6**:282-295.
42. **Rausch JW, Le Grice SF.** 2004. 'Binding, bending and bonding': polypurine tract-primed initiation of plus-strand DNA synthesis in human immunodeficiency virus. *The international journal of biochemistry & cell biology* **36**:1752-1766.
43. **Pollard VW, Malim MH.** 1998. The HIV-1 Rev protein. *Annual review of microbiology* **52**:491-532.
44. **Gentile M, Adrian T, Scheidler A, Ewald M, Dianzani F, Pauli G, Gelderblom HR.** 1994. Determination of the size of HIV using adenovirus type 2 as an internal length marker. *Journal of virological methods* **48**:43-52.
45. **Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, Wyatt RT.** 2008. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. *Nature reviews. Microbiology* **6**:143-155.
46. **Tremblay MJ, Fortin JF, Cantin R.** 1998. The acquisition of host-encoded proteins by nascent HIV-1. *Immunology today* **19**:346-351.
47. **Lee PP, Linial ML.** 1994. Efficient particle formation can occur if the matrix domain of human immunodeficiency virus type 1 Gag is substituted by a myristylation signal. *J Virol* **68**:6644-6654.
48. **Zhou W, Parent LJ, Wills JW, Resh MD.** 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J Virol* **68**:2556-2569.
49. **Zhou W, Resh MD.** 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J Virol* **70**:8540-8548.
50. **Ono A, Orenstein JM, Freed EO.** 2000. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J Virol* **74**:2855-2866.
51. **Spearman P, Horton R, Ratner L, Kuli-Zade I.** 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. *J Virol* **71**:6582-6592.
52. **Scarlata S, Carter C.** 2003. Role of HIV-1 Gag domains in viral assembly. *Biochimica et biophysica acta* **1614**:62-72.
53. **Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO.** 2004. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. *Proc Natl Acad Sci U S A* **101**:14889-14894.
54. **Chukkapalli V, Hogue IB, Boyko V, Hu WS, Ono A.** 2008. Interaction between the human immunodeficiency virus type 1 Gag matrix domain and phosphatidylinositol-(4,5)-bisphosphate is essential for efficient gag membrane binding. *J Virol* **82**:2405-2417.

55. **Lee YM, Tang XB, Cimasky LM, Hildreth JE, Yu XF.** 1997. Mutations in the matrix protein of human immunodeficiency virus type 1 inhibit surface expression and virion incorporation of viral envelope glycoproteins in CD4+ T lymphocytes. *J Virol* **71**:1443-1452.
56. **Owens RJ, Dubay JW, Hunter E, Compans RW.** 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. *Proc Natl Acad Sci U S A* **88**:3987-3991.
57. **Murakami T, Freed EO.** 2000. Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. *J Virol* **74**:3548-3554.
58. **Hourieux C, Brand D, Sizaret PY, Lemiale F, Lebigot S, Barin F, Roingeard P.** 2000. Identification of the glycoprotein 41(TM) cytoplasmic tail domains of human immunodeficiency virus type 1 that interact with Pr55Gag particles. *AIDS research and human retroviruses* **16**:1141-1147.
59. **Freed EO, Martin MA.** 1995. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. *J Virol* **69**:1984-1989.
60. **Mammano F, Kondo E, Sodroski J, Bukovsky A, Gottlinger HG.** 1995. Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains. *J Virol* **69**:3824-3830.
61. **Wyma DJ, Kotov A, Aiken C.** 2000. Evidence for a stable interaction of gp41 with Pr55(Gag) in immature human immunodeficiency virus type 1 particles. *J Virol* **74**:9381-9387.
62. **Celma CC, Manrique JM, Affranchino JL, Hunter E, Gonzalez SA.** 2001. Domains in the simian immunodeficiency virus gp41 cytoplasmic tail required for envelope incorporation into particles. *Virology* **283**:253-261.
63. **Freed EO, Martin MA.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *J Virol* **70**:341-351.
64. **Piller SC, Dubay JW, Derdeyn CA, Hunter E.** 2000. Mutational analysis of conserved domains within the cytoplasmic tail of gp41 from human immunodeficiency virus type 1: effects on glycoprotein incorporation and infectivity. *J Virol* **74**:11717-11723.
65. **Cosson P.** 1996. Direct interaction between the envelope and matrix proteins of HIV-1. *The EMBO journal* **15**:5783-5788.
66. **Kol N, Shi Y, Tsvitov M, Barlam D, Shneck RZ, Kay MS, Rousso I.** 2007. A stiffness switch in human immunodeficiency virus. *Biophysical journal* **92**:1777-1783.
67. **Pang HB, Hevroni L, Kol N, Eckert DM, Tsvitov M, Kay MS, Rousso I.** 2013. Virion stiffness regulates immature HIV-1 entry. *Retrovirology* **10**:4.
68. **Chojnacki J, Staudt T, Glass B, Bingen P, Engelhardt J, Anders M, Schneider J, Muller B, Hell SW, Krausslich HG.** 2012. Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy. *Science* **338**:524-528.
69. **Muranyi W, Malkusch S, Muller B, Heilemann M, Krausslich HG.** 2013. Super-resolution microscopy reveals specific recruitment of HIV-1 envelope proteins to viral assembly sites dependent on the envelope C-terminal tail. *PLoS pathogens* **9**:e1003198.
70. **von Schwedler U, Kornbluth RS, Trono D.** 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc Natl Acad Sci U S A* **91**:6992-6996.
71. **Wright ER, Schooler JB, Ding HJ, Kieffer C, Fillmore C, Sundquist WI, Jensen GJ.** 2007. Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells. *The EMBO journal* **26**:2218-2226.
72. **Briggs JA, Riches JD, Glass B, Bartonova V, Zanetti G, Krausslich HG.** 2009. Structure and assembly of immature HIV. *Proc Natl Acad Sci U S A* **106**:11090-11095.
73. **Alfadhli A, Barklis RL, Barklis E.** 2009. HIV-1 matrix organizes as a hexamer of trimers on membranes containing phosphatidylinositol-(4,5)-bisphosphate. *Virology* **387**:466-472.
74. **Gamble TR, Yoo S, Vajdos FF, von Schwedler UK, Worthylake DK, Wang H, McCutcheon JP, Sundquist WI, Hill CP.** 1997. Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. *Science* **278**:849-853.
75. **Gitti RK, Lee BM, Walker J, Summers MF, Yoo S, Sundquist WI.** 1996. Structure of the amino-terminal core domain of the HIV-1 capsid protein. *Science* **273**:231-235.
76. **Aiken C.** 2006. Viral and cellular factors that regulate HIV-1 uncoating. *Curr Opin HIV AIDS* **1**:194-199.
77. **Dannull J, Surovoy A, Jung G, Moelling K.** 1994. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. *The EMBO journal* **13**:1525-1533.
78. **Cimarelli A, Sandin S, Høglund S, Luban J.** 2000. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. *J Virol* **74**:3046-3057.
79. **Russell RS, Liang C, Wainberg MA.** 2004. Is HIV-1 RNA dimerization a prerequisite for packaging? Yes, no, probably? *Retrovirology* **1**:23.
80. **Rein A, Henderson LE, Levin JG.** 1998. Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: significance for viral replication. *Trends in biochemical sciences* **23**:297-301.
81. **Druillennec S, Caneparo A, de Rocquigny H, Roques BP.** 1999. Evidence of interactions between the nucleocapsid protein NCp7 and the reverse transcriptase of HIV-1. *The Journal of biological chemistry* **274**:11283-11288.

82. **Kondo E, Gottlinger HG.** 1996. A conserved LXXLF sequence is the major determinant in p6gag required for the incorporation of human immunodeficiency virus type 1 Vpr. *J Virol* **70**:159-164.
83. **Zhu P, Chertova E, Bess J, Jr., Lifson JD, Arthur LO, Liu J, Taylor KA, Roux KH.** 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc Natl Acad Sci U S A* **100**:15812-15817.
84. **Yuste E, Reeves JD, Doms RW, Desrosiers RC.** 2004. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J Virol* **78**:6775-6785.
85. **Chertova E, Bess JW, Jr., Crise BJ, Sowder IR, Schaden TM, Hilburn JM, Hoxie JA, Benveniste RE, Lifson JD, Henderson LE, Arthur LO.** 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol* **76**:5315-5325.
86. **Mager PP.** 2001. The active site of HIV-1 protease. *Medicinal research reviews* **21**:348-353.
87. **Navia MA, Fitzgerald PM, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP.** 1989. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature* **337**:615-620.
88. **Zybarth G, Carter C.** 1995. Domains upstream of the protease (PR) in human immunodeficiency virus type 1 Gag-Pol influence PR autoprocessing. *J Virol* **69**:3878-3884.
89. **Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-1790.
90. **Temin HM, Baltimore D.** 1972. RNA-directed DNA synthesis and RNA tumor viruses. *Advances in virus research* **17**:129-186.
91. **Verma IM, Temple GF, Fan H, Baltimore D.** 1972. In vitro synthesis of DNA complementary to rabbit reticulocyte 10S RNA. *Nature: New biology* **235**:163-167.
92. **Baltimore D, Smoler DF.** 1972. Association of an endoribonuclease with the avian myeloblastosis virus deoxyribonucleic acid polymerase. *The Journal of biological chemistry* **247**:7282-7287.
93. **Amacker M, Hubscher U.** 1998. Chimeric HIV-1 and feline immunodeficiency virus reverse transcriptases: critical role of the p51 subunit in the structural integrity of heterodimeric lentiviral DNA polymerases. *Journal of molecular biology* **278**:757-765.
94. **Tsurutani N, Kubo M, Maeda Y, Ohashi T, Yamamoto N, Kannagi M, Masuda T.** 2000. Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells. *J Virol* **74**:4795-4806.
95. **Zamborlini A, Coiffic A, Beauclair G, Delelis O, Paris J, Koh Y, Magne F, Giron ML, Tobaly-Tapiero J, Deprez E, Emiliani S, Engelman A, de The H, Saib A.** 2011. Impairment of human immunodeficiency virus type-1 integrase SUMOylation correlates with an early replication defect. *The Journal of biological chemistry* **286**:21013-21022.
96. **Zheng Y, Yao X.** 2013. Posttranslational modifications of HIV-1 integrase by various cellular proteins during viral replication. *Viruses* **5**:1787-1801.
97. **Cai M, Zheng R, Caffrey M, Craigie R, Clore GM, Gronenborn AM.** 1997. Solution structure of the N-terminal zinc binding domain of HIV-1 integrase. *Nature structural biology* **4**:567-577.
98. **Lins RD, Briggs JM, Straatsma TP, Carlson HA, Greenwald J, Choe S, McCammon JA.** 1999. Molecular dynamics studies on the HIV-1 integrase catalytic domain. *Biophysical journal* **76**:2999-3011.
99. **Chen JC, Krucinski J, Miercke LJ, Finer-Moore JS, Tang AH, Leavitt AD, Stroud RM.** 2000. Crystal structure of the HIV-1 integrase catalytic core and C-terminal domains: a model for viral DNA binding. *Proc Natl Acad Sci U S A* **97**:8233-8238.
100. **Mahlknecht U, Dichamp I, Varin A, Van Lint C, Herbein G.** 2008. NF-kappaB-dependent control of HIV-1 transcription by the second coding exon of Tat in T cells. *Journal of leukocyte biology* **83**:718-727.
101. **Zhu Y, Pe'ery T, Peng J, Ramanathan Y, Marshall N, Marshall T, Amendt B, Mathews MB, Price DH.** 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. *Genes & development* **11**:2622-2632.
102. **Dingwall C, Ernberg I, Gait MJ, Green SM, Heaphy S, Karn J, Lowe AD, Singh M, Skinner MA, Valerio R.** 1989. Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. *Proc Natl Acad Sci U S A* **86**:6925-6929.
103. **Van Lint C, Bouchat S, Marcello A.** 2013. HIV-1 transcription and latency: an update. *Retrovirology* **10**:67.
104. **Zheng L, Yang YD, Lu GC, Salvato MS.** 2005. Extracellular HIV Tat and Tat cysteine rich peptide increase CCR5 expression in monocytes. *Journal of Zhejiang University. Science. B* **6**:668-672.
105. **Malim MH, Hauber J, Le SY, Maizel JV, Cullen BR.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338**:254-257.
106. **Malim MH, Tiley LS, McCarn DF, Rusche JR, Hauber J, Cullen BR.** 1990. HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. *Cell* **60**:675-683.
107. **Fornerod M, Ohno M, Yoshida M, Mattaj JW.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**:1051-1060.
108. **Kao S, Akari H, Khan MA, Dettenhofer M, Yu XF, Strebel K.** 2003. Human immunodeficiency virus type 1 Vif is efficiently packaged into virions during productive but not chronic infection. *J Virol* **77**:1131-1140.

109. **Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D.** 2004. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *The Journal of biological chemistry* **279**:7792-7798.
110. **Ohagen A, Gabuzda D.** 2000. Role of Vif in stability of the human immunodeficiency virus type 1 core. *J Virol* **74**:11055-11066.
111. **Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, Gendelman HE, Ratner L, Stevenson M, Emerman M.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* **91**:7311-7315.
112. **Sawaya BE, Khalili K, Gordon J, Taube R, Amini S.** 2000. Cooperative interaction between HIV-1 regulatory proteins Tat and Vpr modulates transcription of the viral genome. *The Journal of biological chemistry* **275**:35209-35214.
113. **Jowett JB, Planelles V, Poon B, Shah NP, Chen ML, Chen IS.** 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. *J Virol* **69**:6304-6313.
114. **Muthumani K, Hwang DS, Desai BM, Zhang D, Dayes N, Green DR, Weiner DB.** 2002. HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. *The Journal of biological chemistry* **277**:37820-37831.
115. **Willey RL, Maldarelli F, Martin MA, Strebel K.** 1992. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *J Virol* **66**:226-234.
116. **Fujita K, Omura S, Silver J.** 1997. Rapid degradation of CD4 in cells expressing human immunodeficiency virus type 1 Env and Vpu is blocked by proteasome inhibitors. *The Journal of general virology* **78 (Pt 3)**:619-625.
117. **Schubert U, Anton LC, Bacik I, Cox JH, Bour S, Bennink JR, Orlowski M, Strebel K, Yewdell JW.** 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J Virol* **72**:2280-2288.
118. **Douglas JL, Gustin JK, Viswanathan K, Mansouri M, Moses AV, Fruh K.** 2010. The great escape: viral strategies to counter BST-2/tetherin. *PLoS pathogens* **6**:e1000913.
119. **Evans DT, Serra-Moreno R, Singh RK, Guatelli JC.** 2010. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends in microbiology* **18**:388-396.
120. **Jouvenet N, Neil SJ, Zhadina M, Zang T, Kratovac Z, Lee Y, McNatt M, Hatzioannou T, Bieniasz PD.** 2009. Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. *J Virol* **83**:1837-1844.
121. **Kuhl BD, Sloan RD, Donahue DA, Bar-Magen T, Liang C, Wainberg MA.** 2010. Tetherin restricts direct cell-to-cell infection of HIV-1. *Retrovirology* **7**:115.
122. **Le Tortorec A, Neil SJ.** 2009. Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *J Virol* **83**:11966-11978.
123. **Serra-Moreno R, Jia B, Breed M, Alvarez X, Evans DT.** 2011. Compensatory changes in the cytoplasmic tail of gp41 confer resistance to tetherin/BST-2 in a pathogenic nef-deleted SIV. *Cell host & microbe* **9**:46-57.
124. **Neil SJ, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451**:425-430.
125. **Miller MD, Feinberg MB, Greene WC.** 1994. The HIV-1 nef gene acts as a positive viral infectivity factor. *Trends in microbiology* **2**:294-298.
126. **Adnan S, Balamurugan A, Trocha A, Bennett MS, Ng HL, Ali A, Brander C, Yang OO.** 2006. Nef interference with HIV-1-specific CTL antiviral activity is epitope specific. *Blood* **108**:3414-3419.
127. **Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL.** 2008. HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells. *PLoS pathogens* **4**:e1000131.
128. **Chaudhuri R, Lindwasser OW, Smith WJ, Hurley JH, Bonifacino JS.** 2007. Downregulation of CD4 by human immunodeficiency virus type 1 Nef is dependent on clathrin and involves direct interaction of Nef with the AP2 clathrin adaptor. *J Virol* **81**:3877-3890.
129. **Leonard JA, Filzen T, Carter CC, Schaefer M, Collins KL.** 2011. HIV-1 Nef disrupts intracellular trafficking of major histocompatibility complex class I, CD4, CD8, and CD28 by distinct pathways that share common elements. *J Virol* **85**:6867-6881.
130. **Swigut T, Shohdy N, Skowronski J.** 2001. Mechanism for down-regulation of CD28 by Nef. *The EMBO journal* **20**:1593-1604.
131. **El-Far M, Isabelle C, Chomont N, Bourbonniere M, Fonseca S, Ancuta P, Peretz Y, Chouikh Y, Halwani R, Schwartz O, Madrenas J, Freeman GJ, Routy JP, Haddad EK, Sekaly RP.** 2013. Down-Regulation of CTLA-4 by HIV-1 Nef Protein. *PloS one* **8**:e54295.
132. **Verity EE, Zotos D, Wilson K, Chatfield C, Lawson VA, Dwyer DE, Cunningham A, Learmont J, Dyer W, Sullivan J, Churchill M, Wesselingh SL, Gabuzda D, Gorry PR, McPhee DA.** 2007. Viral phenotypes and antibody responses in long-term survivors infected with attenuated human immunodeficiency virus type 1 containing deletions in the nef and long terminal repeat regions. *J Virol* **81**:9268-9278.
133. **Gorry PR, McPhee DA, Verity E, Dyer WB, Wesselingh SL, Learmont J, Sullivan JS, Roche M, Zaunders JJ, Gabuzda D, Crowe SM, Mills J, Lewin SR, Brew BJ, Cunningham AL, Churchill MJ.** 2007. Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. *Retrovirology* **4**:66.
134. **Turner BG, Summers MF.** 1999. Structural biology of HIV. *Journal of molecular biology* **285**:1-32.

