Institute of Molecular Virology
Ulm University Medical Center
Director: Prof. Dr. Frank Kirchhoff

The role of Vpu-mediated tetherin antagonism in HIV-1 fitness

Dissertation
To obtain the Doctoral Degree of Human Biology (Dr. biol. hum.)
At the Faculty of Medicine, University of Ulm

Presented by
Dorota Janina Kmieć
Miastko
2018

Present Dean: Prof. Dr. Thomas Wirth

1st reviewer: Prof. Dr. Frank Kirchhoff

2nd reviewer: Prof. Dr. med. Christian Sinzger

Date of graduation: 22nd of June 2018

„A scientist in his workroom is not merely a technician; he is also a child mesmerized by the phenomena of Nature, as thrilling as a fairy tale."

Maria Sklodowska-Curie

Contents

Abbreviations

A	Adenine
aa	Amino acid
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
APOBEC3G	Apolipoprotein B mRNA editing enzyme catalytic subunit 3G protein
Bis-Tris	Bis(2-hydroxyethyl) amino tris (hydroxymethyl) methane
BSA	Bovine serum albumin
BST-2	Bone Marrow Stromal Cell Antigen 2; Tetherin
bp	Base pair
β-TrCP	β-transducin repeat containing protein
C	Cytosine
CA	Cell associated
$CaCl_2$	Calcium dichloride
CC	Chronic virus
CCR5	C-C chemokine receptor type 5, CD195
CD1d	Cluster of differentiation 1d
CD4	Cluster of differentiation 4
cDNA	Complementary deoxyribonucleic acid
CF	Cell free
CMV	Cytomegalovirus
Co-IP	Co-Immunoprecipitation
CTRL	Control
CXCR4	C-X-C chemokine receptor type 4
Da	Dalton
DC	Dendritic cell
DEAE	Diethylaminoethyl
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco's Phosphate-Buffered Saline
d.p.i.	Days post infection
DRK	German Red Cross (Deutsches Rotes Kreuz)
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
E. coli	*Escherichia coli*
et al.	*et alia*

e.g.	*exempli gratia*
EGFR	Epidermal growth factor receptor
Env	Viral envelope protein
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FL	Firefly luciferase
g	Gram
g	Gravitational force
G	Guanine
Gag	Group specific antigen
GBP5	Guanylate Binding Protein 5
GL	*Gaussia* luciferase
GMP-AMP	Guanosine monophosphate–adenosine monophosphate
gp120	Envelope glycoprotein of HIV, 120 kDa
gp41	Envelope glycoprotein of HIV, 41 kDa
GPI	Glycophosphatidylinositol
HAART	Highly Active Anti-Retroviral Therapy
HBS	HEPES buffered saline
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonicbacid
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HSV	Herpes Simplex Virus
HTSX	Heterosexual
i.e.	*id est*
ICAM-1	Intercellular Adhesion Molecule 1
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IgG	Immunoglobulin G
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit β
IL	Interleukin
IMC	Infectious molecular clone
IRES	Internal ribosomal entry site
IRF7	Interferon regulatory factor 7
ISG	Interferon-stimulated gene
IκB	Inhibitor of κB
kDa	Kilodalton

LAB	Laboratory-adapted
LB	Lysogeny broth
LTR	Long Terminal Repeat
m	Mutant
MFI	Mean Fluorescence Intensity
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
Mut	Mutant
MyD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NaF	Sodium fluoride
Nef	Negative factor
NF-κB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
NIH	National Institutes of Health
NK	Natural Killer cell
NKT	Natural Killer T cell
NP40	Nonidet P40
ns	Not significant
NTB-A	T and B cell antigen
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
Pol	Polymerase
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
R5	CCR5 co-receptor-tropic virus
RCF	Relative centrifugal force
Rev	Regulator of virion expression
RIG-I	Retinoic acid-inducible gene I
RLU/pg	Relative light units/picogram
RLU/s	Relative light units/second
RNA	Ribonucleic acid
RPM	Rotations per minute
RPMI-1640	Roswell Park Memorial Institute medium