135. **Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**:763-767.
136. **McDougal JS, Maddon PJ, Dalgleish AG, Clapham PR, Littman DR, Godfrey M, Maddon DE, Chess L, Weiss RA, Axel R.** 1986. The T4 glycoprotein is a cell-surface receptor for the AIDS virus. *Cold Spring Harbor symposia on quantitative biology* **51 Pt 2**:703-711.
137. **Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**:1955-1958.
138. **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**:661-666.
139. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**:872-877.
140. **Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**:1135-1148.
141. **Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**:667-673.
142. **Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, Sodroski J.** 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**:1949-1953.
143. **Bosch ML, Earl PL, Fargnoli K, Picciafuoco S, Giombini F, Wong-Staal F, Franchini G.** 1989. Identification of the fusion peptide of primate immunodeficiency viruses. *Science* **244**:694-697.
144. **Freed EO, Myers DJ, Risser R.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proc Natl Acad Sci U S A* **87**:4650-4654.
145. **Brasseur R, Vandenbranden M, Cornet B, Burny A, Ruyschaert JM.** 1990. Orientation into the lipid bilayer of an asymmetric amphipathic helical peptide located at the N-terminus of viral fusion proteins. *Biochimica et biophysica acta* **1029**:267-273.
146. **Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC.** 1997. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**:426-430.
147. **Chan DC, Fass D, Berger JM, Kim PS.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**:263-273.
148. **Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB.** 2009. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell* **137**:433-444.
149. **Permanyer M, Ballana E, Este JA.** 2010. Endocytosis of HIV: anything goes. *Trends in microbiology* **18**:543-551.
150. **Hulme AE, Perez O, Hope TJ.** 2011. Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. *Proc Natl Acad Sci U S A* **108**:9975-9980.
151. **Jiang M, Mak J, Ladha A, Cohen E, Klein M, Rovinski B, Kleiman L.** 1993. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. *J Virol* **67**:3246-3253.
152. **Mak J, Jiang M, Wainberg MA, Hammarskjold ML, Rekosh D, Kleiman L.** 1994. Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles. *J Virol* **68**:2065-2072.
153. **Fassati A, Goff SP.** 2001. Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. *J Virol* **75**:3626-3635.
154. **Warren K, Warrilow D, Meredith L, Harrich D.** 2009. Reverse Transcriptase and Cellular Factors: Regulators of HIV-1 Reverse Transcription. *Viruses* **1**:873-894.
155. **Warrilow D, Tachedjian G, Harrich D.** 2009. Maturation of the HIV reverse transcription complex: putting the jigsaw together. *Reviews in medical virology* **19**:324-337.
156. **Gilboa E, Goff S, Shields A, Yoshimura F, Mitra S, Baltimore D.** 1979. In vitro synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. *Cell* **16**:863-874.
157. **Gilboa E, Mitra SW, Goff S, Baltimore D.** 1979. A detailed model of reverse transcription and tests of crucial aspects. *Cell* **18**:93-100.
158. **Hu WS, Temin HM.** 1990. Retroviral recombination and reverse transcription. *Science* **250**:1227-1233.
159. **Auxilien S, Keith G, Le Grice SF, Darlix JL.** 1999. Role of post-transcriptional modifications of primer tRNA(Lys)3 in the fidelity and efficacy of plus strand DNA transfer during HIV-1 reverse transcription. *The Journal of biological chemistry* **274**:4412-4420.
160. **Hameau L, Jeusset J, Lafosse S, Coulaud D, Delain E, Unge T, Restle T, Le Cam E, Mirambeau G.** 2001. Human immunodeficiency virus type 1 central DNA flap: dynamic terminal product of plus-strand displacement dna synthesis catalyzed by reverse transcriptase assisted by nucleocapsid protein. *J Virol* **75**:3301-3313.
161. **Levin JG, Mitra M, Mascarenhas A, Musier-Forsyth K.** 2010. Role of HIV-1 nucleocapsid protein in HIV-1 reverse transcription. *RNA biology* **7**:754-774.
162. **LaFemina RL, Callahan PL, Cordingley MG.** 1991. Substrate specificity of recombinant human immunodeficiency virus integrase protein. *J Virol* **65**:5624-5630.

163. **Sirven A, Pflumio F, Zennou V, Titeux M, Vainchenker W, Coulombel L, Dubart-Kupperschmitt A, Charneau P.** 2000. The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood* **96**:4103-4110.
164. **Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P.** 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**:173-185.
165. **Ao Z, Yao X, Cohen EA.** 2004. Assessment of the role of the central DNA flap in human immunodeficiency virus type 1 replication by using a single-cycle replication system. *J Virol* **78**:3170-3177.
166. **Kankia BI, Musier-Forsyth K.** 2007. The HIV-1 central DNA flap region contains a "flapping" third strand. *Biophysical chemistry* **127**:64-68.
167. **De Rijck J, Debysers Z.** 2006. The central DNA flap of the human immunodeficiency virus type 1 is important for viral replication. *Biochemical and biophysical research communications* **349**:1100-1110.
168. **Arhel NJ, Souquere-Besse S, Charneau P.** 2006. Wild-type and central DNA flap defective HIV-1 lentiviral vector genomes: intracellular visualization at ultrastructural resolution levels. *Retrovirology* **3**:38.
169. **Cherepanov P, Maertens G, Proost P, Devreese B, Van Beeumen J, Engelborghs Y, De Clercq E, Debysers Z.** 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *The Journal of biological chemistry* **278**:372-381.
170. **Maertens G, Cherepanov P, Pluymers W, Busschots K, De Clercq E, Debysers Z, Engelborghs Y.** 2003. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *The Journal of biological chemistry* **278**:33528-33539.
171. **Vincent KA, York-Higgins D, Quiroga M, Brown PO.** 1990. Host sequences flanking the HIV provirus. *Nucleic acids research* **18**:6045-6047.
172. **Vink C, Groenink M, Elgersma Y, Fouchier RA, Tersmette M, Plasterk RH.** 1990. Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. *J Virol* **64**:5626-5627.
173. **Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F.** 2002. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**:521-529.
174. **Suzuki Y, Chew ML, Suzuki Y.** 2012. Role of host-encoded proteins in restriction of retroviral integration. *Frontiers in microbiology* **3**:227.
175. **Jordan A, Defechereux P, Verdin E.** 2001. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *The EMBO journal* **20**:1726-1738.
176. **Siliciano RF, Greene WC.** 2011. HIV latency. *Cold Spring Harbor perspectives in medicine* **1**:a007096.
177. **Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA.** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *The EMBO journal* **9**:1551-1560.
178. **Nabel G, Baltimore D.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326**:711-713.
179. **Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ.** 1993. A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *The EMBO journal* **12**:3551-3558.
180. **Kwon H, Pelletier N, DeLuca C, Genin P, Cisternas S, Lin R, Wainberg MA, Hiscott J.** 1998. Inducible expression of IkappaBalpha repressor mutants interferes with NF-kappaB activity and HIV-1 replication in Jurkat T cells. *The Journal of biological chemistry* **273**:7431-7440.
181. **Wu Y.** 2004. HIV-1 gene expression: lessons from provirus and non-integrated DNA. *Retrovirology* **1**:13.
182. **Malim MH, Cullen BR.** 1991. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. *Cell* **65**:241-248.
183. **Truant R, Cullen BR.** 1999. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Molecular and cellular biology* **19**:1210-1217.
184. **Karn J, Stoltzfus CM.** 2012. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harbor perspectives in medicine* **2**:a006916.
185. **Campbell S, Fisher RJ, Towler EM, Fox S, Issaq HJ, Wolfe T, Phillips LR, Rein A.** 2001. Modulation of HIV-like particle assembly in vitro by inositol phosphates. *Proc Natl Acad Sci U S A* **98**:10875-10879.
186. **Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG.** 2006. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci U S A* **103**:2641-2646.
187. **Park J, Morrow CD.** 1992. The nonmyristylated Pr160gag-pol polyprotein of human immunodeficiency virus type 1 interacts with Pr55gag and is incorporated into viruslike particles. *J Virol* **66**:6304-6313.
188. **Srinivasakumar N, Hammarskjold ML, Rekosh D.** 1995. Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. *J Virol* **69**:6106-6114.
189. **Simons K, Gerl MJ.** 2010. Revitalizing membrane rafts: new tools and insights. *Nature reviews. Molecular cell biology* **11**:688-699.
190. **Yu X, Yuan X, Matsuda Z, Lee TH, Essex M.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J Virol* **66**:4966-4971.
191. **Bachand F, Yao XJ, Hrimech M, Rougeau N, Cohen EA.** 1999. Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 gag precursor. *The Journal of biological chemistry* **274**:9083-9091.

192. **Huwent I, Hong SS, Fournier C, Gay B, Tournier J, Carriere C, Courcou M, Vigne R, Spire B, Boulanger P.** 1998. Interaction and co-encapsulation of human immunodeficiency virus type 1 Gag and Vif recombinant proteins. *The Journal of general virology* **79** (Pt 5):1069-1081.
193. **Kleiman L, Halwani R, Javanbakht H.** 2004. The selective packaging and annealing of primer tRNA^{Lys3} in HIV-1. *Current HIV research* **2**:163-175.
194. **Usami Y, Popov S, Popova E, Inoue M, Weissenhorn W, H GG.** 2009. The ESCRT pathway and HIV-1 budding. *Biochemical Society transactions* **37**:181-184.
195. **Strickler JE, Gorniak J, Dayton B, Meek T, Moore M, Magaard V, Malinowski J, Debouck C.** 1989. Characterization and autoprocessing of precursor and mature forms of human immunodeficiency virus type 1 (HIV 1) protease purified from *Escherichia coli*. *Proteins* **6**:139-154.
196. **Louis JM, Wondrak EM, Kimmel AR, Wingfield PT, Nashed NT.** 1999. Proteolytic processing of HIV-1 protease precursor, kinetics and mechanism. *The Journal of biological chemistry* **274**:23437-23442.
197. **Wieggers K, Rutter G, Kottler H, Tessmer U, Hohenberg H, Krausslich HG.** 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. *J Virol* **72**:2846-2854.
198. **Pettit SC, Henderson GJ, Schiffer CA, Swanstrom R.** 2002. Replacement of the P1 amino acid of human immunodeficiency virus type 1 Gag processing sites can inhibit or enhance the rate of cleavage by the viral protease. *J Virol* **76**:10226-10233.
199. **Wyma DJ, Jiang J, Shi J, Zhou J, Lineberger JE, Miller MD, Aiken C.** 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. *J Virol* **78**:3429-3435.
200. **Jiang J, Aiken C.** 2007. Maturation-dependent human immunodeficiency virus type 1 particle fusion requires a carboxyl-terminal region of the gp41 cytoplasmic tail. *J Virol* **81**:9999-10008.
201. **Murakami T, Ablan S, Freed EO, Tanaka Y.** 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. *J Virol* **78**:1026-1031.
202. **Fun A, Wensing AM, Verheyen J, Nijhuis M.** 2012. Human Immunodeficiency Virus Gag and protease: partners in resistance. *Retrovirology* **9**:63.
203. **Royce RA, Sena A, Cates W, Jr., Cohen MS.** 1997. Sexual transmission of HIV. *N Engl J Med* **336**:1072-1078.
204. **Quinn TC, Wawer MJ, Sewankambo N, Serwadda D, Li C, Wabwire-Mangen F, Meehan MO, Lutalo T, Gray RH.** 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* **342**:921-929.
205. **Fideli US, Allen SA, Musonda R, Trask S, Hahn BH, Weiss H, Mulenga J, Kasolo F, Vermund SH, Aldrovandi GM.** 2001. Virologic and immunologic determinants of heterosexual transmission of human immunodeficiency virus type 1 in Africa. *AIDS research and human retroviruses* **17**:901-910.
206. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* **105**:7552-7557.
207. **Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F, Lutalo T, Li X, vanCott T, Quinn TC, Rakai Project T.** 2001. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* **357**:1149-1153.
208. **Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**:367-377.
209. **McLaren PJ, Ball TB, Wachih C, Jaoko W, Kelvin DJ, Danesh A, Kimani J, Plummer FA, Fowke KR.** 2010. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *The Journal of infectious diseases* **202 Suppl 3**:S339-344.
210. **Laga M, Manoka A, Kivuvu M, Malele B, Tuliza M, Nzila N, Goeman J, Behets F, Batter V, Alary M, et al.** 1993. Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. *AIDS* **7**:95-102.
211. **Wawer MJ, Gray RH, Sewankambo NK, Serwadda D, Li X, Laeyendecker O, Kiwanuka N, Kigozi G, Kiddugavu M, Lutalo T, Nalugoda F, Wabwire-Mangen F, Meehan MP, Quinn TC.** 2005. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. *The Journal of infectious diseases* **191**:1403-1409.
212. **Miller WC, Rosenberg NE, Rutstein SE, Powers KA.** 2010. Role of acute and early HIV infection in the sexual transmission of HIV. *Curr Opin HIV AIDS* **5**:277-282.
213. **Bangham CR.** 2009. CTL quality and the control of human retroviral infections. *European journal of immunology* **39**:1700-1712.
214. **Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, Boden D, Racz P, Markowitz M.** 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *The Journal of experimental medicine* **200**:761-770.

215. **Coffin J, Swanstrom R.** 2013. HIV pathogenesis: dynamics and genetics of viral populations and infected cells. *Cold Spring Harbor perspectives in medicine* **3**:a012526.
216. **Grossman Z, Meier-Schellersheim M, Paul WE, Picker LJ.** 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nature medicine* **12**:289-295.
217. **Picker LJ.** 2006. Immunopathogenesis of acute AIDS virus infection. *Current opinion in immunology* **18**:399-405.
218. **Veazey RS, Marx PA, Lackner AA.** 2003. Vaginal CD4+ T cells express high levels of CCR5 and are rapidly depleted in simian immunodeficiency virus infection. *The Journal of infectious diseases* **187**:769-776.
219. **Banchereau J, Steinman RM.** 1998. Dendritic cells and the control of immunity. *Nature* **392**:245-252.
220. **Steinman RM.** 1991. The dendritic cell system and its role in immunogenicity. *Annual review of immunology* **9**:271-296.
221. **Turville SG, Santos JJ, Frank I, Cameron PU, Wilkinson J, Miranda-Saksena M, Dable J, Stossel H, Romani N, Piatak M, Jr., Lifson JD, Pope M, Cunningham AL.** 2004. Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* **103**:2170-2179.
222. **McDonald D, Wu L, Bohks SM, KewalRamani VN, Unutmaz D, Hope TJ.** 2003. Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science* **300**:1295-1297.
223. **Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, Dandekar S.** 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* **77**:11708-11717.
224. **Grossman Z, Feinberg MB, Paul WE.** 1998. Multiple modes of cellular activation and virus transmission in HIV infection: a role for chronically and latently infected cells in sustaining viral replication. *Proc Natl Acad Sci U S A* **95**:6314-6319.
225. **Douek DC, Picker LJ, Koup RA.** 2003. T cell dynamics in HIV-1 infection. *Annual review of immunology* **21**:265-304.
226. **Richman DD, Wrin T, Little SJ, Petropoulos CJ.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* **100**:4144-4149.
227. **McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF.** 2010. The immune response during acute HIV-1 infection: clues for vaccine development. *Nature reviews. Immunology* **10**:11-23.
228. **Steinhauer DA, Holland JJ.** 1987. Rapid evolution of RNA viruses. *Annual review of microbiology* **41**:409-433.
229. **Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**:123-126.
230. **Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD.** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science* **271**:1582-1586.
231. **Okoye AA, Picker LJ.** 2013. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. *Immunological reviews* **254**:54-64.
232. **Jiang W, Lederman MM, Hunt P, Sieg SF, Haley K, Rodriguez B, Landay A, Martin J, Sinclair E, Asher AI, Deeks SG, Douek DC, Brenchley JM.** 2009. Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *The Journal of infectious diseases* **199**:1177-1185.
233. **Biancotto A, Iglehart SJ, Vanpouille C, Condack CE, Lisco A, Ruecker E, Hirsch I, Margolis LB, Grivel JC.** 2008. HIV-1 induced activation of CD4+ T cells creates new targets for HIV-1 infection in human lymphoid tissue ex vivo. *Blood* **111**:699-704.
234. **Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS.** 1983. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med* **309**:453-458.
235. **Ng VL.** 1996. B-lymphocytes and autoantibody profiles in HIV disease. *Clinical reviews in allergy & immunology* **14**:367-384.
236. **Tersmette M, Gruters RA, de Wolf F, de Goede RE, Lange JM, Schellekens PT, Goudsmit J, Huisman HG, Miedema F.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. *J Virol* **63**:2118-2125.
237. **Gougeon ML.** 2005. To kill or be killed: how HIV exhausts the immune system. *Cell death and differentiation* **12 Suppl 1**:845-854.
238. **Garg H, Mohl J, Joshi A.** 2012. HIV-1 induced bystander apoptosis. *Viruses* **4**:3020-3043.
239. **Ahr B, Robert-Hebmann V, Devaux C, Biard-Piechaczyk M.** 2004. Apoptosis of uninfected cells induced by HIV envelope glycoproteins. *Retrovirology* **1**:12.
240. **Gonzalez-Scarano F, Martin-Garcia J.** 2005. The neuropathogenesis of AIDS. *Nature reviews. Immunology* **5**:69-81.
241. **Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**:1529-1531.
242. **Beignon AS, McKenna K, Skoberne M, Manches O, DaSilva I, Kavanagh DG, Larsson M, Gorelick RJ, Lifson JD, Bhardwaj N.** 2005. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *The Journal of clinical investigation* **115**:3265-3275.

243. **Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F, Colombo S, Brown EE, Shupert WL, Phair J, Goedert JJ, Buchbinder S, Kirk GD, Telenti A, Connors M, O'Brien SJ, Walker BD, Parham P, Deeks SG, McVicar DW, Carrington M.** 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nature genetics* **39**:733-740.
244. **Gray CM, Lawrence J, Schapiro JM, Altman JD, Winters MA, Crompton M, Loi M, Kundu SK, Davis MM, Merigan TC.** 1999. Frequency of class I HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). *Journal of immunology* **162**:1780-1788.
245. **Peretz Y, Ndongala ML, Boulet S, Boulassel MR, Rouleau D, Cote P, Longpre D, Routy JP, Falutz J, Tremblay C, Tsoukas CM, Sekaly RP, Bernard NF.** 2007. Functional T cell subsets contribute differentially to HIV peptide-specific responses within infected individuals: correlation of these functional T cell subsets with markers of disease progression. *Clinical immunology* **124**:57-68.
246. **Grant MD, Smail FM, Rosenthal KL.** 1993. Lysis of CD4+ lymphocytes by non-HLA-restricted cytotoxic T lymphocytes from HIV-infected individuals. *Clinical and experimental immunology* **93**:356-362.
247. **Yang OO, Kalams SA, Trocha A, Cao H, Luster A, Johnson RP, Walker BD.** 1997. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol* **71**:3120-3128.
248. **Soghoian DZ, Jessen H, Flanders M, Sierra-Davidson K, Cutler S, Pertel T, Ranasinghe S, Lindqvist M, Davis I, Lane K, Rychert J, Rosenberg ES, Piechocka-Trocha A, Brass AL, Brenchley JM, Walker BD, Streeck H.** 2012. HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome. *Science translational medicine* **4**:123ra125.
249. **Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barre-Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A.** 2007. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci U S A* **104**:6776-6781.
250. **Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Protocol GPI, Kaminsky S, Zamb T, Moyle M, Koff WC, Pognard P, Burton DR.** 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* **326**:285-289.
251. **Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, Lehrman JK, Boaz M, Tarragona-Fiol T, Miiro G, Birungi J, Pozniak A, McPhee DA, Manigart O, Karita E, Inwoley A, Jaoko W, Dehovitz J, Bekker LG, Pitisuttithum P, Paris R, Walker LM, Pognard P, Wrin T, Fast PE, Burton DR, Koff WC.** 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *J Virol* **83**:7337-7348.
252. **Proffy AT, Salinas PA, Eckler LI, Dunlop NM, Nara PL, Putney SD.** 1990. Epitopes recognized by the neutralizing antibodies of an HIV-1-infected individual. *Journal of immunology* **144**:4641-4647.
253. **Curlin ME, Zion R, Hawes SE, Liu Y, Deng W, Gottlieb GS, Zhu T, Mullins JI.** 2010. HIV-1 envelope subregion length variation during disease progression. *PLoS pathogens* **6**:e1001228.
254. **Fenyo EM, Esbjornsson J, Medstrand P, Jansson M.** 2011. Human immunodeficiency virus type 1 biological variation and coreceptor use: from concept to clinical significance. *J Intern Med* **270**:520-531.
255. **Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM.** 2003. Antibody neutralization and escape by HIV-1. *Nature* **422**:307-312.
256. **Descours B, Cribier A, Chable-Bessia C, Ayinde D, Rice G, Crow Y, Yatim A, Schwartz O, Laguet N, Benkirane M.** 2012. SAMHD1 restricts HIV-1 reverse transcription in quiescent CD4(+) T-cells. *Retrovirology* **9**:87.
257. **Pan X, Baldauf HM, Keppler OT, Fackler OT.** 2013. Restrictions to HIV-1 replication in resting CD4+ T lymphocytes. *Cell research* **23**:876-885.
258. **Goldstone DC, Ennis-Adeniran V, Hedden JJ, Groom HC, Rice GI, Christodoulou E, Walker PA, Kelly G, Haire LF, Yap MW, de Carvalho LP, Stoye JP, Crow YJ, Taylor IA, Webb M.** 2011. HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature* **480**:379-382.
259. **Baldauf HM, Pan X, Erikson E, Schmidt S, Daddacha W, Burggraf M, Schenkova K, Ambiel I, Wabnitz G, Gramberg T, Panitz S, Flory E, Landau NR, Sertel S, Rutsch F, Lasitschka F, Kim B, Konig R, Fackler OT, Keppler OT.** 2012. SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells. *Nature medicine*.
260. **Lahouassa H, Daddacha W, Hofmann H, Ayinde D, Logue EC, Dragin L, Bloch N, Maudet C, Bertrand M, Gramberg T, Pancino G, Priet S, Canard B, Laguet N, Benkirane M, Transy C, Landau NR, Kim B, Margottin-Goguet F.** 2012. SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nature immunology* **13**:223-228.
261. **Sheehy AM, Gaddis NC, Choi JD, Malim MH.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**:646-650.
262. **Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L.** 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**:94-98.
263. **Sheehy AM, Gaddis NC, Malim MH.** 2003. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nature medicine* **9**:1404-1407.

264. **Tokarev A, Skasko M, Fitzpatrick K, Guatelli J.** 2009. Antiviral activity of the interferon-induced cellular protein BST-2/tetherin. *AIDS research and human retroviruses* **25**:1197-1210.
265. **Goujon C, Moncorge O, Bauby H, Doyle T, Ward CC, Schaller T, Hue S, Barclay WS, Schulz R, Malim MH.** 2013. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature* **502**:559-562.
266. **Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, Schoggins JW, Rice CM, Yamashita M, Hatziioannou T, Bieniasz PD.** 2013. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature* **502**:563-566.
267. **Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J.** 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427**:848-853.
268. **Nisole S, Stoye JP, Saib A.** 2005. TRIM family proteins: retroviral restriction and antiviral defence. *Nature reviews. Microbiology* **3**:799-808.
269. **Yap MW, Nisole S, Stoye JP.** 2005. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Current biology : CB* **15**:73-78.
270. **Ezzell C.** 1987. AZT given the green light for clinical treatment of AIDS. *Nature* **326**:430.
271. **Cihlar T, Ray AS.** 2010. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral research* **85**:39-58.
272. **Adamson CS.** 2012. Protease-Mediated Maturation of HIV: Inhibitors of Protease and the Maturation Process. *Molecular biology international* **2012**:604261.
273. **McCull DJ, Chen X.** 2010. Strand transfer inhibitors of HIV-1 integrase: bringing IN a new era of antiretroviral therapy. *Antiviral research* **85**:101-118.
274. **Tilton JC, Doms RW.** 2010. Entry inhibitors in the treatment of HIV-1 infection. *Antiviral research* **85**:91-100.
275. **Piatak M, Jr., Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM, Lifson JD.** 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259**:1749-1754.
276. **McGowan I.** 2006. Microbicides: a new frontier in HIV prevention. *Biologicals : journal of the International Association of Biological Standardization* **34**:241-255.
277. **Zhu J, Paul WE.** 2008. CD4 T cells: fates, functions, and faults. *Blood* **112**:1557-1569.
278. **Paul WE, Seder RA.** 1994. Lymphocyte responses and cytokines. *Cell* **76**:241-251.
279. **Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM.** 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* **24**:677-688.
280. **Sakaguchi S.** 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual review of immunology* **22**:531-562.
281. **El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, Valentine F, Littman DR, Unutmaz D.** 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *The Journal of infectious diseases* **201**:843-854.
282. **Moreno-Fernandez ME, Zapata W, Blackard JT, Franchini G, Chougnet CA.** 2009. Human regulatory T cells are targets for human immunodeficiency Virus (HIV) infection, and their susceptibility differs depending on the HIV type 1 strain. *J Virol* **83**:12925-12933.
283. **Haase AT.** 2010. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* **464**:217-223.
284. **Klatzmann D, Barre-Sinoussi F, Nugeyre MT, Danquet C, Vilmer E, Griscelli C, Brun-Veziret F, Rouzioux C, Gluckman JC, Chermann JC, et al.** 1984. Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. *Science* **225**:59-63.
285. **Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR.** 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci U S A* **94**:1925-1930.
286. **Sleasman JW, Aleixo LF, Morton A, Skoda-Smith S, Goodenow MM.** 1996. CD4+ memory T cells are the predominant population of HIV-1-infected lymphocytes in neonates and children. *AIDS* **10**:1477-1484.
287. **Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, Nguyen PL, Khoruts A, Larson M, Haase AT, Douek DC.** 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *The Journal of experimental medicine* **200**:749-759.
288. **Gordon S, Taylor PR.** 2005. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology* **5**:953-964.
289. **Koning FA, Goujon C, Bauby H, Malim MH.** 2011. Target cell-mediated editing of HIV-1 cDNA by APOBEC3 proteins in human macrophages. *J Virol* **85**:13448-13452.
290. **Cassetta L, Kajaste-Rudnitski A, Coradin T, Saba E, Chiara GD, Barbagallo M, Graziano F, Alfano M, Cassol E, Vicenzi E, Poli G.** 2013. M1 polarization of human monocyte-derived macrophages restricts pre-and post-integration steps of HIV-1 replication. *AIDS*.
291. **Cassetta L, Cassol E, Poli G.** 2011. Macrophage polarization in health and disease. *TheScientificWorldJournal* **11**:2391-2402.
292. **Smith PD, Meng G, Salazar-Gonzalez JF, Shaw GM.** 2003. Macrophage HIV-1 infection and the gastrointestinal tract reservoir. *Journal of leukocyte biology* **74**:642-649.
293. **Collin M, Gordon S.** 1994. The kinetics of human immunodeficiency virus reverse transcription are slower in primary human macrophages than in a lymphoid cell line. *Virology* **200**:114-120.
294. **Ayinde D, Casartelli N, Schwartz O.** 2012. Restricting HIV the SAMHD1 way: through nucleotide starvation. *Nature reviews. Microbiology* **10**:675-680.

295. **Musher DM, Watson DA, Nickeson D, Gyorkey F, Lahart C, Rossen RD.** 1990. The effect of HIV infection on phagocytosis and killing of *Staphylococcus aureus* by human pulmonary alveolar macrophages. *The American journal of the medical sciences* **299**:158-163.
296. **Kedzierska K, Crowe SM.** 2002. The role of monocytes and macrophages in the pathogenesis of HIV-1 infection. *Current medicinal chemistry* **9**:1893-1903.
297. **Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M.** 2007. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *The Journal of cell biology* **177**:329-341.
298. **Bennett AE, Narayan K, Shi D, Hartnell LM, Gousset K, He H, Lowekamp BC, Yoo TS, Bliss D, Freed EO, Subramaniam S.** 2009. Ion-abrasion scanning electron microscopy reveals surface-connected tubular conduits in HIV-infected macrophages. *PLoS pathogens* **5**:e1000591.
299. **Koppensteiner H, Banning C, Schneider C, Hohenberg H, Schindler M.** 2012. Macrophage internal HIV-1 is protected from neutralizing antibodies. *J Virol* **86**:2826-2836.
300. **Gaudin R, Berre S, Cunha de Alencar B, Decalf J, Schindler M, Gobert FX, Jouve M, Benaroch P.** 2013. Dynamics of HIV-containing compartments in macrophages reveal sequestration of virions and transient surface connections. *PLoS one* **8**:e69450.
301. **Liu YJ.** 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106**:259-262.
302. **Randolph GJ, Beaulieu S, Lebecque S, Steinman RM, Muller WA.** 1998. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282**:480-483.
303. **Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, Figdor CG.** 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**:575-585.
304. **Gurney KB, Elliott J, Nassanian H, Song C, Soilleux E, McGowan I, Anton PA, Lee B.** 2005. Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa. *J Virol* **79**:5762-5773.
305. **Lambert AA, Gilbert C, Richard M, Beaulieu AD, Tremblay MJ.** 2008. The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. *Blood* **112**:1299-1307.
306. **Turville SG, Cameron PU, Handley A, Lin G, Pohlmann S, Doms RW, Cunningham AL.** 2002. Diversity of receptors binding HIV on dendritic cell subsets. *Nature immunology* **3**:975-983.
307. **Turville S, Wilkinson J, Cameron P, Dable J, Cunningham AL.** 2003. The role of dendritic cell C-type lectin receptors in HIV pathogenesis. *Journal of leukocyte biology* **74**:710-718.
308. **van der Vlist M, van der Aar AM, Gringhuis SI, Geijtenbeek TB.** 2011. Innate signaling in HIV-1 infection of dendritic cells. *Curr Opin HIV AIDS* **6**:348-352.
309. **Guo Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blixt O, Taylor ME, Weis WI, Drickamer K.** 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nature structural & molecular biology* **11**:591-598.
310. **Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW.** 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci U S A* **96**:5215-5220.
311. **Hosmalin A, McIlroy D, Cheynier R, Clauvel JP, Oksenhendler E, Wain-Hobson S, Debre P, Autran B.** 1995. Splenic interdigitating dendritic cells in humans: characterization and HIV infection frequency in vivo. *Advances in experimental medicine and biology* **378**:439-441.
312. **Pichlmair A, Reis e Sousa C.** 2007. Innate recognition of viruses. *Immunity* **27**:370-383.
313. **Izquierdo-Useros N, Esteban O, Rodriguez-Plata MT, Erkizia I, Prado JG, Blanco J, Garcia-Parajo MF, Martinez-Picado J.** 2011. Dynamic imaging of cell-free and cell-associated viral capture in mature dendritic cells. *Traffic* **12**:1702-1713.
314. **Vasiliver-Shamis G, Dustin ML, Hioe CE.** 2010. HIV-1 Virological Synapse is not Simply a Copycat of the Immunological Synapse. *Viruses* **2**:1239-1260.
315. **Jolly C, Mitar I, Sattentau QJ.** 2007. Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells. *J Virol* **81**:13916-13921.
316. **Coleman CM, Gelais CS, Wu L.** 2013. Cellular and viral mechanisms of HIV-1 transmission mediated by dendritic cells. *Advances in experimental medicine and biology* **762**:109-130.
317. **Eisele E, Siliciano RF.** 2012. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* **37**:377-388.
318. **Pantaleo G, Graziosi C, Butini L, Pizzo PA, Schnittman SM, Kotler DP, Fauci AS.** 1991. Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc Natl Acad Sci U S A* **88**:9838-9842.
319. **Chun TW, Nickle DC, Justement JS, Meyers JH, Roby G, Hallahan CW, Kottlilil S, Moir S, Mican JM, Mullins JI, Ward DJ, Kovacs JA, Mannon PJ, Fauci AS.** 2008. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *The Journal of infectious diseases* **197**:714-720.
320. **Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DF, Thompson KA, Gabuzda D, McArthur JC, Pardo CA, Wesselingh SL.** 2006. Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. *Journal of neurovirology* **12**:146-152.

321. **Overbaugh J, Anderson RJ, Ndinya-Achola JO, Kreiss JK.** 1996. Distinct but related human immunodeficiency virus type 1 variant populations in genital secretions and blood. *AIDS research and human retroviruses* **12**:107-115.
322. **Gupta P, Mellors J, Kingsley L, Riddler S, Singh MK, Schreiber S, Cronin M, Rinaldo CR.** 1997. High viral load in semen of human immunodeficiency virus type 1-infected men at all stages of disease and its reduction by therapy with protease and nonnucleoside reverse transcriptase inhibitors. *J Virol* **71**:6271-6275.
323. **Ho DD, Rota TR, Hirsch MS.** 1986. Infection of monocyte/macrophages by human T lymphotropic virus type III. *The Journal of clinical investigation* **77**:1712-1715.
324. **Pace MJ, Graf EH, Agosto LM, Mexas AM, Male F, Brady T, Bushman FD, O'Doherty U.** 2012. Directly infected resting CD4+T cells can produce HIV Gag without spreading infection in a model of HIV latency. *PLoS pathogens* **8**:e1002818.
325. **Bohnlein E, Lowenthal JW, Siekevitz M, Ballard DW, Franza BR, Greene WC.** 1988. The same inducible nuclear proteins regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. *Cell* **53**:827-836.
326. **Kao SY, Calman AF, Luciw PA, Peterlin BM.** 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**:489-493.
327. **Peterlin BM, Price DH.** 2006. Controlling the elongation phase of transcription with P-TEFb. *Molecular cell* **23**:297-305.
328. **Schiralli Lester GM, Henderson AJ.** 2012. Mechanisms of HIV Transcriptional Regulation and Their Contribution to Latency. *Molecular biology international* **2012**:614120.
329. **Earl PL, Doms RW, Moss B.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc Natl Acad Sci U S A* **87**:648-652.
330. **Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *The Journal of biological chemistry* **265**:10373-10382.
331. **Bernstein HB, Tucker SP, Hunter E, Schutzbach JS, Compans RW.** 1994. Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. *J Virol* **68**:463-468.
332. **Montefiori DC, Robinson WE, Jr., Mitchell WM.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **85**:9248-9252.
333. **Li H, Chien PC, Jr., Tuen M, Visciano ML, Cohen S, Blais S, Xu CF, Zhang HT, Hioe CE.** 2008. Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes. *Journal of immunology* **180**:4011-4021.
334. **Checkley MA, Luttgé BG, Freed EO.** 2011. HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. *Journal of molecular biology* **410**:582-608.
335. **Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk HD, Garten W.** 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature* **360**:358-361.
336. **Frey G, Peng H, Rits-Volloch S, Morelli M, Cheng Y, Chen B.** 2008. A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. *Proc Natl Acad Sci U S A* **105**:3739-3744.
337. **Willey RL, Rutledge RA, Dias S, Folks T, Theodore T, Buckler CE, Martin MA.** 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. *Proc Natl Acad Sci U S A* **83**:5038-5042.
338. **Palmer C, Balfe P, Fox D, May JC, Frederiksson R, Fenyo EM, McKeating JA.** 1996. Functional characterization of the V1V2 region of human immunodeficiency virus type 1. *Virology* **220**:436-449.
339. **Sagar M, Wu X, Lee S, Overbaugh J.** 2006. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. *J Virol* **80**:9586-9598.
340. **Raska M, Novak J.** 2010. Involvement of envelope-glycoprotein glycans in HIV-1 biology and infection. *Archivum immunologiae et therapeuticae experimentalis* **58**:191-208.
341. **Zolla-Pazner S.** 2004. Identifying epitopes of HIV-1 that induce protective antibodies. *Nature reviews. Immunology* **4**:199-210.
342. **Olshevsky U, Helseth E, Furman C, Li J, Haseltine W, Sodroski J.** 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *J Virol* **64**:5701-5707.
343. **Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**:648-659.
344. **Wyatt R, Sullivan N, Thali M, Repke H, Ho D, Robinson J, Posner M, Sodroski J.** 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *J Virol* **67**:4557-4565.
345. **Shioda T, Levy JA, Cheng-Mayer C.** 1992. Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **89**:9434-9438.

346. **Shioda T, Oka S, Ida S, Nokihara K, Toriyoshi H, Mori S, Takebe Y, Kimura S, Shimada K, Nagai Y, et al.** 1994. A naturally occurring single basic amino acid substitution in the V3 region of the human immunodeficiency virus type 1 env protein alters the cellular host range and antigenic structure of the virus. *J Virol* **68**:7689-7696.
347. **Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR, Parren PW.** 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol* **75**:10892-10905.
348. **Hunter E, Swanstrom R.** 1990. Retrovirus envelope glycoproteins. *Current topics in microbiology and immunology* **157**:187-253.
349. **da Silva ES, Mulinge M, Bercoff DP.** 2013. The frantic play of the concealed HIV envelope cytoplasmic tail. *Retrovirology* **10**:54.
350. **Eisenberg D, Wesson M.** 1990. The most highly amphiphilic alpha-helices include two amino acid segments in human immunodeficiency virus glycoprotein 41. *Biopolymers* **29**:171-177.
351. **Kliger Y, Shai Y.** 1997. A leucine zipper-like sequence from the cytoplasmic tail of the HIV-1 envelope glycoprotein binds and perturbs lipid bilayers. *Biochemistry* **36**:5157-5169.
352. **Yang C, Spies CP, Compans RW.** 1995. The human and simian immunodeficiency virus envelope glycoprotein transmembrane subunits are palmitoylated. *Proc Natl Acad Sci U S A* **92**:9871-9875.
353. **Viard M, Ablan SD, Zhou M, Veenstra TD, Freed EO, Raviv Y, Blumenthal R.** 2008. Photoinduced reactivity of the HIV-1 envelope glycoprotein with a membrane-embedded probe reveals insertion of portions of the HIV-1 Gp41 cytoplasmic tail into the viral membrane. *Biochemistry* **47**:1977-1983.
354. **Chernomordik L, Chanturiya AN, Suss-Toby E, Nora E, Zimmerberg J.** 1994. An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. *J Virol* **68**:7115-7123.
355. **Miller MA, Cloyd MW, Liebmann J, Rinaldo CR, Jr., Islam KR, Wang SZ, Mietzner TA, Montelaro RC.** 1993. Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virology* **196**:89-100.
356. **Cleveland SM, McLain L, Cheung L, Jones TD, Hollier M, Dimmock NJ.** 2003. A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its exposure on the surface of the virion. *The Journal of general virology* **84**:591-602.
357. **Kennedy RC, Henkel RD, Pauletti D, Allan JS, Lee TH, Essex M, Dreesman GR.** 1986. Antiserum to a synthetic peptide recognizes the HTLV-III envelope glycoprotein. *Science* **231**:1556-1559.
358. **Hollier MJ, Dimmock NJ.** 2005. The C-terminal tail of the gp41 transmembrane envelope glycoprotein of HIV-1 clades A, B, C, and D may exist in two conformations: an analysis of sequence, structure, and function. *Virology* **337**:284-296.
359. **Steckbeck JD, Sun C, Sturgeon TJ, Montelaro RC.** 2010. Topology of the C-terminal tail of HIV-1 gp41: differential exposure of the Kennedy epitope on cell and viral membranes. *PLoS one* **5**:e15261.
360. **Waheed AA, Ablan SD, Roser JD, Sowder RC, Schaffner CP, Chertova E, Freed EO.** 2007. HIV-1 escape from the entry-inhibiting effects of a cholesterol-binding compound via cleavage of gp41 by the viral protease. *Proc Natl Acad Sci U S A* **104**:8467-8471.
361. **Steckbeck JD, Sun C, Sturgeon TJ, Montelaro RC.** 2013. Detailed Topology Mapping Reveals Substantial Exposure of the "Cytoplasmic" C-Terminal Tail (CTT) Sequences in HIV-1 Env Proteins at the Cell Surface. *PLoS one* **8**:e65220.
362. **Postler TS, Martinez-Navio JM, Yuste E, Desrosiers RC.** 2012. Evidence against extracellular exposure of a highly immunogenic region in the C-terminal domain of the simian immunodeficiency virus gp41 transmembrane protein. *J Virol* **86**:1145-1157.
363. **Liu S, Kondo N, Long Y, Xiao D, Iwamoto A, Matsuda Z.** 2010. Membrane topology analysis of HIV-1 envelope glycoprotein gp41. *Retrovirology* **7**:100.
364. **Lu L, Zhu Y, Huang J, Chen X, Yang H, Jiang S, Chen YH.** 2008. Surface exposure of the HIV-1 env cytoplasmic tail LLP2 domain during the membrane fusion process: interaction with gp41 fusion core. *The Journal of biological chemistry* **283**:16723-16731.
365. **Wilk T, Pfeiffer T, Bosch V.** 1992. Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. *Virology* **189**:167-177.
366. **Akari H, Fukumori T, Adachi A.** 2000. Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions. *J Virol* **74**:4891-4893.
367. **Kalia V, Sarkar S, Gupta P, Montelaro RC.** 2003. Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. *J Virol* **77**:3634-3646.
368. **Murakami T, Freed EO.** 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. *Proc Natl Acad Sci U S A* **97**:343-348.
369. **Sandrin V, Cosset FL.** 2006. Intracellular versus cell surface assembly of retroviral pseudotypes is determined by the cellular localization of the viral glycoprotein, its capacity to interact with Gag, and the expression of the Nef protein. *The Journal of biological chemistry* **281**:528-542.