RT	Reverse transcriptase
SAMHD1	SAM domain and HD domain-containing protein 1
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERINC5	Serine incorporator 5
SHC1	Src Homology Domain Containing Transforming Protein 1
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SIVcpz	SIV infecting chimpanzees
SIVgor	SIV infecting gorillas
SIVsm/SIVsmm	SIV infecting sooty mangabeys
S.O.C.	Super Optimal Catabolite repression medium
SOE	Splicing by overlap extension
ss	Single stranded
STING	Stimulator of interferon genes
SV40	Simian virus 40
T	Thymine
TAE	Tris-acetate-EDTA
TAL	TATA-like
Tat	Trans-activator of transcription
TF	Transmitted founder virus
TGN	Trans-Golgi network
TK	Thymidine kinase
TLR	Toll-like receptor
TMB	3,3', 5,5'- tetramethylbenzidine
TMD	Transmembrane domain
TMDmut	Transmembrane domain mutant
TRIM5α	Tripartite Motif Containing protein 5 alpha
Tris	Trisaminomethane
UNAIDS	United Nations Programme on HIV/AIDS
v/v	volume per volume
Vif	Viral infectivity factor
Vpr	Viral protein r
Vpu	Viral protein unique to HIV-1
VS	Virus stock
VSV-g	Vesicular stomatitis virus glycoprotein
w/v	Weight per volume
WHO	World Health Organization
WT	Wild type
X4	CXCR4 co-receptor-tropic virus

Amino acid code

Letter abbreviation	Abbreviation	Name	Nucleotide sequence
A	Ala	Alanine	GCT, GCC, GCA, GCG
C	Cys	Cysteine	TGT, TGC
D	Asp	Aspartic acid	GAT, GAC
E	Glu	Glutamic acid	GAA, GAG
F	Phe	Phenylalanine	TTT, TTC
G	Gly	Glycine	GGT, GGC, GGA, GGG
H	His	Histidine	CAT, CAC
I	Ile	Isoleucine	ATT, ATC, ATA
K	Lys	Lysine	AAA, AAG
L	Leu	Leucine	CTT, CTC, CTA, CTG, TTA, TTG
M	Met	Methionine	ATG
N	Asn	Asparagine	AAT, AAC
P	Pro	Proline	CCT, CCC, CCA, CCG
Q	Gln	Glutamine	CAA, CAG
R	Arg	Arginine	CGT, CGC, CGA, CGG, AGA, AGG
S	Ser	Serine	TCT, TCC, TCA, TCG, AGT, AGC
T	Thr	Threonine	ACT, ACC, ACA, ACG
V	Val	Valine	GTT, GTC, GTA, GTG
W	Trp	Tryptophan	TGG
Y	Tyr	Tyrosine	TAT, TAC

Part of the results presented in my thesis have been previously published in:

Kmiec D, Iyer SS, Stürzel CM, Sauter D, Hahn BH, Kirchhoff F. Vpu-Mediated Counteraction of Tetherin Is a Major Determinant of HIV-1 Interferon Resistance. MBio. 2016 Aug 16;7(4). doi: 10.1128/mBio.00934-16