370. **Emerson V, Haller C, Pfeiffer T, Fackler OT, Bosch V.** 2010. Role of the C-terminal domain of the HIV-1 glycoprotein in cell-to-cell viral transmission between T lymphocytes. *Retrovirology* **7**:43.
371. **Sandrin V, Russell SJ, Cosset FL.** 2003. Targeting retroviral and lentiviral vectors. *Current topics in microbiology and immunology* **281**:137-178.
372. **Leung K, Kim JO, Ganesh L, Kabat J, Schwartz O, Nabel GJ.** 2008. HIV-1 assembly: viral glycoproteins segregate quantally to lipid rafts that associate individually with HIV-1 capsids and virions. *Cell host & microbe* **3**:285-292.
373. **Jorgenson RL, Vogt VM, Johnson MC.** 2009. Foreign glycoproteins can be actively recruited to virus assembly sites during pseudotyping. *J Virol* **83**:4060-4067.
374. **Cronin J, Zhang XY, Reiser J.** 2005. Altering the tropism of lentiviral vectors through pseudotyping. *Current gene therapy* **5**:387-398.
375. **Murakami T.** 2012. Retroviral env glycoprotein trafficking and incorporation into virions. *Molecular biology international* **2012**:682850.
376. **Bhattacharya J, Repik A, Clapham PR.** 2006. Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes. *J Virol* **80**:5292-5300.
377. **Lindwasser OW, Resh MD.** 2001. Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. *J Virol* **75**:7913-7924.
378. **Ono A, Waheed AA, Freed EO.** 2007. Depletion of cellular cholesterol inhibits membrane binding and higher-order multimerization of human immunodeficiency virus type 1 Gag. *Virology* **360**:27-35.
379. **Rowell JF, Stanhope PE, Siliciano RF.** 1995. Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. *Journal of immunology* **155**:473-488.
380. **Ohno H, Aguilar RC, Fournier MC, Hennecke S, Cosson P, Bonifacino JS.** 1997. Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. *Virology* **238**:305-315.
381. **Ohno H, Fournier MC, Poy G, Bonifacino JS.** 1996. Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. *The Journal of biological chemistry* **271**:29009-29015.
382. **Batonick M, Favre M, Boge M, Spearman P, Honing S, Thali M.** 2005. Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2. *Virology* **342**:190-200.
383. **Camus G, Segura-Morales C, Molle D, Lopez-Verges S, Begon-Pescia C, Cazevielle C, Schu P, Bertrand E, Berlioz-Torrent C, Basyuk E.** 2007. The clathrin adaptor complex AP-1 binds HIV-1 and MLV Gag and facilitates their budding. *Molecular biology of the cell* **18**:3193-3203.
384. **Bauby H, Lopez-Verges S, Hoeffel G, Delcroix-Genete D, Janvier K, Mammano F, Hosmalin A, Berlioz-Torrent C.** 2010. TIP47 is required for the production of infectious HIV-1 particles from primary macrophages. *Traffic* **11**:455-467.
385. **Lopez-Verges S, Camus G, Blot G, Beauvois R, Benarous R, Berlioz-Torrent C.** 2006. Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. *Proc Natl Acad Sci U S A* **103**:14947-14952.
386. **Checkley MA, Luttge BG, Mercredi PY, Kyere SK, Donlan J, Murakami T, Summers MF, Cocklin S, Freed EO.** 2013. Reevaluation of the requirement for TIP47 in human immunodeficiency virus type 1 envelope glycoprotein incorporation. *J Virol* **87**:3561-3570.
387. **Beaumont E, Vendrame D, Verrier B, Roch E, Biron F, Barin F, Mammano F, Brand D.** 2009. Matrix and envelope coevolution revealed in a patient monitored since primary infection with human immunodeficiency virus type 1. *J Virol* **83**:9875-9889.
388. **Lodge R, Lalonde JP, Lemay G, Cohen EA.** 1997. The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. *The EMBO journal* **16**:695-705.
389. **Edwards TG, Wyss S, Reeves JD, Zolla-Pazner S, Hoxie JA, Doms RW, Baribaud F.** 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. *J Virol* **76**:2683-2691.
390. **Wyss S, Dimitrov AS, Baribaud F, Edwards TG, Blumenthal R, Hoxie JA.** 2005. Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail. *J Virol* **79**:12231-12241.
391. **Zhu Y, Lu L, Chao L, Chen YH.** 2007. Important changes in biochemical properties and function of mutated LLP12 domain of HIV-1 gp41. *Chemical biology & drug design* **70**:311-318.
392. **Chen SS, Lee SF, Wang CT.** 2001. Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope transmembrane protein gp41. *J Virol* **75**:9925-9938.
393. **Abrahamyan LG, Mkrtychyan SR, Binley J, Lu M, Melikyan GB, Cohen FS.** 2005. The cytoplasmic tail slows the folding of human immunodeficiency virus type 1 Env from a late prebundle configuration into the six-helix bundle. *J Virol* **79**:106-115.
394. **Lee SF, Wang CT, Liang JY, Hong SL, Huang CC, Chen SS.** 2000. Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41. *The Journal of biological chemistry* **275**:15809-15819.

395. **Egan MA, Carruth LM, Rowell JF, Yu X, Siliciano RF.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. *J Virol* **70**:6547-6556.
396. **Berlioz-Torrent C, Shacklett BL, Erdtmann L, Delamarre L, Bouchaert I, Sonigo P, Dokhelar MC, Benarous R.** 1999. Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. *J Virol* **73**:1350-1361.
397. **Boge M, Wyss S, Bonifacino JS, Thali M.** 1998. A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 envelope glycoprotein via interaction with the AP-2 clathrin adaptor. *The Journal of biological chemistry* **273**:15773-15778.
398. **LaBranche CC, Sauter MM, Haggarty BS, Vance PJ, Romano J, Hart TK, Bugelski PJ, Marsh M, Hoxie JA.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. *J Virol* **69**:5217-5227.
399. **Dong X, Li H, Derdowski A, Ding L, Burnett A, Chen X, Peters TR, Dermody TS, Woodruff E, Wang JJ, Spearman P.** 2005. AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. *Cell* **120**:663-674.
400. **Liu L, Sutton J, Woodruff E, Villalta F, Spearman P, Dong X.** 2012. Defective HIV-1 particle assembly in AP-3-deficient cells derived from patients with Hermansky-Pudlak syndrome type 2. *J Virol* **86**:11242-11253.
401. **Byland R, Vance PJ, Hoxie JA, Marsh M.** 2007. A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein. *Molecular biology of the cell* **18**:414-425.
402. **Wyss S, Berlioz-Torrent C, Boge M, Blot G, Honing S, Benarous R, Thali M.** 2001. The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]. *J Virol* **75**:2982-2992.
403. **Bhakta SJ, Shang L, Prince JL, Claiborne DT, Hunter E.** 2011. Mutagenesis of tyrosine and di-leucine motifs in the HIV-1 envelope cytoplasmic domain results in a loss of Env-mediated fusion and infectivity. *Retrovirology* **8**:37.
404. **Blot G, Janvier K, Le Panse S, Benarous R, Berlioz-Torrent C.** 2003. Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity. *J Virol* **77**:6931-6945.
405. **DenBoer LM, Hardy-Smith PW, Hogan MR, Cockram GP, Audas TE, Lu R.** 2005. Luman is capable of binding and activating transcription from the unfolded protein response element. *Biochemical and biophysical research communications* **331**:113-119.
406. **Liang G, Audas TE, Li Y, Cockram GP, Dean JD, Martyn AC, Kokame K, Lu R.** 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Molecular and cellular biology* **26**:7999-8010.
407. **Blot G, Lopez-Verges S, Treand C, Kubat NJ, Delcroix-Genete D, Emiliani S, Benarous R, Berlioz-Torrent C.** 2006. Luman, a new partner of HIV-1 TMgp41, interferes with Tat-mediated transcription of the HIV-1 LTR. *Journal of molecular biology* **364**:1034-1047.
408. **Audas TE, Li Y, Liang G, Lu R.** 2008. A novel protein, Luman/CREB3 recruitment factor, inhibits Luman activation of the unfolded protein response. *Molecular and cellular biology* **28**:3952-3966.
409. **Zhang H, Wang L, Kao S, Whitehead IP, Hart MJ, Liu B, Duus K, Burrige K, Der CJ, Su L.** 1999. Functional interaction between the cytoplasmic leucine-zipper domain of HIV-1 gp41 and p115-RhoGEF. *Current biology* : **CB 9**:1271-1274.
410. **Wang L, Zhang H, Solski PA, Hart MJ, Der CJ, Su L.** 2000. Modulation of HIV-1 replication by a novel RhoA effector activity. *Journal of immunology* **164**:5369-5374.
411. **Postler TS, Desrosiers RC.** 2012. The cytoplasmic domain of the HIV-1 glycoprotein gp41 induces NF-kappaB activation through TGF-beta-activated kinase 1. *Cell host & microbe* **11**:181-193.
412. **Griffin GE, Leung K, Folks TM, Kunkel S, Nabel GJ.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature* **339**:70-73.
413. **Alcami J, Lain de Lera T, Folgueira L, Pedraza MA, Jacque JM, Bachelerie F, Noriega AR, Hay RT, Harrich D, Gaynor RB, et al.** 1995. Absolute dependence on kappa B responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. *The EMBO journal* **14**:1552-1560.
414. **Schiavoni I, Trapp S, Santarcangelo AC, Piacentini V, Pugliese K, Baur A, Federico M.** 2004. HIV-1 Nef enhances both membrane expression and virion incorporation of Env products. A model for the Nef-dependent increase of HIV-1 infectivity. *The Journal of biological chemistry* **279**:22996-23006.
415. **Ishikawa H, Sasaki M, Noda S, Koga Y.** 1998. Apoptosis induction by the binding of the carboxyl terminus of human immunodeficiency virus type 1 gp160 to calmodulin. *J Virol* **72**:6574-6580.
416. **Micoli KJ, Pan G, Wu Y, Williams JP, Cook WJ, McDonald JM.** 2000. Requirement of calmodulin binding by HIV-1 gp160 for enhanced FAS-mediated apoptosis. *The Journal of biological chemistry* **275**:1233-1240.
417. **Srinivas SK, Srinivas RV, Anantharamaiah GM, Compans RW, Segrest JP.** 1993. Cytosolic domain of the human immunodeficiency virus envelope glycoproteins binds to calmodulin and inhibits calmodulin-regulated proteins. *The Journal of biological chemistry* **268**:22895-22899.

418. **Kim EM, Lee KH, Kim JW.** 1999. The cytoplasmic domain of HIV-1 gp41 interacts with the carboxyl-terminal region of alpha-catenin. *Molecules and cells* **9**:281-285.
419. **Kim JT, Kim EM, Lee KH, Choi JE, Jhun BH, Kim JW.** 2002. Leucine zipper domain of HIV-1 gp41 interacted specifically with alpha-catenin. *Biochemical and biophysical research communications* **291**:1239-1244.
420. **Emerson V, Holtkotte D, Pfeiffer T, Wang IH, Schnolzer M, Kempf T, Bosch V.** 2010. Identification of the cellular prohibitin 1/prohibitin 2 heterodimer as an interaction partner of the C-terminal cytoplasmic domain of the HIV-1 glycoprotein. *J Virol* **84**:1355-1365.
421. **Evans DT, Tillman KC, Desrosiers RC.** 2002. Envelope glycoprotein cytoplasmic domains from diverse lentiviruses interact with the prenylated Rab acceptor. *J Virol* **76**:327-337.
422. **Blancou P, Evans DT, Desrosiers RC.** 2005. PRA1 co-localizes with envelope but does not influence primate lentivirus production, infectivity or envelope incorporation. *The Journal of general virology* **86**:1785-1790.
423. **Day JR, Munk C, Guatelli JC.** 2004. The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. *J Virol* **78**:1069-1079.
424. **Lambele M, Labrosse B, Roch E, Moreau A, Verrier B, Barin F, Roingeard P, Mammano F, Brand D.** 2007. Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of envelope glycoproteins and viral assembly. *J Virol* **81**:125-140.
425. **Steckbeck JD, Craigo JK, Barnes CO, Montelaro RC.** 2011. Highly conserved structural properties of the C-terminal tail of HIV-1 gp41 protein despite substantial sequence variation among diverse clades: implications for functions in viral replication. *The Journal of biological chemistry* **286**:27156-27166.
426. **Ramakrishnan R, Mehta R, Sundaravaradan V, Davis T, Ahmad N.** 2006. Characterization of HIV-1 envelope gp41 genetic diversity and functional domains following perinatal transmission. *Retrovirology* **3**:42.
427. **Pollakis G, Abebe A, Kliphuis A, Chalaby MI, Bakker M, Mengistu Y, Brouwer M, Goudsmit J, Schuitemaker H, Paxton WA.** 2004. Phenotypic and genotypic comparisons of CCR5- and CXCR4-tropic human immunodeficiency virus type 1 biological clones isolated from subtype C-infected individuals. *J Virol* **78**:2841-2852.
428. **Rangel HR, Weber J, Chakraborty B, Gutierrez A, Marotta ML, Mirza M, Kiser P, Martinez MA, Este JA, Quinones-Mateu ME.** 2003. Role of the human immunodeficiency virus type 1 envelope gene in viral fitness. *J Virol* **77**:9069-9073.
429. **Jacobs GB, Bock S, Schuch A, Moschall R, Schrom EM, Zahn J, Reuter C, Preiser W, Rethwilm A, Engelbrecht S, Kerkau T, Bodem J.** 2012. Construction of a high titer infectious HIV-1 subtype C proviral clone from South Africa. *Viruses* **4**:1830-1843.
430. **Bhattacharya J, Peters PJ, Clapham PR.** 2004. Human immunodeficiency virus type 1 envelope glycoproteins that lack cytoplasmic domain cysteines: impact on association with membrane lipid rafts and incorporation onto budding virus particles. *J Virol* **78**:5500-5506.
431. **Rouso I, Mixon MB, Chen BK, Kim PS.** 2000. Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. *Proc Natl Acad Sci U S A* **97**:13523-13525.
432. **Lodge R, Gottlinger H, Gabuzda D, Cohen EA, Lemay G.** 1994. The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells. *J Virol* **68**:4857-4861.
433. **Dubay JW, Roberts SJ, Hahn BH, Hunter E.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. *J Virol* **66**:6616-6625.
434. **Yu X, Yuan X, McLane MF, Lee TH, Essex M.** 1993. Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. *J Virol* **67**:213-221.
435. **Monel B, Beaumont E, Vendrame D, Schwartz O, Brand D, Mammano F.** 2012. HIV cell-to-cell transmission requires the production of infectious virus particles and does not proceed through env-mediated fusion pores. *J Virol* **86**:3924-3933.
436. **Durham ND, Yewdall AW, Chen P, Lee R, Zony C, Robinson JE, Chen BK.** 2012. Neutralization resistance of virological synapse-mediated HIV-1 infection is regulated by the gp41 cytoplasmic tail. *J Virol* **86**:7484-7495.
437. **Gavegnano C, Kennedy EM, Kim B, Schinazi RF.** 2012. The Impact of Macrophage Nucleotide Pools on HIV-1 Reverse Transcription, Viral Replication, and the Development of Novel Antiviral Agents. *Molecular biology international* **2012**:625983.
438. **Gousset K, Ablan SD, Coren LV, Ono A, Soheilian F, Nagashima K, Ott DE, Freed EO.** 2008. Real-time visualization of HIV-1 GAG trafficking in infected macrophages. *PLoS pathogens* **4**:e1000015.
439. **Groot F, Welsch S, Sattentau QJ.** 2008. Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. *Blood* **111**:4660-4663.
440. **Jolly C, Sattentau QJ.** 2004. Retroviral spread by induction of virological synapses. *Traffic* **5**:643-650.
441. **Welsch S, Groot F, Krausslich HG, Keppler OT, Sattentau QJ.** 2011. Architecture and regulation of the HIV-1 assembly and holding compartment in macrophages. *J Virol* **85**:7922-7927.
442. **Welsch S, Keppler OT, Habermann A, Allespach I, Krijnse-Locker J, Krausslich HG.** 2007. HIV-1 buds predominantly at the plasma membrane of primary human macrophages. *PLoS pathogens* **3**:e36.
443. **Gaudin R, de Alencar BC, Jouve M, Berre S, Le Boudier E, Schindler M, Varthaman A, Gobert FX, Benaroch P.** 2012. Critical role for the kinesin KIF3A in the HIV life cycle in primary human macrophages. *The Journal of cell biology* **199**:467-479.

444. **Cymer F, Veerappan A, Schneider D.** 2012. Transmembrane helix-helix interactions are modulated by the sequence context and by lipid bilayer properties. *Biochimica et biophysica acta* **1818**:963-973.
445. **Unterreitmeier S, Fuchs A, Schaffler T, Heym RG, Frishman D, Langosch D.** 2007. Phenylalanine promotes interaction of transmembrane domains via GxxxG motifs. *Journal of molecular biology* **374**:705-718.
446. **Prakash A, Janosi L, Doxastakis M.** 2011. GxxxG motifs, phenylalanine, and cholesterol guide the self-association of transmembrane domains of ErbB2 receptors. *Biophysical journal* **101**:1949-1958.
447. **Davis MR, Jiang J, Zhou J, Freed EO, Aiken C.** 2006. A mutation in the human immunodeficiency virus type 1 Gag protein destabilizes the interaction of the envelope protein subunits gp120 and gp41. *J Virol* **80**:2405-2417.

ANNEXE

HIV-1 Tropism Determination Using a Phenotypic Env Recombinant Viral Assay Highlights Overestimation of CXCR4-Usage by Genotypic Prediction Algorithms for CRRF01_AE and CRF02_AG

Martin Mulinge¹, Morgane Lemaire¹, Jean-Yves Servais¹, Arkadiusz Rybicki¹, Daniel Struck¹, Eveline Santos da Silva¹, Chris Verhofstede³, Yolanda Lie⁴, Carole Seguin-Devaux¹, Jean-Claude Schmit^{1,2}, Danielle Perez Bercoff^{1*}

1 Laboratory of Retrovirology, Centre Recherche Public de la Santé, Luxembourg, Luxembourg, **2** Service National des Maladies Infectieuses, Centre Hospitalier de Luxembourg, Luxembourg, Luxembourg, **3** AIDS Reference Laboratory, Ghent University, Ghent, Belgium, **4** Monogram Biosciences Inc., South San Francisco, California, United States of America

Abstract

Background: Human Immunodeficiency virus type-1 (HIV) entry into target cells involves binding of the viral envelope (Env) to CD4 and a coreceptor, mainly CCR5 or CXCR4. The only currently licensed HIV entry inhibitor, maraviroc, targets CCR5, and the presence of CXCR4-using strains must be excluded prior to treatment. Co-receptor usage can be assessed by phenotypic assays or through genotypic prediction. Here we compared the performance of a phenotypic Env-Recombinant Viral Assay (RVA) to the two most widely used genotypic prediction algorithms, Geno2Pheno_[coreceptor] and webPSSM.

Methods: Co-receptor tropism of samples from 73 subtype B and 219 non-B infections was measured phenotypically using a luciferase-tagged, NL4-3-based, RVA targeting Env. In parallel, tropism was inferred genotypically from the corresponding V3-loop sequences using Geno2Pheno_[coreceptor] (5–20% FPR) and webPSSM-R5X4. For discordant samples, phenotypic outcome was retested using co-receptor antagonists or the validated Trofile[®] Enhanced-Sensitivity-Tropism-Assay.