1. INTRODUCTION

1.1 Origin of HIV

The Human Immunodeficiency Virus (HIV) accounts for ~1 million deaths each year (2016, UNAIDS) and remains one of the biggest health concerns worldwide. HIV causes a progressive impairment of the immune system, termed Acquired Immune Deficiency Syndrome (AIDS). Cross-species transmission of HIV and its initial spread within the human population is estimated to have taken place in the 20th century in West Central Africa (Korber *et al*., 2000). Genetic similarity between HIV type 1 (HIV-1) and simian immunodeficiency viruses (SIV) infecting great apes strongly suggests that HIV-1 viruses emerged from SIVs infecting chimpanzees and gorillas and were probably acquired through contaminated bush meat (Sharp and Hahn, 2011). At least four independent cross-species transmissions from apes occurred, giving rise to HIV-1 groups M, N, O, and P (Fig.1). The second type of HIV (HIV-2) characterized by slower disease progression (Vidya Vijayan *et al.,* 2017), originated from at least nine zoonotic transmissions of SIVsmm infecting sooty mangabeys (Ayouba *et al*., 2013). These monkeys have a high prevalence of SIV (up to 60 % in wild populations; Santiago *et al*., 2005) and are often kept as pets or hunted for meat, which might partly explain their relatively frequent transmission to humans.

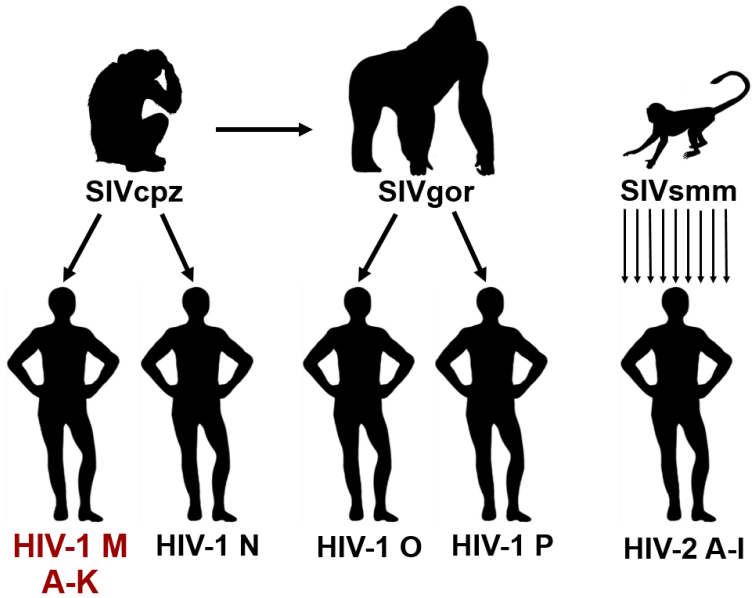


Figure 1: Origins of Human Immunodeficiency Viruses. SIVcpz infecting chimpanzees (*Pan troglodytes troglodytes*) was transmitted to humans on at least two occasions giving rise to the HIV-1 group N and the pandemic group M (which differentiated into subtypes A-K). SIVcpz was also transmitted to gorillas (*Gorilla gorilla*), giving rise to SIVgor (Takehisa *et al.*, 2009), which led to the emergence of HIV-1 groups O and P. SIVsmm infecting sooty mangabeys (*Cercocebus atys*) was transmitted on at least 9 occasions, giving rise to HIV-2 groups A-I (Visseaux *et al*., 2016).

More than 95 % of HIV infections are caused by HIV-1 group M (mainly by subtypes A, B and C; UNAIDS) which is thus largely responsible for the AIDS pandemic. Despite the development of effective chemotherapeutic agents complementarily targeting viral replication (HAART - highly active antiretroviral therapy), there is still no cure for HIV/AIDS. Once HIV infects a cell, its genetic material integrates into the host DNA. Even following extended administration of HAART, virus present in the long-lived memory cells can restore high viral loads shortly after treatment cessation (Davey *et al*., 1999; Maenza *et al*., 2015).