Results: The lower detection limit of the RVA was 2.5% and 5% for X4 and R5 minority variants respectively. A phenotype/genotype result was obtained for 210 samples. Overall, concordance of phenotypic results with Geno2Pheno_[coreceptor] was 85.2% and concordance with webPSSM was 79.5%. For subtype B, concordance with Geno2pheno_[coreceptor] was 94.4% and concordance with webPSSM was 79.6%. High concordance of genotypic tools with phenotypic outcome was seen for subtype C (90% for both tools). Main discordances involved CRF01_AE and CRF02_AG for both algorithms (CRF01_AE: 35.9% discordances with Geno2Pheno_[coreceptor] and 28.2% with webPSSM; CRF02_AG: 20.7% for both algorithms). Genotypic prediction overestimated CXCR4-usage for both CRFs. For webPSSM, 40% discordance was observed for subtype A.

Conclusions: Phenotypic assays remain the most accurate for most non-B subtypes and new subtype-specific rules should be developed for non-B subtypes, as research studies more and more draw conclusions from genotypically-inferred tropism, and to avoid unnecessarily precluding patients with limited treatment options from receiving maraviroc or other entry inhibitors.

Citation: Mulinge M, Lemaire M, Servais J-Y, Rybicki A, Struck D, et al. (2013) HIV-1 Tropism Determination Using a Phenotypic Env Recombinant Viral Assay Highlights Overestimation of CXCR4-Usage by Genotypic Prediction Algorithms for CRRF01_AE and CRF02_AG. PLoS ONE 8(5): e60566. doi:10.1371/journal.pone.0060566

Editor: Zhiwei Chen, The University of Hong Kong, Hong Kong

Received: November 23, 2012; **Accepted:** February 28, 2013; **Published:** May 8, 2013

Copyright: © 2013 Mulinge et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Luxembourg Ministry of Research and Education Grant #MCESR LRTV20081003. MM and ESS were supported by PhD scholarships from the Fonds National de la Recherche (# AFR grant PHD08-074 and AFR grant PHD-09-115, respectively). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: One of the authors, Yolanda Lie, is an employee of a commercial company, Monogram Bioscience. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: danielle.perezbercoff@crp-sante.lu

Introduction

Entry of the Human Immunodeficiency Virus type 1 (HIV-1) into target cells is a three-step process involving sequential interactions between the viral envelope glycoprotein trimer (Env) with the CD4 receptor and one of two coreceptors, CCR5 or

CXCR4 [1–7]. Binding to the CD4 receptor induces a series of conformational changes within Env that expose the third hypervariable region (V3-loop), which in turn binds the coreceptor, ultimately leading to the so-called “fusion-active” state required for fusion of the viral and cellular membranes [8]. The V3-loop, which is the main determinant of coreceptor binding,

therefore largely accounts for viral tropism [9,10], and viral strains are classified as R5, when using the CCR5 coreceptor for viral entry, X4 when using CXCR4, and dual-tropic or mixed (R5X4) when using both coreceptors [11]. Other regions of Env, and namely the V1/V2 loops and the constant region C4, have been shown to also participate in viral tropism [12,13].

R5 strains are generally predominant during the early stages of infection and are thought to be preferentially transmitted by distinct, not yet fully elucidated processes [14,15]. As infection progresses, viral strains feature increased variability within the infected host, and particularly, Envs acquire broadened coreceptor usage. At late stages of infection, X4 strains become dominant in 50% of patients infected with subtype B strains [16], but subtype-related specificities have been reported [17–20]. X4 strains replicate more rapidly than R5 strains *in vitro* and have been associated with increased cytopathicity. *In vivo*, the appearance of X4 strains correlates with a sharp decline of CD4⁺ T cells and the onset of AIDS defining symptoms [21].

With the advent of entry inhibitors targeting CCR5, such as maraviroc, monitoring coreceptor usage has become prerequisite to the prescription of such entry inhibitors, in order to exclude the presence of X4 or R5/X4 variants [22–24]. Under maraviroc selective pressure, pre-existing X4 or DM strains can be selected. CCR5 is a cellular target and resistance to maraviroc most often arises through the re-emergence of archived minority X4 strains rather than through a coreceptor usage switch or through the acquisition of mutations that allow gp120 to engage with drug-bound CCR5 [25–28]. Viral coreceptor usage can be measured *in vitro* by phenotypic and genotypic assays [29]. Various phenotypic assays based on different techniques are currently available, including the Trofile® Enhanced-Sensitivity-Trofile-Assay (ESTA) (Monogram Biosciences, South San Francisco, CA) [30], the Virco phenotypic test (Virco BVBA, Mechelen, Belgium) and others [30–33], which are based on pseudovirions, and assays based on recombinant viruses, among which are the Phenoscript test (VIRalliance, France) [34] and the Toulouse Tropism Test [35]. These assays, their design and performance are summarized in Table 1. The Trofile assay is the most widely used in the clinic. It features a high sensitivity in detecting X4 minority variants [30]. Nonetheless, because phenotypic tests are expensive, time consuming and require specialized laboratories, more interest has been driven toward genotypic testing. Genotypic assays are based on predictions of coreceptor usage from the V3-loop sequence using bioinformatic tools and algorithms. They are currently preferred in Europe due to their accessibility, rapid turnaround and low cost [36,37]. Many prediction tools are available, with similar specificities and sensitivities despite the fact that they are based on different algorithms involving the 11/25 rule, the number of positively charged AA, the overall net charge of the V3 loop, or combinations thereof. Among them, Geno2Pheno_[coreceptor] [38] and webPSSM [39] are the most widely used. European Guidelines for HIV patient management currently recommend the use of Geno2Pheno_[coreceptor] with a 10% false positive rate (FPR) cut-off, which has been shown to provide the best balance between specificity and sensitivity for predicting R5 or X4/R5X4 tropism [40–43]. The major caveat of such algorithms however lies in the fact that they are based on V3-loop sequences from subtypes B and C mainly, and inadequacies requiring fine-tuning or subtype-specific rules have been reported [19,44–46].

This study evaluates the performance of an in-house Env recombinant viral assay (Env-RVA) targeting the full HIV Env ectodomain, in comparison to Geno2Pheno_[coreceptor] and webPSSM (Fig. 1). Concordance between the RVA and

Geno2Pheno_[coreceptor] with a false positive cutoff set at 10% was 85.2% and concordance with webPSSM was 79.6%. Discordant results most often involved non-B subtypes, particularly subtype A1 for webPSSM and CRF01_AE and CRF02_AG for both algorithms. Repeat experiments in the presence of coreceptor antagonists and, when possible, testing the sample using the Trofile ESTA, confirmed phenotypic results of the RVA. Taken together, these results highlight that prediction algorithms are not always accurate for predicting tropism of some subtypes, particularly CRF01_AE and CRF02_AG and underscore the usefulness of maintaining phenotypic testing as well as to adapt algorithms for certain subtypes and recombinant forms.

Materials and Methods

Study Population

Plasma samples from 292 patients infected with HIV-1 subtypes B (73), A1 (17), C (21), D (15), F (15), G (55), CRF01_AE (42) and CRF02_AG (54) were included in the study. Left-over plasma obtained from samples for routine clinical tests was used. Ethical approval for use of left-over plasma was obtained from the Comité National d’Ethique pour la Recherche in Luxembourg for HIV assay validation in Luxembourg for HIV assay validation without patient informed consent. The IRB waived the need for written informed consent from the participants for this study. HIV-1 subtypes were determined from HIV-1 PR-RT and Env sequences spanning the V3-loop using COMET (www.comet.retrovirology.lu) and the REGA HIV subtyping tool [47]. CD4 counts ranged from 11 cells/mm³ to 1460 cells/mm³ (mean: 391 and median: 356 cells/mm³). Plasma viral load (VL) (Abbott m2000 RealTime HIV-1 assay) ranged from 466 to 1,350,000 RNA copies/ml for all subtypes, with a mean and median of 14,055 and 71,115 RNA copies/ml respectively. 26/73 subtype B samples with VL <10³ RNA copies/mL were also included to assess the RVA’s performance for VL <10³ RNA copies/ml.

Cell Culture

HEK293T cells were obtained from ATCC and were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum, 1% Glutamate, 50 µg/ml Penicillin and 50 µg/ml Streptomycin. U87.CD4.CCR5 and U87.CD4.CXCR4 cells were obtained from the NIH AIDS Reagent Program and were maintained in DMEM containing 10% Fetal Bovine Serum, 1% Glutamate, 300 µg/ml Geneticin and 1 µg/ml Puromycin to maintain CD4 and co-receptor expression respectively. All media supplements and antibiotics were sourced from Invitrogen, Belgium.

Env Amplification

One ml of plasma or of Env-recombinant virus supernatant was centrifuged at 24,000×g for 1 hour at 4°C and viral RNA was extracted from the pellet using the Qiagen Viral RNA extraction kit (Qiagen, Belgium) according to the manufacturer’s instructions. Viral cDNA was synthesized in a one-step RT-PCR reaction using forward primer KVL008 and reverse primer KVL009 [48] in 50 µl mix containing 5 µl viral RNA, 20 µM of each primer, 1 µl SuperScript III One-Step RT-PCR with Platinum *Taq* High Fidelity mix and 8 units RNaseOUT (all from Invitrogen, Merelbeke, Belgium) under the following conditions: initial denaturation at 94°C for 2 mins and 40 amplification cycles (94°C for 15 s, 60°C for 30 s, 68°C for 4 mins) followed by a final 10 mins extension step at 68°C. 2 µl of the amplified cDNA was further amplified using forward primer MM1 FP (5′-GCCTTAGGCATCTCTTATGGCAGGAAGAAG-3′)

Table 1. Characteristics of phenotypic assays developed for determination of HIV-1 coreceptor usage.

Assay	System	Env target	Producer cells	Target cells	readout	Detection limit	reference	
ESTA	Pseudovirions	Full Env	Hek293T	U87.CD4.R5/R4	Luciferase	0.3% X4	[30]	
Virco	Recombinant viral particles	gp120 (NH2-V4)	Hek293T	U87.CD4.R5/R4	eGFP	<10% X4	[32]	
Commercial assays	Phenoscript	Recombinant viral particles	V1-V3	Hek293T	U373MG.CD4.R5/X4	β -Galactosidase	5–10% X4	[34]
	PhenXR	Recombinant viral particles	V1-V3	HeLa	SX22-HeLaR5/X4	β -Galactosidase	1% X4	[71]
	Toulouse tropism test (TTT)	Recombinant viral particles	Env Ectodomain	Hek293T	U87.CD4.R5/R4	Luciferase	0.5% X4	[35]
Non-commercial assays	Recombinant viral particles	Full Env	Hek293T	U87.CD4.R5/R4 or GHOST. CD4.R5/X4	Luciferase	1% X4	[72]	
	Pseudovirions	Full Env	Hek293T	U87.CD4.R5/R4	Luciferase	1% X4 (high VL) 5% X4 (low VL)	[33]	
	CRP Env-RVA	Recombinant viral particles	Env ectodomain	Hek293T	U87.CD4.R5/R4	Luciferase	2.5% X4 5% R5	

Abbreviations: ESTA: Enhanced Sensitivity Trofile Assay; Env: Envelope; eGFP: enhanced Green Fluorescent protein; X4: CXCR4-using strains; R5:CCR5-using strains. doi:10.1371/journal.pone.0060566.t001

and reverse primer rec HR1-2_RP (5'-CTCTCTCTTC-ACCTTCTTCTTC-3') [27] in a 50 μ l reaction mix containing 2 mM MgSO₄, 0.2 mM of each dNTP, 20 μ M of each primer, 2.5 Units Platinum *Taq* High Fidelity DNA polymerase. The amplification conditions were: initial denaturation step at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, extension at 68°C for 3 min, and a final extension step at 68°C for 10 min. Amplification was verified by agarose gel electrophoresis. To avoid PCR selection, five independent amplifications were performed in parallel for each sample and were pooled for recombinant virus production and sequencing.

Sequencing

To circumvent a potential primer-linked bias, the V3-loop was sequenced directly from the same Env ectodomain amplicon used to produce Env-recombinant viruses (Fig. 1). Sequencing was performed using the BigDye Terminator v3.1 dye on Applied Biosystems 3500 xL DX genetic analyzer (Applied Biosystems Europe BV, Belgium), using sense primers 6951 and 6990 and reverse primers 7336 [49]. For sequencing of viral supernatants, viral RNA was extracted and amplified as described previously and a nested PCR was performed using 2 μ l of the Env cDNA, using primers KK1 [50] and DR8 [51] (400 nM each) in a mix containing 5 μ l 10 \times PCR Gold Buffer II, 20 μ M dNTPs, 200 μ M MgCl₂ and 0.5 μ l AmpliTaq Gold DNA polymerase (Applied Biosystems), in the following cycling conditions: denaturing for 10 mins at 95°C, followed by 40 amplification cycles (15 sec 95°C, 30 sec 55.5°C, 1 min 72°C) and a final 10 mins extension step at 72°C. For those viral supernatants that could not be sequenced using this method because of inadequate viral content, the viral RNA was amplified and sequenced using primers KK1 and DR8 (400 nM each) in a one-step RT-PCR reaction containing 10 μ l viral RNA, 1.5 μ l of each primer, 10 μ l 5 \times buffer, 40 μ M dNTPs, 0.1 μ l RNase inhibitor and 2 μ l Qiagen *Taq* (Qiagen), as follows: RT: 30 mins at 50°C, denaturation: 15 mins 95°C, 40 cycles of amplification (15 sec 95°C, 30 sec 55.5°C, 1 min 72°C) and a final 5 min extension step at 72°C, followed by an inner PCR using the

same conditions as above. V3-loop sequences are available under EMBL Nucleotide Sequence Database with accession numbers: HE972342-HE972511 and JN407569, JN407585, JN407591, JN407601, JN407602, JN407608, JN407609, JN407611, JN407624, JN407629, JN407632, JN407661, JN407676, JN407696, JN407704, JN407706, JN407709, JN407713, JN407726, JN407738, JN407740, JN407745, JN407747, JN407805, JN407808, JN407810, JN407813, JN407814, JN407816, JN407817, JN407836, JN407872, JN407949, JN407971, JN407987, JN407991, JN408004, JN408005, JN408022, JN408023, JN408027, JN408043 and JN408058.

Genotyping

The V3 nucleotide sequences were submitted to the Geno2-Pheno_[coreceptor] algorithm (<http://coreceptor.bioinf.mpi-inf.mpg.de>) setting the FPR set at 5%, 10%, 15% and 20% and to webPSSM (<http://indra.mullins.microbiol.washington.edu/PSSM/>) using the X4/R5 matrix. The webPSSM subtype C SINSI matrix was used for subtype C. When mixtures were present in the viral population, all possible combinations were submitted independently to webPSSM and the results were reported as numbers of R5, X4 or R5/X4 clones for comparison with the phenotypic assay.

Production of Recombinant Viruses

pNL4.3 Δ EC.Luc has been described elsewhere [27]. Briefly, pNL4.3 Δ EC.Luc is a NL4-3-derived plasmid deleted of the Env ectodomain (6225–8314), containing a AfeI restriction site in the place of Env for linearization and harboring a *firefly luciferase* gene in the place of *nef*. For Env-recombinant viral production, 70% confluent HEK293T cells were co-transfected with Afe I-linearized pNL4.3 Δ EC.Luc (Westburg, Netherlands) and patient-derived Env PCR amplicons using Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) according to manufacturer's instructions. HIV-1 NL4-3 (X4) and NL-AD8 (R5) were used as positive controls. Cell-free culture supernatants were collected 48

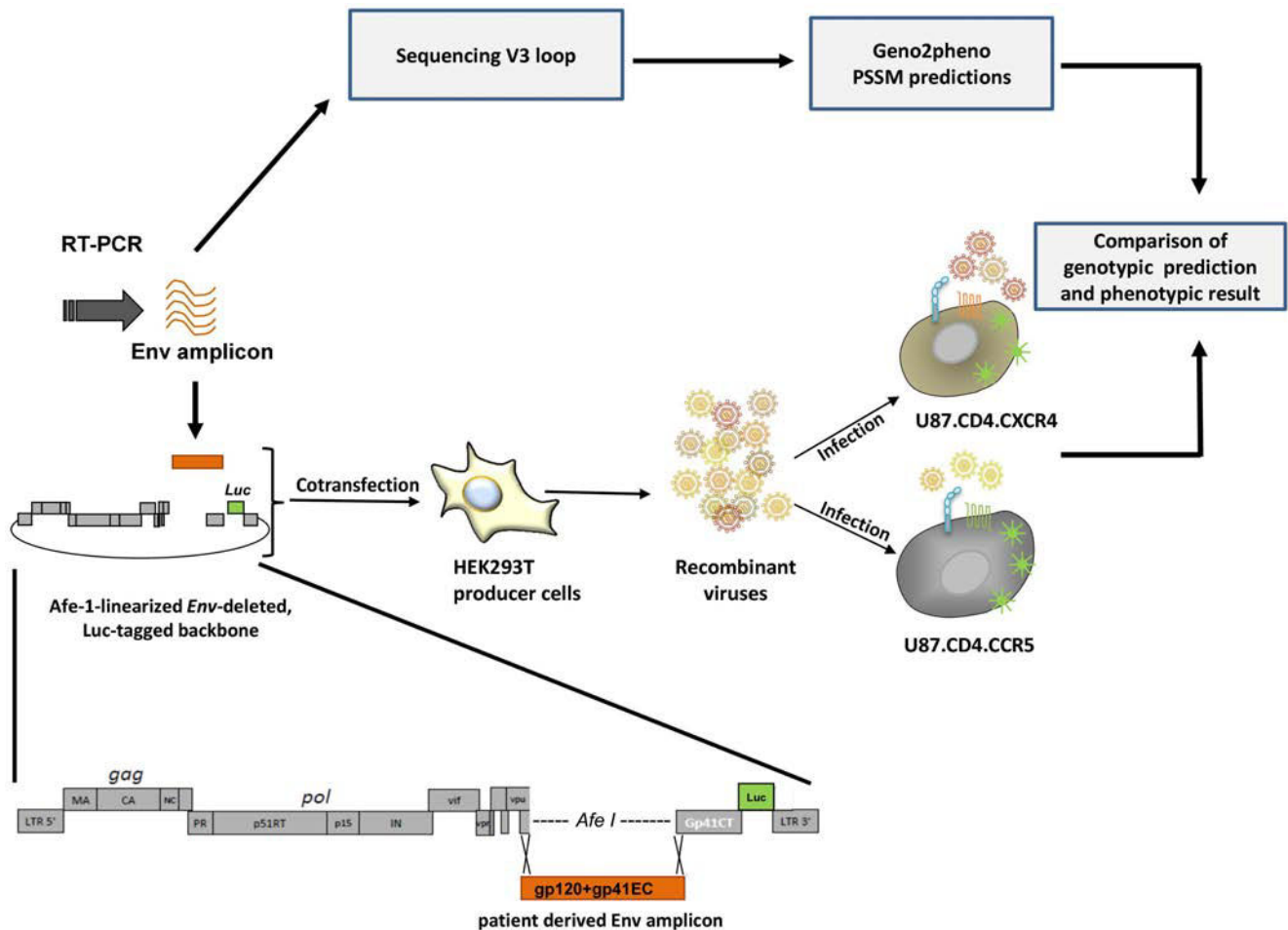


Figure 1. Study design/RVA design. Viral RNA was extracted from patient plasma RT-PCR amplified. Env amplicons spanning the Env ectodomain were further amplified through an inner PCR. Five independent PCRs were pooled to minimize PCR-selection. Recombinant viruses were produced by co-transfecting HEK293T cells with Afe I-linearized, luciferase-tagged, Env-deleted, viral backbone and patient-derived PCR amplicon. Normalized amounts of recombinant viruses were used to infect U87.CD4.CCR5 or U87.CD4.CXCR4 indicator cells. Infection was monitored by quantifying luminescence in the cell lysates. Depending on the outcome of the infection, viruses were classified as either CCR5 tropic, CXCR4 tropic or dual/mixed. The same patient-derived PCR amplicon used for viral production was sequenced and tropism inferred by Geno2Pheno_[coreceptor] and webPSSM algorithms. The phenotypic and genotypic results were compared. Abbreviations: Env EC: Env ectodomain; gp41-TM-CT: gp41 Transmembrane+cytoplasmic tail.
doi:10.1371/journal.pone.0060566.g001

hours post-transfection, clarified by centrifugation and stored at -80°C until use (Fig. 1). Viral production was determined by quantifying p24 capsid protein using a p24 ELISA test (Perkin Elmer, Amsterdam, Netherlands). Virus lacking an envelope produced by transfecting the sole linearized backbone was used to assess background noise.