SIV and HIV genomes have a similar organization, are very compact (~9500 bp) and contain 8 to 9 genes (Fig.2). Viral genomes are flanked by long terminal repeats (LTR), which play an important role in viral DNA integration into the host genome and gene expression. Essential structural proteins are encoded by *gag* (matrix protein p17, capsid protein p24, nucleocapsid protein p7, spacer peptides 1 and 2 and p6 protein) and *env* (surface glycoproteins gp120 and gp41), whereas *pol* is the source of enzymatic proteins (reverse transcriptase – RT, RNAse H, integrase – IN, and viral protease – PR). In addition, these viruses express two regulatory proteins: the trans-activator Tat, which regulates the transcription of viral RNA and the regulator of expression of viral proteins Rev, which controls viral protein synthesis. There is also a group of accessory proteins, which are dispensable for viral replication in some cell lines, but important for enhancing *in vivo* fitness of the virus: Nef, Vpr, Vif and Vpu. The latter is found in HIV-1 and its direct precursors, but not in HIV-2 and SIVsmm, where a different accessory protein is present (Vpx).

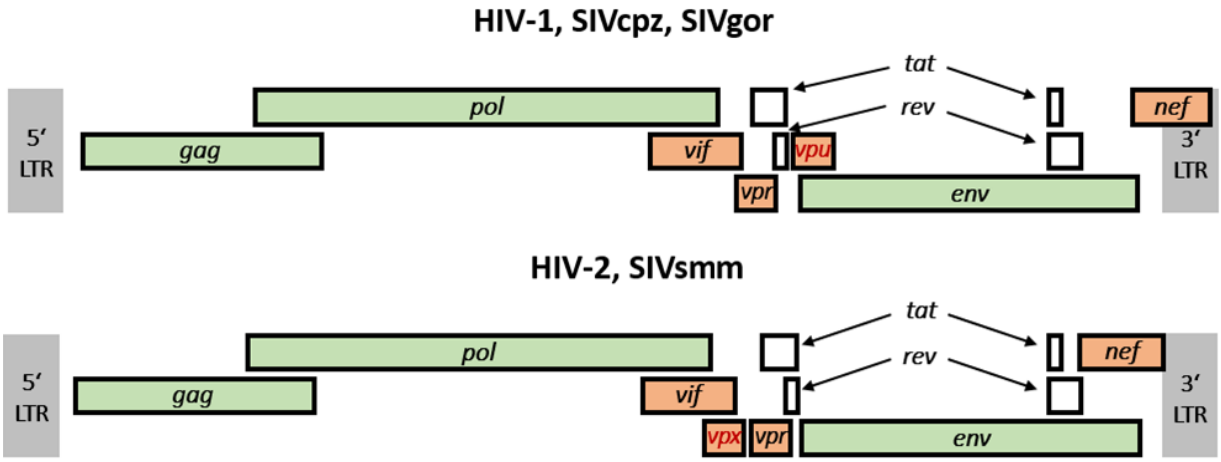


Figure 2: Genomic organization of HIV-1, HIV-2 and their SIV precursors. Viral genes shown in green encode for structural and enzymatic proteins, white indicates regulatory genes whereas accessory genes are coloured in orange. Differences between HIV-1 and HIV-2 genomes include the presence of a *vpu* or *vpx* gene, respectively, as well as a significant overlap between the *env* and *nef* genes in case of HIV-2 and SIVsmm.

1.2 Viral replication cycle

Although HIV has the ability to productively infect a variety of different cell types, which may also act as viral reservoirs (Kandathil *et al*., 2016), its main *in vivo* targets are CD4+ T cells. These cells play an important role in humoral and cellular immune responses and the loss of CD4+ T cells directly correlates with immune dysfunction observed in AIDS patients. HIV enters susceptible cells by binding of the viral envelope glycoprotein (Env) to the primary receptor CD4 (Dalgleish *et al*., 1984) and a chemokine co-receptor (CCR5 or less often CXCR4; Björndal *et al*., 1997) (Fig.3). This interaction induces conformational changes within Env (Sattentau *et al*., 1991), which lead to the fusion of viral and host membranes and delivery of the HIV capsid into the cell cytoplasm. There, the reverse transcription of viral RNA into DNA takes place, followed by capsid uncoating (Cosnefroy *et al*., 2016), formation of the viral pre-integration complex (PIC) and transport of the viral genome through the nuclear pore complex (Suzuki and Craigie, 2007). Viral integrase and LTR mediate insertion of the reverse transcribed HIV genome into the host DNA. HIV has been shown to preferentially insert its genome into active genes (Schröder *et al.*, 2002), which is thought to promote viral gene expression. Initially, only short fully-spliced mRNAs encoding the viral regulatory proteins Tat and Rev are produced (Kim *et al*., 1989). These two proteins mediate and control efficient transcription of other viral mRNAs and their nuclear export, respectively (reviewed in Karn *et al*., 2012). Following viral mRNA translation and processing of HIV-1 proteins, progeny virions assemble at the cell surface. Plasma membrane wraps around the budding virion and upon its fission, viral progeny is released into the extracellular space. Finally, viral protease present in the virions cleaves the precursor Gag and Gag-Pol polyproteins, which results in capsid formation and virion maturation (late stages of replication reviewed in Freed, 2015).