Env Recombinant Viral Assay

10^4 U87.CD4.CCR5 or U87.CD4.CXCR4 cells in 96-well plates were infected with Env-recombinant viruses (200 pg p24, quantified by Perkin Elmer kit) by spinoculation at $1200\times g$ for 2 hours at 25°C [52], followed by incubation for 1 hour at 37°C . Medium was replaced and cells were cultured for a further 48 hours, after which luciferase activity was assayed using the Promega Luciferase assay kit (Promega, Leiden, Netherlands) according to manufacturer's instructions. Luminescence readout was performed on a Tecan microplate reader (Tecan, Switzerland) over one second exposure. All infections were performed in triplicate. Recombinant viruses were scored as positive for the

specific coreceptor if the luciferase signal was at least twice the background.

Where used, $1\ \mu\text{M}$ Maraviroc (CCR5 antagonist) or $1\ \mu\text{M}$ AMD3100 (CXCR4 antagonist) were added to the cells, the plate was centrifuged at $400\times g$ for 10 mins at 25°C and incubated at 37°C for 15 mins prior to addition of the recombinant viruses and spinoculation.

Trofile ESTA

19 samples (5 subtype B, 2 subtype A1, 2 subtype C, 1 subtype D, 3 CRF01_AE, 6 CRF02_AG) for which phenotypic results differed from the Geno2Pheno_[coreceptor]-inferred results and for which plasma was available were tested in the Trofile[®] ESTA.

Statistical Analyses

In this study, we did not assume the RVA nor the genotypic prediction tools to be the reference test to determine viral tropism, therefore concordance and Cohen kappa values were assessed using Statools (www.stattools.net). Concordance between the

phenotypically measured and genotypically predicted tropism was calculated as follows for each subtype or group of subtypes: Concordance = Number of samples with the same tropism by both assays/Total number of samples tested \times 100. The correlation between tests is usually considered good when kappa $>$ 0.6. Tropism measured using the RVA was considered to be concordant with Geno2Pheno_[coreceptor] prediction if they both detected pure R5 or pure X4. When dual/mixed strains were detected using the RVA, they were considered to be concordant with an X4 genotypic prediction and discordant with a R5 prediction, as Geno2Pheno only predicts the presence of X4 strains. For comparison with webPSSM, results were considered to be concordant if both assays provided the same result, i.e. R5, X4, or Mixed (D/M). D/M samples detected as a purely X4 or purely R5 sample by the other test were considered to be discordant. Sensitivity and specificity were calculated using GraphPad Prism version 5, setting the phenotypic results as 'true'.

Results

Detection of Minority Variants

To determine the threshold for detecting minority variants, mixtures containing known proportions of pNLAD8 (R5) and of pNL4-3 (X4) were PCR-amplified and used to produce R5/X4 mixed Env recombinant viruses. U87.CD4.CCR5 and U87.CD4.CXCR4 indicator cells were infected with 2-fold serial dilutions of the recombinant NLAD8:NL4-3 mixtures (20 pg to 12.5 pg). As reported in Fig. 2A, 2.5% NL4-3 (X4) minority variants could be detected for NL4-3 with signals higher than 200,000 RLU and 5% NL4-3 variants were detectable with pure NL4-3 signals higher than 50,000 RLU. NLAD8 (R5) minority variants were detected down to 5% at the highest viral input and 10% for control values above 50,000 RLU (Fig. 2B). In this study, experiments were included if positive controls (pure NLAD8 and NL4-3) generated infection signals above 50,000 RLU.

Production of Patient-derived Env-recombinant Viruses

Overall PCR amplification success of patient-derived Envs was 87% (254/292 samples included) (Table 2). Amplification success was dependent on both subtype and VL. For subtypes A1, B, C, G, and CRF01_AE and CRF02_AG, amplification was achieved in 83.3–100% of cases, while for subtypes D and F, amplification was successful in 66.7% and 46.7% of cases respectively (Table 2). Of note, for some subtypes (D, F) few samples were available (15) inflating the relative weight of failed amplification compared to the overall rate. Both viral load (VL $<$ 1,000 RNA copies/mL) and non-B subtype compromised amplification success: the use of subtype-specific primers designed to target the most conserved regions of *env* and based on the most frequent polymorphisms did not improve these figures further (data not shown). When stratified for viral load, amplification succeeded in 94.9% of cases for VL $>$ 100,000 RNA copies/mL, 89.7% of cases for VL between 10,001–100,000 RNA copies/mL, 83.8% of cases for VL between 1,000–10,000 RNA copies/mL and decreased to 70% for VL $<$ 1,000 RNA copies/mL (Fig. 3). Recombinant viruses could be produced for 231 Envs, as determined by p24 antigen ELISA in the viral supernatant. Of those, 91.3% were infectious and tropism was tested (Table 2).

Concordance of Env-RVA with Genotypic Prediction

Tropism determined phenotypically using the Env-RVA was compared to tropism inferred by the Geno2Pheno_[coreceptor] and webPSSM prediction algorithms based on the V3-loop sequence. These two algorithms were chosen among all available genotyping

tools because they are the most widely used in the clinic. Results are reported in Table 2. Overall, concordance between the phenotypically measured tropism (RVA) and Geno2Pheno_[coreceptor] (10% FPR cutoff) was 85.2% and concordance of the RVA with webPSSM was 79.5% (Table 2). The overall Cohen kappa value for comparison with Geno2Pheno was 0.6252, ranging from 0.3226 (CRF01_AE) to 0.9180 (subtype G) (Table 2), reflecting good concordance of the phenotypic measure with genotypic prediction. Overall kappa value for comparison of phenotypically measured tropism with webPSSM was lower (0.4544), ranging from 0.0745 (CRF02_AG) to 0.6190 (subtype C with webPSSM subtype C). If detection of the presence of X4 strains is considered rather than full concordance between the phenotype and the genotype inferred by webPSSM, then concordance between these two assays increased to 83.3% (not shown) and Cohen kappa value reached 0.5240, ranging from 0.1509 (subtype A1) to 0.8262 (subtype G) (Table 2). Among discordant samples, in 14 cases the outcome of both prediction algorithms was identical but disagreed with the phenotypic result, while in the remaining cases, the phenotypically measured tropism disagreed with one algorithm only (Table 3). Decreasing the Geno2Pheno_[coreceptor] FPR cutoff to 5% (less sensitive to detect X4) increased concordance to 91.4%, as expected, while augmenting the FPR to 15% and 20% lowered concordance to 81.0% and 79.5% respectively (Table 2).

For all discordant results (FPR cutoff up to 20% for Geno2Pheno_[coreceptor] and webPSSM) for which enough material was available (50/74 samples) the phenotypic assay was repeated using CCR5 and CXCR4 inhibitors (1 μ M Maraviroc and 1 μ M AMD3100 respectively) (not shown). In all but two cases, the use of coreceptor inhibitors confirmed tropism. In one case, detection of X4 minority variants was close to the limit of detection and was not confirmed in the repeated experiment. In the second case, a strictly R5 strain by phenotypic measure and webPSSM, Maraviroc inhibited infection by 3 logs but did not fully inhibit entry in U87.CD4.CCR5 cells. To rule out the possibility that an intrinsic bias in the recombination step of the RVA would result in selection of some strains and in failure to detect some minority X4 strains, we sequenced the V3-loop of Env-recombinant viral particles used to infect U87 indicator cells. For all re-tested samples (50), the V3-loop sequence of recombinant viral supernatants was identical to the V3-loop of the parental PCR amplicon used to produce the recombinant viruses, and when submitted to Geno2Pheno_[coreceptor], a similar or identical FPR was obtained (data not shown), strongly arguing against the hypothesis of a selection due to the recombination process while generating recombinant viruses. Further, for 18 discordant samples, tropism was determined using the Trofile ESTA, which is based on pseudovirions rather than on recombination. Tropism measured using the Trofile ESTA confirmed the RVA results in 16/18 positive tests (Table 3). In one case (CRF01_AE) the RVA failed to detect a minority X4 strain, reflecting the low infectivity of this recombinant, whereas in the second case (CRF02_AG), the RVA detected the presence of low X4 variants which were undetectable using the Trofile ESTA, potentially a consequence of PCR selection due to the sample's plasma viral load being near the limit validated for the assay.

Characterization of Discordant Outcomes

Most discordant cases involved non-B subtypes for Geno2Pheno_[coreceptor] (10%FPR) (5.6% discordant cases for subtype B, Cohen kappa = 0.8591, versus 17.9% for non-B subtypes, Cohen kappa = 0.5446), but not for webPSSM, for which 20.4% (Cohen kappa = 0.3926) and 20.5% (Cohen kappa = 0.4734) discordant cases were recorded for subtype B and for non-B subtypes

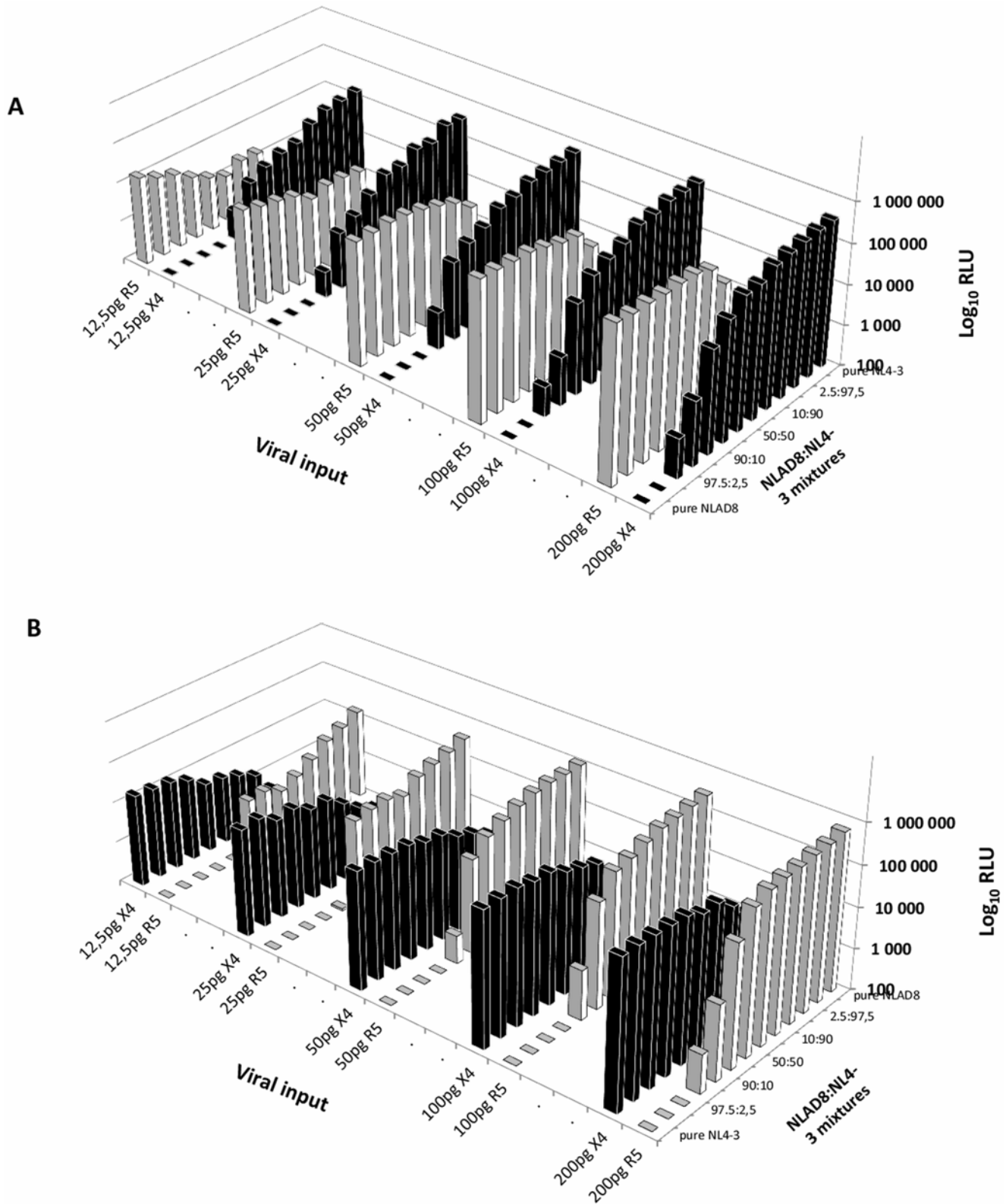


Figure 2. Detection of minority CXCR4 and CCR5 using variants within mixed viral populations. Mixtures containing known proportions of pNLAD8 and pNL4-3 (100:0, i.e. pure NLAD8, 99:1, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, 1:99, 0:100, i.e. pure NL4-3) were PCR-amplified and used to generate recombinant viruses. U87.CD4.CCR5 and U87.CD4.CXCR4 indicator cells were infected with serial 2-fold dilutions (x-axis) of mixtures (z-axis) to determine the threshold for detection of minority variants. Infection was quantified 48 hours after infection by measuring luciferase activity in cell lysates (y-axis). Black bars report infection of U87.CD4.CXCR4 cells and grey bars report infection of U87.CD4.CCR5 cells. Panels A and B report the same data, oriented to focus on NL4-3 minority variants (A) or on NLAD8 minority variants (B).
doi:10.1371/journal.pone.0060566.g002

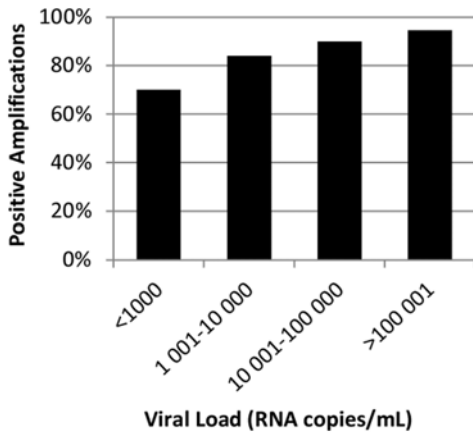


Figure 3. Distribution of PCR amplification success stratified by viral load. The Env ectodomain was amplified from plasma viral RNA by a one-step RT-PCR followed by an inner PCR. Five independent PCR amplifications were pooled to minimize primer-related selection. 292 samples from patients infected with HIV subtypes A1, B, C, D, F, G, CRF01_AE and CRF02_AG were included. Viral load ranged from 466 to 1,350,000 RNA copies/mL.
doi:10.1371/journal.pone.0060566.g003

respectively (Table 2, Fig. 4). For Geno2Pheno_[coreceptor], the highest kappa values were recorded for subtypes G and B, and the lowest value for CRF01_AE. For webPSSM, good kappa values were recorded for subtypes C (using the webPSSM subtype C matrix); using the subtype B X4/R5 matrix, good kappa values were also seen for subtypes D, G, F and CRF01_AE, intermediate for subtypes B, and poor for subtypes A1 and CRF02_AG (Table 2). Of note, kappa values increased to 0.4752 for subtype B when detection of X4 strains was compared rather than absolute

concordance (i.e. D/M and D/M, X4 and X4), probably reflecting tuning of the algorithm for the detection of X4 strains.

The sensitivity and specificity of Geno2Pheno_[coreceptor] and PSSM with respect to the recombinant viral assay (setting the phenotypic measure as ‘true’) was calculated: overall sensitivity and specificity were 88.9% and 84.2% for Geno2Pheno_[coreceptor] (10% FPR cutoff) and 65.2% and 88.4% for webPSSM (considering the ability to detect the presence of X4 variants) (data not shown). For subtype B strains, sensitivity and specificity were 100% and 92.7% respectively for Geno2Pheno_[coreceptor]; for webPSSM, sensitivity and specificity were 46.1% and 95.1% respectively (data not shown). For non-B subtypes, sensitivity was 84.4% for Geno2Pheno_[coreceptor] and 72.7% for webPSSM (data not shown). Specificities ranged from 50% to 100% for Geno2Pheno_[coreceptor] and from 50 to 100% for webPSSM (data not shown), in line with previous reports [53–57].

For subtype B samples, Geno2Pheno_[coreceptor] overestimated X4 usage for all 3 discordant samples. In contrast, webPSSM failed to detect CXCR4 usage for 7/11 samples (Table 3). Neither viral load nor the presence of mixtures could account for failure to detect X4 minor strains. For non-B subtypes, Geno2Pheno_[coreceptor] predicted CXCR4 usage while the phenotypic assay identified strictly R5 strains in most cases, and particularly for CRF01_AE (12/14 cases) and CRF02_AG (6/7) (Table 3). Such skewing towards overestimating the presence of X4 minority variants for these CRFs was maintained when the FPR cut-off was shifted, although these observations did not reach statistical significance using a Fisher’s exact test ($p < 0.05$). Disagreement of the phenotypically measured tropism with webPSSM was observed chiefly for subtype A1 (40% disagreement) and CRF01_AE (28.2% disagreement). For CRF01_AE, webPSSM predicted CXCR4 usage while the phenotypic RVA reported strictly R5 strains in 7/11 cases, and in 2/11 cases, CXCR4 usage detected phenotypically was missed by webPSSM (Table 3). For

Table 2. Distribution of samples, phenotyping and genotyping, and concordance between phenotypic and genotypic tropism.

Samples	Env PCR		RVA result		V3 loop sequence		Pheno/geno paired results	Concordance of RVA with					Cohen kappa values		
	N	%	N	%	N	%		G2P: 5%	G2P: 10%	G2P: 15%	G2P: 20%	PSSM	G2P: 10%	PSSM	PSSM X4
A1	17	100.0%	17	100.0%	15	88.2%	15	93.3%	86.7%	73.3%	73.3%	60.0%	0.4444	0.1743	0.1509
B	73	84.9%	62	100.0%	54	87.1%	54	100.0%	94.4%	90.7%	88.9%	79.6%	0.8591	0.3926	0.4752
C	21	100.0%	21	100.0%	20	95.2%	20	90.0%	90.0%	85.0%	80.0%	90.0%*	0.6078	0.6190*	0.6078*
D	15	66.7%	10	100.0%	7	70.0%	7	85.7%	71.4%	71.4%	71.4%	85.7%	0.3636	0.6111	0.5882
F	15	46.7%	7	100.0%	6	85.7%	6	83.3%	83.3%	83.3%	66.7%	83.3%	0.5714	0.5714	0.5714
G	55	94.5%	44	84.6%	42	80.8%	40	100.0%	97.5%	95.0%	95.0%	87.5%	0.9180	0.5910	0.8268
CRF01_AE	42	95.2%	39	97.5%	40	100.0%	39	79.5%	64.1%	61.5%	61.5%	71.8%	0.3226	0.5153	0.5351
CRF02_AG	54	83.3%	32	71.1%	30	66.7%	29	82.8%	79.3%	72.4%	72.4%	79.3%	0.4494	0.0745	0.2077
All non-B	219	87.7%	170	88.5%	160	83.3%	156	88.5%	82.1%	77.6%	76.3%	79.5%	0.5446	0.4734	0.5411
Total	292	87.0%	232	91.3%	214	84.3%	210	91.4%	85.2%	81.0%	79.5%	79.5%	0.6252	0.4544	0.5240

Distribution of samples per subtype, successful Env PCR amplification, recombinant virus production and sequencing of the V3-loop, and results (concordance and Cohen kappa values) for genotype/phenotype pairs are reported.

N: number of samples. G2P: Geno2Pheno_[coreceptor]. The percentage following G2P indicates the FPR cut-off. Distribution of samples per subtype, successful Env PCR amplification, recombinant virus production and sequencing of the V3-loop, and results (concordance and Cohen kappa values) for genotype/phenotype pairs are reported.

*For subtype C, concordance was determined and with webPSSM subtype C SIN1. Concordance with webPSSM X4/R5 was 75% and Cohen kappa values were poor (−0.0989 for full agreement and −0.1365 when detection of X4 strains was considered).

doi:10.1371/journal.pone.0060566.t002

Table 3. Detail of discordant results between RVA, ESTA, Geno2Pheno_(coreceptor) and webPSSM.

HIV-1 subtype	RVA result	Trofile result	G2P					webPSSM	Discordance	
			5% cutoff	10% cutoff	15% cutoff	20% cutoff	FPR		G2P	PSSM
A1	R5	R5	R5	X4	X4	X4	6.8	X4	G2P 10%	PSSM
A1	R5	N/A	R5	X4	X4	X4	8.5	D/M	G2P 10%	PSSM
A1	R5	R5	R5	R5	X4	X4	10.5	X4	G2P 15%	PSSM
A1	R5	N/A	R5	R5	R5	R5	42.2	D/M	agree	PSSM
A1	R5	N/A	R5	R5	R5	R5	40.3	X4	agree	PSSM
A1	R5	N/A	R5	R5	R5	R5	22.6	D/M	agree	PSSM
B	R5	R5	R5	X4	X4	X4	6.7	R5	G2P 10%	agree
B	R5	failed	R5	X4	X4	X4	6.8	R5	G2P 10%	agree
B	R5	N/A	R5	R5	X4	X4	13.8	R5	G2P 15%	agree
B	R5	N/A	R5	R5	X4	X4	10.5	R5	G2P 15%	agree
B	R5	N/A	R5	R5	R5	X4	17.6	R5	G2P 20%	agree
B	R5	R5	R5	X4	X4	X4	6.9	R5	G2P 10%	agree
B	D/M	D/M	X4	X4	X4	X4	4.6	R5	agree	PSSM
B	R5	N/A	R5	R5	R5	R5	26.2	D/M	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	0.7	D/M	agree	PSSM partial
B	R5	N/A	R5	R5	R5	R5	72.8	D/M	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.7	R5	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.8	D/M	agree	PSSM partial
B	X4	N/A	X4	X4	X4	X4	4.7	R5	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.8	R5	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.7	R5	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.8	R5	agree	PSSM
B	X4	N/A	X4	X4	X4	X4	3.8	R5	agree	PSSM
C	D/M	N/A	R5	X4	X4	X4	6.9	R5	G2P 5%	agree
C	R5	R5	R5	X4	X4	X4	9.6	R5	G2P 10%	agree
C	R5	N/A	R5	R5	X4	X4	10.9	X4	G2P 15%	PSSM
C	R5	N/A	R5	R5	R5	X4	17.9	R5	G2P 20%	agree
C	D/M	N/A	R5	R5	R5	R5	28.8	R5	G2P 5%	PSSM
D	R5	R5	X4	X4	X4	X4	4.7	X4	G2P 5%	PSSM
D	R5	R5	R5	X4	X4	X4	6.8	R5	G2P 10%	agree
G	R5	N/A	R5	X4	X4	X4	5	R5	G2P 10%	agree
G	R5	N/A	R5	R5	X4	X4	13.2	R5	G2P 15%	agree
G	D/M	N/A	R5	X4	X4	X4	6.8	R5	G2P 5%	PSSM
G	D/M	N/A	X4	X4	X4	X4	1.3	X4	agree	PSSM partial
G	D/M	N/A	X4	X4	X4	X4	1.7	X4	agree	PSSM partial
G	D/M	N/A	X4	X4	X4	X4	1.1	X4	agree	PSSM partial
G	R5	N/A	R5	R5	R5	R5	26.9	D/M	agree	PSSM
F	D/M	N/A	R5	X4	X4	X4	6.9	D/M	G2P 5%	agree
F	R5	N/A	R5	R5	X4	X4	14.4	R5	G2P 15%	agree
F	R5	N/A	R5	R5	R5	X4	17.5	R5	G2P 20%	agree
F	D/M	N/A	X4	X4	X4	X4	1.7	R5	agree	PSSM
AE	R5	R5	R5	X4	X4	X4	5.3	R5	G2P 10%	agree
AE	R5	N/A	R5	X4	X4	X4	7.9	D/M	G2P 10%	PSSM
AE	R5	N/A	X4	X4	X4	X4	1.8	X4	G2P 5%	PSSM
AE	R5	N/A	X4	X4	X4	X4	2.7	D/M	G2P 5%	PSSM
AE	R5	D/M	X4	X4	X4	X4	1.8	X4	G2P 5%	PSSM
AE	D/M	N/A	X4	X4	X4	X4	1.7	X4	agree	PSSM partial
AE	R5	N/A	R5	X4	X4	X4	9.6	R5	G2P 10%	agree

Table 3. Cont.

HIV-1 subtype	RVA result	Trofile result	G2P					webPSSM	Discordance	
			5% cutoff	10% cutoff	15% cutoff	20% cutoff	FPR		G2P	PSSM
AE	R5	N/A	R5	X4	X4	X4	5.7	R5	G2P 10%	agree
AE	R5	N/A	R5	X4	X4	X4	5.7	R5	G2P 10%	agree
AE	D/M	N/A	R5	X4	X4	X4	8.7	D/M	G2P 10%	agree
AE	R5	N/A	R5	X4	X4	X4	5	D/M	G2P 10%	PSSM
AE	R5	N/A	R5	R5	X4	X4	10.5	R5	G2P 15%	agree
AE	R5	N/A	R5	R5	X4	X4	10.5	R5	G2P 15%	agree
AE	R5	N/A	R5	R5	X4	X4	12	R5	G2P 15%	agree
AE	R5	N/A	X4	X4	X4	X4	4.1	D/M	G2P 5%	PSSM
AE	D/M	N/A	R5	R5	R5	R5	77.2	R5	G2P 5%	PSSM
AE	R5	N/A	X4	X4	X4	X4	4.7	X4	G2P 5%	PSSM
AE	D/M	N/A	R5	R5	X4	X4	10.5	R5	G2P 5–10%	PSSM
AE	D/M	N/A	X4	X4	X4	X4	1.7	X4	agree	PSSM partial
AG	R5	R5	R5	X4	X4	X4	6.4	R5	G2P 10%	agree
AG	R5	R5	R5	X4	X4	X4	9.6	R5	G2P 10%	agree
AG	R5	N/A	R5	R5	X4	X4	13.8	R5	G2P 15%	agree
AG	R5	R5	R5	R5	X4	X4	10.2	R5	G2P 15%	agree
AG	R5	R5	X4	X4	X4	X4	4.8	R5	G2P 5%	agree
AG	R5	N/A	X4	X4	X4	X4	2.6	R5	G2P 5%	agree
AG	R5	N/A	R5	X4	X4	X4	6.8	R5	G2P 10%	agree
AG	D/M	R5	R5	R5	R5	R5	40.7	R5	G2P 5%	PSSM
AG	D/M	N/A	R5	X4	X4	X4	5.8	R5	G2P 5%	PSSM
AG/G	R5	R5	R5	R5	X4	X4	10.1	R5	G2P 15%	agree
AG	D/M	N/A	X4	X4	X4	X4	1.7	R5	agree	PSSM
AG	D/M	N/A	X4	X4	X4	X4	0.5	X4	agree	PSSM partial
AG	R5	N/A	R5	R5	R5	R5	39.6	D/M	agree	PSSM
AG	D/M	N/A	X4	X4	X4	X4	5.3	R5	agree	PSSM

G2P: Geno2Pheno; FPR: False Positive Rate; D/M: dual mixed N/A = sample was not analyzed; PSSM ‘partial’ refers to samples for which webPSSM agrees with the phenotypic result for the detection of X4 variants, but not on the presence or absence of CCR5-using variants.
doi:10.1371/journal.pone.0060566.t003

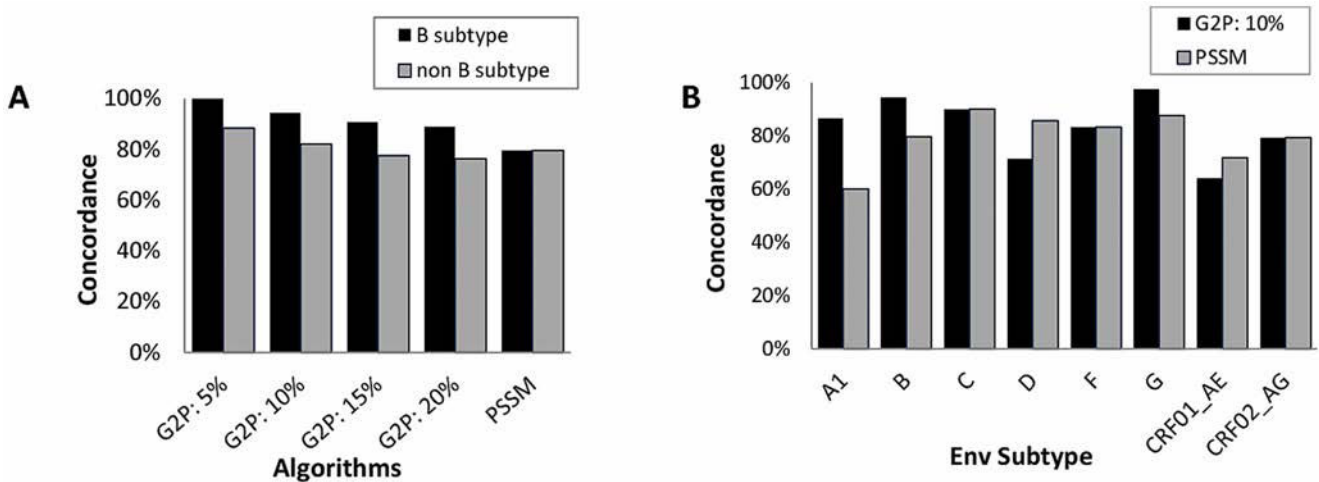


Figure 4. Concordance between tropism measured phenotypically and inferred genotypically using the Geno2pheno_(coreceptor) and webPSSM algorithms. (A) Concordance for subtype B (black bars) and non-B subtype (grey bars) strains with Geno2Pheno (G2P) at different FPR cutoffs and webPSSM. (B) Concordance with Geno2pheno_(coreceptor) with a FPR set at 10% (black bars) and webPSSM (grey bars) for different HIV-1 subtypes. The webPSSM X4/R5 matrix was used for all subtypes, except for subtype C, for which the subtype C SI/NSI matrix was used.
doi:10.1371/journal.pone.0060566.g004

subtype A1, CXCR4 usage was overestimated in all cases (6/6 discordant cases) (Table 3).

Overall, both Geno2Pheno_[coreceptor] and webPSSM overestimate the presence of X4 viruses for CRF01_AE. A similar trend was also observed for Geno2Pheno_[coreceptor] in the case of CRF02_AG and for webPSSM in the case of subtype A1, while webPSSM underestimates the presence of X4 for subtype B. Taken together, these results point to an inadequacy of the genotypic prediction algorithms in correctly inferring tropism for some subtypes CRF01_AE and CRF02_AG, and for subtype A1 in the case of webPSSM.

Discussion

In this study, the performance of an in-house Env-recombinant viral assay for determining viral tropism was evaluated in comparison to genotypic prediction using 2 widely used algorithms, Geno2Pheno_[coreceptor] [38] and webPSSM [39] on a majority of non-B subtypes. Overall, we found good concordance between our phenotypic assay and these algorithms, as reflected by 85.2% concordance of the phenotype with Geno2Pheno_[coreceptor] and 79.5% with webPSSM and relative kappa values of 0.6252 and 0.4544 respectively. The highest genotypic/phenotypic concordance was generally found for subtypes G, B and C strains, whereas, despite improved rules, substantial discordances involved non-B subtypes and CRFs. This is in line with previous studies reporting the performance of genotypic tools, mainly webPSSM (X4/R5 and SINSE matrices), Wetcat, Geno2Pheno_[coreceptor] or the 11/25 rule, compared to the Phenoscript test or to the Trofile phenotypic assay [53,58,59] measuring the sensitivity and specificity of these algorithms to detect the presence of X4 strains. In order to compare our findings with previous reports which evaluated the reliability of different genotypic prediction tools for detecting X4 strains, sensitivity and specificity of these algorithms with respect to the recombinant viral assay were calculated, although this approach presents the intrinsic drawback of setting the phenotypic assay as the standard. We found overall good sensitivities with Geno2Pheno and webPSSM, similar to previous reports on the sensitivity and specificity ranges of different bioinformatics tools for subtype B and some non-B strains [53–58]. Of note however, despite similar conclusions, concordance of different genotypic prediction tools with phenotypic assays did not always agree on which algorithm performed best, probably reflecting differences in the panel of viruses and of subtypes included, primer selection, and subsequent comparison with different phenotypic assays targeting the full Env or just the V1–V3 portion. Although the purpose of this study was not to compare the performance of different bioinformatics tools for detecting X4 minority variants but rather to validate our in-house RVA, our findings confirm the high reliability of genotypic prediction tools for detecting the tropism of subtype B strains [53,54,56], but also highlight incongruent results for many non-B strains. This has been addressed by webPSSM by developing a specific matrix for subtype C. Concordance of webPSSM with phenotypic results increased from 75% using the webPSSM X4/R5 matrix (based on subtype B) (data not shown) to 90% using the webPSSM subtype C matrix; likewise Cohen kappa values shifted from negative (data not shown) to >0.6 using both algorithms respectively. Therefore, it is important to consider subtype when assessing the presence of X4 strains in the clinical context prior to maraviroc prescription. Our findings strongly argue in favor of using multiple genotypic prediction tools and to consider maintaining phenotypic testing for those subtypes for which coreceptor usage determination using genotypic tests features low concordance with phenotypic mea-

asures, and for which prediction algorithms have not been tuned specifically, i.e. non-B, and non-C subtypes.

In this study, genotypic prediction featured the highest discordance with CRF01_AE and CRF02_AG and subtype A1. Of note, Geno2Pheno_[coreceptor] and webPSSM did not always predict the same tropism, as previously reported [60]. Geno2Pheno_[coreceptor] tended to overestimate the presence of CXCR4 usage (Table 3), while WebPSSM overestimated CXCR4-usage for subtype A1, but not for subtype B or CRF01_AE and CRF02_AG (Table 3). It may be important to keep in mind that when mixtures are present in the V3-loop, all possible combinations are genotyped, and the algorithm provides a tropism prediction for each possible clone. In this scenario, webPSSM would infer tropism for sequences that do not exist in the viral population, eventually leading to an overestimation of dual tropic variants, whereas the phenotypic assays only measures existing strains. Although this phenomenon does not account for mistakenly assigned coreceptor usage, (e.g. strict X4 rather than strict R5), it could explain the improved kappa values recorded for some subtypes when the reliability in detecting the presence of CXCR4-using variants is compared. Such an improvement was particularly marked for subtypes B, G and CRF02_AG (Table 2).

Poor specificity has previously been reported for tropism prediction of subtype D strains by Geno2Pheno_[coreceptor] [46] and for CRF02_AG [19,45], for which specific determinants have been described to improve the algorithm. We therefore subjected our samples to the rules provided by Raymond [45] and those proposed shortly after by Esbjörnsson [19] for CRF02_AG: the Raymond rules, which combine the 11/25 rule and the net charge rule (R/K at position 11 and/or K at position 25, or R at position 25+ net charge $\geq +5$ or the net charge $\geq +6$) [45,61] resulted in 7 discordant cases (20.7%) (3 R5 samples scored as X4 and 3 X4 samples scored as R5) and the Esbjörnsson rule (net charge $\geq +5$ and total charged AA ≥ 8) [19] in 10 discordances (34.5%) (4 missed X4 calls and 6 X4 calls for R5 viruses). Therefore, the Raymond rules slightly improved concordance of genotypic prediction with phenotypic measure for CRF02_AG compared to Geno2Pheno_[coreceptor] and webPSSM while the Esbjörnsson rules further increased false positive X4 calls on our samples. While this manuscript was under revision, Raymond et al. reported similarly low sensitivity and specificity of Geno2Pheno_[coreceptor] (10% FPR cutoff) for CRF01_AE, and proposed a new rule combining the 11/25 rule and disruption of the potential N-glycosylation site PNG (N₆NT₈) within the V3-loop [62]. Using a similar approach, we found that in our samples, the presence of a positively charged AA (K or R) at positions 11 or 25 was relatively rare (R/K at position 11 in 6/40 sequences and K at position 25 in one sequence), but reliably translated into CXCR4 usage measured phenotypically. Position 11 hosted a S in 30/40 sequences and a G in 3/40 sequences; position 25 displayed a negatively charged AA (D or E) in 35 sequences. The total number of positively charged AA, total net charge, or total charge, which was generally high (>+5), did not provide further support for sorting CXCR4-using strains in our samples, in agreement with the findings reported by Raymond et al. [62]. In 6/8 phenotypically X4 strains in which positions 11 and 25 were not positively charged, the PNG (N₆NT₈) was (or was likely to be, due to mixtures) disrupted and the net charge was $\geq +4$, as reported by Raymond et al. [62]. The Raymond rules improved concordance of the phenotypic test with genotypic prediction from 61.4% with Geno2Pheno_[coreceptor] and 71.8% with webPSSM to 87.2% (34/39) for this CRF. Nonetheless, with these rules (11 K/R and/or 25 K or disrupted PNG+net charge $\geq +4$ [62]) in 2/25 cases, phenotypically R5 samples were scored as X4 and 2/14

phenotypically X4 samples were predicted to be R5, suggesting that other criteria apply to this CRF and larger scale studies combining phenotypic testing to genotypic tuning combining the 11/25 rule to the PNG and to charge will be needed to further improve sensitivity and specificity of prediction tools. We cannot exclude that in our study, the use of bulk sequences may impact the reliability of prediction rules, whereas the rules by Raymond were partially based on clonal samples, where the relative weight of each position is absolute rather than being relative to its proportion within the quasispecies.