The mature HIV-1 virion has a spherical shape with a diameter of ~150 nm (Briggs *et al*., 2003) and contains 7–14 trimeric complexes of viral envelope protein in its cell-derived membrane (Chertova *et al*., 2002). Outer envelope is anchored by the transmembrane protein gp41, which is associated with the viral matrix protein p17. Viral cone-shaped capsid assembled from the p24 capsid protein encloses multiple viral proteins (RT, RNAse H, IN, PR, Vif, Vpr, and Nef) as well as two copies of single-stranded RNA genome bound to the nucleocapsid (Sundquist *et al*., 2012).

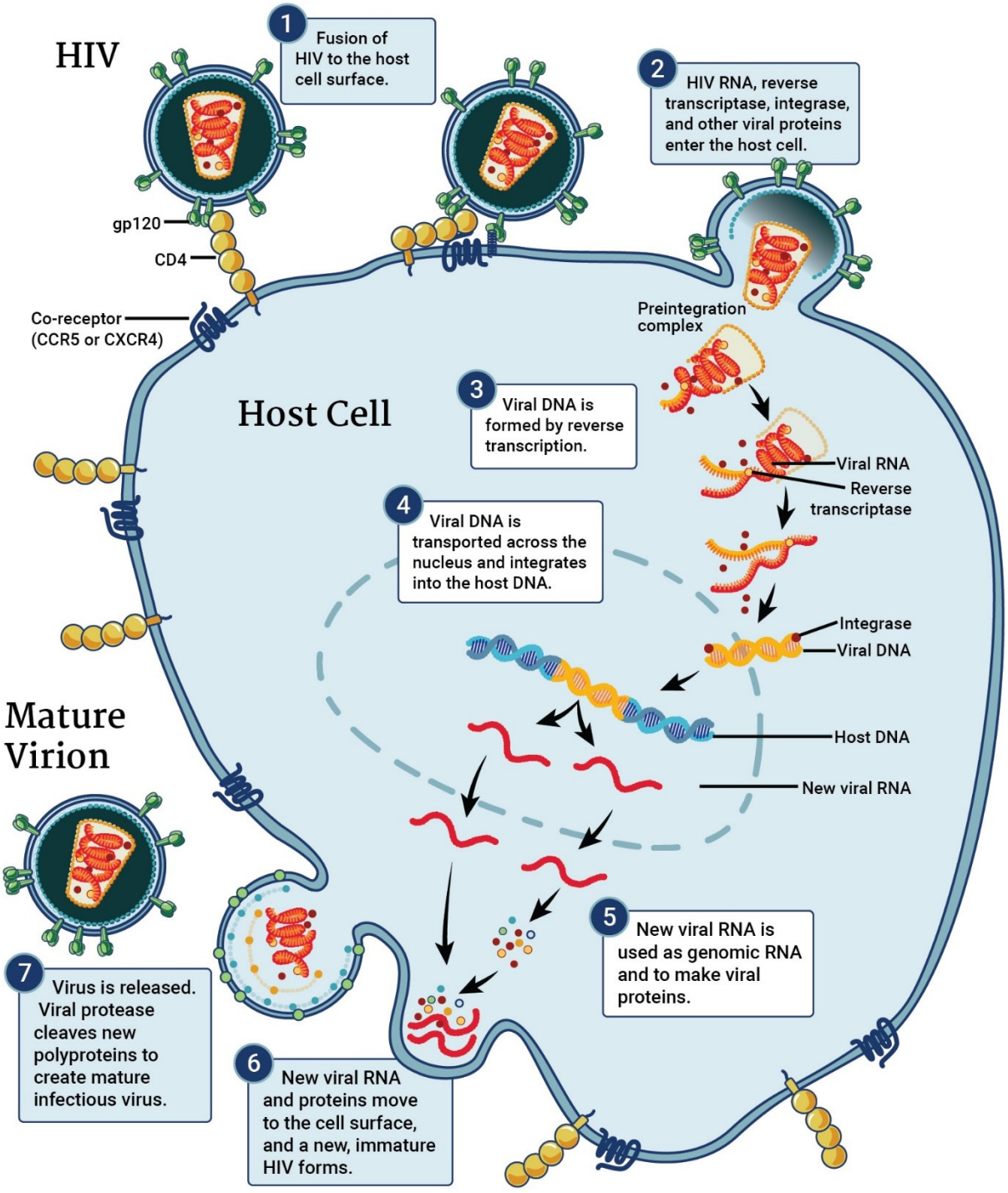


Figure 3: HIV replication cycle. Binding of the viral envelope gp120 to the CD4 receptor and the co-receptor (mainly CCR5 or CXCR4) induces membrane fusion (1). This results in capsid release (2). Reverse transcription (3) converts viral RNA genome into DNA, which then imported into the cell nucleus. Integration of viral DNA into the host cell genome is mediated by the viral Integrase (4). Transcriptional activation of viral gene expression is regulated by Tat whereas Rev mediates the nuclear export of viral RNA, which is then translated and processed (5). Virions containing two copies of RNA genome assemble at the plasma membrane (6). Following the release, virions undergo maturation (7). Diagram reproduced from https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle. Figure licence: Creative Commons Attribution Generic 2.0 (CC-BY 2.0) https://creativecommons.org/licenses/by/2.0/legalcode

1.3 HIV transmission and disease progression