Three technical reasons could account for discordant results between genotypic prediction and phenotypically determined tropism: PCR selection, a bias/selection arising from the recombination step of the RVA, inadequately inferred tropism by genotypic tools, consequent to subtype-related specificities not taken into account by the algorithm, either within the V3 loop or in other regions of Env. To minimize a potential impact of PCR selection, 5 independent PCR reactions were pooled and the same amplicon used to produce recombinant viruses was sequenced. Nonetheless, whereas comparison of the Env-RVA and genotypic prediction by Geno2Pheno and webPSSM were based on the use of the same PCR pool, the Trofile ESTA was performed using an independent plasma tube and different PCR primers, eventually translating into PCR selection. Hence, it is not possible to exclude that failure to pick-up X4 minority variants by the RVA (1 CRF01_AE) or by the Trofile ESTA (1 CRF02_AG), reflect PCR selection, particularly in the case of poorly infectious recombinant or pseudotyped particles. Selection during the recombination step was ruled out as the V3-loop sequences from viral supernatants (after the recombination step) were identical to the parental PCR amplicon that served to produce recombinant viruses. When assessed, tropism of CRF01_AE and CRF02_AG strains assessed using the Trofile ESTA matched the phenotype determined by the RVA and disagreed with the Geno2Pheno_[coreceptor] prediction in all but two cases (Table 3). The Trofile ESTA was chosen among all available tropism phenotypic tests because it is a high sensitivity and specificity single cycle pseudovirus assay [30]. Lastly, repeat experiments in the presence of CCR5 and CXCR4 inhibitors confirmed the phenotypic results, strongly indicating that the Geno2Pheno_[coreceptor] and webPSSM algorithms require more specific improvements for some subtypes, particularly CRF01_AE and CRF02_AG, and that the RVA described here reliably indicates coreceptor usage. It is known that the V3-loop is not the sole Env determinant of HIV-1 co-receptor usage. Sequence changes within the V1, V2 and C4 regions of gp120 [13,63–67], as well as the level of glycosylation [68–70] can also profoundly impact coreceptor usage. In this study, it is not possible to rule out that some discordant results arise from the comparison of coreceptor usage predictions based on the V3-loop sequence only to a phenotypic assay taking into account the whole Env ectodomain.

Various commercial and non-commercial phenotypic assays have been developed over the last 10 years to measure tropism. These are based on different approaches to produce recombinant viruses, including homologous recombination, pseudotyping, or a combination of both; they target different parts of Env, ranging

from the V1–V3 region only (e.g. Phenoscript [34], PhenXR [71]) to the full Env (e.g. ESTA [30]); the producer and the target cells, as well as the readout (virally-encoded *luciferase* or *GFP* reporter gene (e.g. ESTA [30], TTT [35], the Virco Assay [32], two non-commercial assays utilizing recombinant particles and pseudovirions respectively [33,72], and the RVA presented here), or target cell line containing a LTR- β -Galactosidase reporter which is activated upon infection (e.g. Phenoscript [34], PhenXR [71]) further distinguish these tests. These are recorded in Table 1. The RVA presented here mostly resembles the TTT assay in the design of the backbone, production of recombinant viral particles through homologous recombination and location of Luciferase reporter in the place of Nef [35]. The TTT showed high performance in terms of Env amplification and production of recombinant viruses, particularly for subtypes that were difficult to amplify using our primers (D and F), likely due to primer location. The TTT group also selected one U87-CD4.CXCR4 cell clone with high expression of CXCR4 to increase the limit of detection of X4 minority variants [35]. In our design, we verified the expression of CXCR4 and only maintained cells in culture for a limited number of passages. To ensure the lower limit of detection of X4 strains was maintained, we tested the performance of the RVA using serial dilutions and systematically discarded experiments where the lower threshold of infection of U87.CD4.CXCR4 cells by NL4-3 did not reach 100,000 RLU (Fig. 2). It would be interesting to compare the performance of different phenotypic assays using a large panel of samples, although this could not be performed here because of insufficient plasma from the sample to allow independent testing and repeat experiments in different laboratories.

Taken together, the findings reported here strongly support the need for further large-scale studies to improve prediction models and/or to appeal to more than one algorithm when non-B subtypes are involved. The use of phenotypic measurements could nevertheless be required in cases where different algorithms point to potential difficulties in inferring the correct tropism. Such a confirmation is important in the clinical set-up as a false prediction of X4 variants may lead to exclusion of patients who could have benefited from prescription of CCR5 inhibitors while false prediction of R5 only variants may lead to selection and reemergence of X4-strains under maraviroc pressure (Baatz et al., 2011; Kuhmann and Hartley, 2008; Pugach et al., 2007; Westby et al., 2007).

Acknowledgments

The authors are deeply grateful to Christos Petropoulos and Laura Napolitano for enriching discussions and critical reading of the manuscript, and to Olivier Collignon for support with statistical analyses.

Author Contributions

Conceived and designed the experiments: MM DPB. Performed the experiments: MM ML JYS AR YL. Analyzed the data: MM DS JYS YL ESS DPB. Contributed reagents/materials/analysis tools: CV CSD JCS. Wrote the paper: MM DPB. Critically read and helped improve the manuscript: CV YL ESS CSD.

References

- Dalglish AG (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312: 763–767.
- Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381: 661–666.
- Oberlin E (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line adapted HIV-1. *Nature* 382: 833–835.
- Feng Y, Broder CC, Kennedy PE, Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. *Science* 272: 872–877.
- Choe H (1996) The [beta]-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85: 1135–1148.
- Dragic T (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381: 667–673.

7. Alkhatib G, Combadiere C, Broder C, Feng Y, Kennedy P, et al. (1996) CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272: 1955–1958.
8. Weissenhorn W, Dessen A, Harrison S, Skehel J, Wiley D (1997) Atomic structure of the ectodomain from HIV-1 gp120. *Nature* 387: 426–430.
9. Cardozo T (2007) Structural basis of co-receptor selectivity by the HIV-1 V3 loop. *AIDS Res Hum Retroviruses* 23: 415–426.
10. Shioda T, Levy JA, Cheng-Mayer C (1992) Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 89: 9434–9438.
11. Berger E, Doms R, Fenyo E, Korber B, Littman D, et al. (1998) A new classification for HIV-1. *Nature* 391: 240.
12. Hartley O, Klasse PJ, Sattentau QJ, Moore JP (2005) V3: HIV's Switch-Hitter. *AIDS Research and Human Retroviruses* 21: 171–189.
13. Pastore C, Nedellec R, Ramos A, Pontow S, Ratner L, et al. (2006) Human immunodeficiency virus type 1 coreceptor switching: V1/V2 gain-of-fitness mutations compensate for V3 loss-of-fitness mutations. *J Virol* 80: 750–758.
14. Chalmet K, Dauwe K, Foquet L, Baatz F, Seguin-Devaux C, et al. (2011) Presence of CXCR4-Using HIV-1 in Patients With Recently Diagnosed Infection: Correlates and Evidence for Transmission. *Journal of Infectious Diseases*.
15. Schuitemaker H, van 't Wout A, Lusso P (2011) Clinical significance of HIV-1 coreceptor usage. *Journal of Translational Medicine* 9: 1–17.
16. Bratt G, Karlsson A, Leandersson AC, Albert J, Wahren B, et al. (1998) Treatment history and baseline viral load, but not viral tropism or CCR-5 genotype, influence prolonged antiviral efficacy of highly active antiretroviral treatment. *AIDS* 12: 2193–2202.
17. Bjornald A, Deng H, Jansson M, Fiore J, Colognesi C, et al. (1997) Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 71: 7478–7487.
18. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR (1997) Change in coreceptor use correlates with disease progression in HIV-1 infected individuals. *J Exp Med* 185: 621–628.
19. Esbjornsson J, Mansson F, Martinez-Arias W, Vincic E, Biague A, et al. (2010) Frequent CXCR4 tropism of HIV-1 subtype A and CRF02_AG during late-stage disease - indication of an evolving epidemic in West Africa. *Retrovirology* 7: 23.
20. Fenyö EM, Esbjornsson J, Medstrand P, Jansson M (2011) Human immunodeficiency virus type 1 biological variation and coreceptor use: from concept to clinical significance. *Journal of Internal Medicine* 270: 520–531.
21. Blaak H (2000) In vivo HIV-1 infection of CD45RA+CD4+ T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4+ T cell decline. *Proc Natl Acad Sci USA* 97: 1269–1274.
22. Fatkenheuer G, Nelson M, Lazzarin A, Konourina I, Hoepelman A, et al. (2008) Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. *N Engl J Med* 359: 1442–1455.
23. Gulick R, Lalezari J, Goodrich J, Clumeck N, DeJesus E, et al. (2008) Maraviroc for previously treated patients with R5 HIV-1 infection. *N Engl J Med* 359: 1429–1441.
24. Harrigan PR, Geretti AM (2011) Genotypic tropism testing: evidence-based or leap of faith. *AIDS* 25: 257–264.
25. Kuhmann SE, Hartley O (2008) Targeting chemokine receptors in HIV: A status report. *Annual Review of Pharmacology and Toxicology*. 425–461.
26. Westby M, Smith-Burchnell C, Mori J, Lewis M, Mosley M, et al. (2007) Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* 81: 2359–2371.
27. Baatz F, Nijhuis M, Lemaire M, Riedijk M, Wensing AMJ, et al. (2011) Impact of the HIV-1 Genetic Context outside HR1/HR2 on Resistance to the Fusion Inhibitor Enfuvirtide and Viral Infectivity in Clinical Isolates. *PLoS ONE* 6: e21535.
28. Pugach P, Marozsan AJ, Ketas TJ, Landes EL, Moore JP, et al. (2007) HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. *Virology* 361: 212–228.
29. Rose JD, Rhea AM, Weber J, Quinones-Mateu ME (2009) Current tests to evaluate HIV-1 coreceptor tropism. *Current Opinion in HIV & AIDS* 4: 136–142.
30. Reeves JD, Coakley E, Petropoulos CJ, Whitcomb JM (2009) An Enhanced-Sensitivity Profile™ HIV Coreceptor Tropism Assay for Selecting Patients for Therapy with Entry Inhibitors Targeting CCR5: A Review of Analytical and Clinical Studies. *The Journal of Viral Entry* 3: 94–102.
31. Whitcomb JM, Huang W, Franssen S, Limoli K, Toma J, et al. (2007) Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. *Antimicrob Agents Chemother* 51: 566–575.
32. Van Baelen K, Vandenbroucke I, Rondelez E, Van Eygen V, Vermeiren H, et al. (2007) HIV-1 coreceptor usage determination in clinical isolates using clonal and population-based genotypic and phenotypic assays. *J Virol Methods* 146: 61–73.
33. Lin NH, Negusse DM, Beroukhim R, Giguel F, Lockman S, et al. (2010) The design and validation of a novel phenotypic assay to determine HIV-1 coreceptor usage of clinical isolates. *Journal of Virological Methods* 169: 39–46.
34. Trouplin V, Salvatori F, Cappello F, Obry V, Brelot A, et al. (2001) Determination of coreceptor usage of human immunodeficiency virus type 1 from patient plasma samples by using a recombinant phenotypic assay. *J Virol* 75: 251–259.
35. Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, et al. (2010) Development and performance of a new recombinant virus phenotypic entry assay to determine HIV-1 coreceptor usage. *Journal of Clinical Virology* 47: 126–130.
36. Asboe D, Aitken C, Boffito M, Booth C, Cane P, et al. (2012) British HIV Association guidelines for the routine investigation and monitoring of adult HIV-1-infected individuals 2011. *HIV Medicine* 13: 1–44.
37. Delobel P, Nugyere M-T, Cazabat M, Pasquier C, Marchou B, et al. (2007) Population-Based Sequencing of the V3 Region of env for Predicting the Coreceptor Usage of Human Immunodeficiency Virus Type 1 Quasispecies. *Journal of Clinical Microbiology* 45: 1572–1580.
38. Beerenwinkel N, Däumer M, Oette M, Korn K, Hoffmann D, et al. (2003) Geno2pheno: estimating phenotypic drug resistance from HIV-1 genotypes. *Nucleic Acids Research* 31: 3850–3855.
39. Jensen M, Li F, van 't Wout A, Nickle D, Shriner D, et al. (2003) Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loopsequences. *J Virol* 77: 13376–13388.
40. Vandekerckhove LPR, Wensing AMJ, Kaiser R, Brun-Vézinet F, Clotet B, et al. (2011) European guidelines on the clinical management of HIV-1 tropism testing. *The Lancet Infectious Diseases* 11: 394–407.
41. McGovern RA, Harrigan PR, Swenson LC (2010) Genotypic Inference of HIV-1 Tropism Using Population-based Sequencing of V3. *J Vis Exp*: e2531.
42. Recordon-Pinson P, Soulie C, Flandre P (2010) Evaluation of the genotypic prediction of HIV-1 coreceptor use versus a phenotypic assay and correlation with the virological response to maraviroc: the ANRS GenoTropism study. *Antimicrob Agents Chemother* 54: 3335–3340.
43. Swenson LC, Moores A, Low AJ, Thielen A, Dong W, et al. (2010) Improved detection of CXCR4-using HIV by V3 genotyping: application of population-based and "deep" sequencing to plasma RNA and proviral DNA. *J Acquir Immune Defic Syndr* 54: 506–510.
44. Garrido C, Roulet V, Chueca N, Poveda E, Aguilera A, et al. (2008) Evaluation of Eight Different Bioinformatics Tools To Predict Viral Tropism in Different Human Immunodeficiency Virus Type 1 Subtypes. *Journal of Clinical Microbiology* 46: 887–891.
45. Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, et al. (2009) Genotypic Prediction of HIV-1 CRF02-AG Tropism. *J Clin Microbiol* 47: 2292–2294.
46. Raymond S, Delobel P, Chaix ML, Cazabat M, Encinas S, et al. (2011) Genotypic prediction of HIV-1 subtype D tropism. *Retrovirology* 8: 56.
47. de Oliveira T, Deforche K, Cassol S, Salminen M, Paraskevis D, et al. (2005) An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics* 21: 3797–3800.
48. Van Laethem K, Schrooten Y, Lemey P, Wijngaerden EV, Wit SD, et al. (2005) A genotypic resistance assay for the detection of drug resistance in the human immunodeficiency virus type 1 envelope gene. *Journal of Virological Methods* 123: 25–34.
49. Verhofstede C, Brudney D, Reynaerts J, Vaira D, Franssen K, et al. (2011) Concordance between HIV-1 genotypic coreceptor tropism predictions based on plasma RNA and proviral DNA. *HIV Medicine* 12: 544–552.
50. Batra M, Tien PC, Shafer RW, Contag CH, Katzenstein DA (2000) HIV type 1 envelope subtype C sequences from recent seroconverters in Zimbabwe. *AIDS Res Hum Retroviruses* 16: 973–979.
51. Chakrabarti AK, Caruso L, Ding M, Shen C, Buchanan W, et al. (2009) Detection of HIV-1 RNA/DNA and CD4 mRNA in feces and urine from chronic HIV-1 infected subjects with and without anti-retroviral therapy. *AIDS Res Ther* 6: 20.
52. O'Doherty U, Swiggard WJ, Malim MH (2000) Human Immunodeficiency Virus Type 1 Spine Infection Enhances Infection through Virus Binding. *Journal of virology* 74: 10074–10080.
53. Chueca N, Garrido C, Álvarez M, Poveda E, de Dios Luna J, et al. (2009) Improvement in the determination of HIV-1 tropism using the V3 gene sequence and a combination of bioinformatic tools. *Journal of Medical Virology* 81: 763–767.
54. Seclén E, Garrido C, Gonzalez MdM, Gonzalez-Lahoz J, de Mendoza C, et al. (2010) High sensitivity of specific genotypic tools for detection of X4 variants in antiretroviral-experienced patients suitable to be treated with CCR5 antagonists. *Journal of Antimicrobial Chemotherapy* 65: 1486–1492.
55. Qu S, Ma L, Yuan L, Xu W, Hong K, et al. (2010) Co-receptor usage and prediction of V3 genotyping algorithms in HIV-1 subtype B' from paid blood donors experienced anti-retroviral therapy in Chinese central province. *Virology* 7: 280.
56. Delgado E, Fernandez-Garcia A, Vega Y, Cuevas T, Pinilla M, et al. (2012) Evaluation of genotypic tropism prediction tests compared with in vitro coreceptor usage in HIV-1 primary isolates of diverse subtypes. *Journal of Antimicrobial Chemotherapy* 67: 25–31.
57. Poveda E, Seclén E, Gonzalez MdM, Garcia F, Chueca N, et al. (2009) Design and validation of new genotypic tools for easy and reliable estimation of HIV tropism before using CCR5 antagonists. *J Antimicrob Chemother* 63: 1006–1010.

58. Poveda E, Briz V, Roulet V, del Mar Gonzalez M, Faudon J-L, et al. (2007) Correlation between a phenotypic assay and three bioinformatic tools for determining HIV co-receptor use. *AIDS* 21: 1487–1490. doi:10.1097/QAD.1480-b1013e32826fb32741.
59. de Mendoza C, Van Baelen K, Poveda E, Rondelez E, Zahonero N, et al. (2008) Performance of a population-based HIV-1 tropism phenotypic assay and correlation with V3 genotypic prediction tools in recent HIV-1 seroconverters. *J Acquir Immune Defic Syndr* 48: 241–244.
60. Rodriguez JJ, Seclen E, Poveda E, Varela E, Regueiro B, et al. (2011) [Variability in HIV viral tropism determination using different genotypic algorithms in patients infected with B versus non-B HIV-1 subtypes]. *Enferm Infecc Microbiol Clin* 29: 4–8.
61. Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, et al. (2008) Correlation between genotypic predictions based on V3 sequences and phenotypic determination of HIV-1 tropism. *AIDS* 22: F11–F16.
62. Raymond S, Delobel P, Rogez S, Encinas S, Bruel P, et al. (2012) Genotypic Prediction of HIV-1 CRF01-AE Tropism. *J Clin Microbiol* 5: 5.
63. Hoffman NG, Seillier-Moiseiwitsch F, Ahn J, Walker JM, Swanstrom R (2002) Variability in the Human Immunodeficiency Virus Type 1 gp120 Env Protein Linked to Phenotype-Associated Changes in the V3 Loop. *Journal of Virology* 76: 3852–3864.
64. Huang CC (2005) Structure of a V3-containing HIV-1 gp120 core. *Science* 310: 1025–1028.
65. Nabatov AA, Pollakis G, Linnemann T, Kliphuis A, Chalaby MIM, et al. (2004) Intrapatient Alterations in the Human Immunodeficiency Virus Type 1 gp120 V1V2 and V3 Regions Differentially Modulate Coreceptor Usage, Virus Inhibition by CC/CXC Chemokines, Soluble CD4, and the b12 and 2G12 Monoclonal Antibodies. *Journal of Virology* 78: 524–530.
66. Zolla-Pazner S, Cardozo T (2010) Structure-function relationships of HIV-1 envelope sequence-variable regions refocus vaccine design. *Nat Rev Immunol* 10: 527–535.
67. Ross TM, Cullen BR (1998) The ability of HIV type 1 to use CCR-3 as a coreceptor is controlled by envelope V1/V2 sequences acting in conjunction with a CCR-5 tropic V3 loop. *Proc Natl Acad Sci U S A* 95: 7682–7686.
68. Clevestig P, Maljkovic I, Casper C, Carlenor E, Lindgren S, et al. (2005) The X4 phenotype of HIV type 1 evolves from R5 in two children of mothers, carrying X4, and is not linked to transmission. *AIDS Res Hum Retroviruses* 21: 371–378.
69. McCaffrey RA, Saunders C, Hensel M, Stamatatos L (2004) N-Linked Glycosylation of the V3 Loop and the Immunologically Silent Face of gp120 Protects Human Immunodeficiency Virus Type 1 SF162 from Neutralization by Anti-gp120 and Anti-gp41 Antibodies. *Journal of Virology* 78: 3279–3295.
70. Pollakis G, Kang S, Kliphuis A, Chalaby MIM, Goudsmit J, et al. (2001) N-Linked Glycosylation of the HIV Type-1 gp120 Envelope Glycoprotein as a Major Determinant of CCR5 and CXCR4 Coreceptor Utilization. *Journal of Biological Chemistry* 276: 13433–13441.
71. Hamy F, Vidal V, Hubert S, Klimkait T (2007) Hybridization-based assay and replicative phenotyping as diagnostic platform for determination of coreceptor tropism. 5th European HIV drug resistance workshop: Abstract 60.
72. Gonzalez N, Perez-Olmeda M, Mateos E, Cascajero A, Alvarez A, et al. (2010) A sensitive phenotypic assay for the determination of human immunodeficiency virus type 1 tropism. *J Antimicrob Chemother* 65: 2493–2501.