Currently, around 37 million people worldwide live infected with HIV, passing it to further ~2 million individuals each year (UNAIDS, 2016). Transmission might result from the exchange of contaminated body fluids, including blood or breast milk as well as organ/tissue transplants. However, in most cases, the virus is transmitted through the sexual/mucosal route. Despite high viral loads of up to 10^7 RNA copies/ml in blood and semen (Gupta *et al*., 1997; Little *et al*., 1999) and a high diversity of viral quasi-species in the donor, in ~80 % of sexual transmission cases, only a single virus, called transmitted founder (TF) is responsible for the establishment of infection in the recipient (Wilen *et al.*, 2011). Selection pressure represented by this transmission bottleneck is one of the reasons why the risk of acquiring the virus is only between 0.04 % - 1.5 % per sexual intercourse (Patel *et al*., 2014). A TF virus has to overcome multiple donor and recipient barriers to successfully establish an infection. These include unfavourably low vaginal pH (Aldunate *et al*., 2013), natural antimicrobials such as mannose-binding lectin (Saifuddin *et al*., 2000) present in blood and genital mucosa and variable availability of target cells (reviewed in Iwasaki, 2010).

It is still not clear, which viral characteristics are specifically important during the transmission. TF viruses almost always utilize CCR5 as an entry co-receptor (Wilen *et al*., 2011; Parker *et al*., 2013) and readily infect CD4+ T cells but not macrophages, which express lower levels of the entry receptor CD4 (Isaacman-Beck *et al*., 2009). TF viruses also show higher *in vivo* fitness (Carlson *et al*., 2014), enhanced infectivity as well as partial resistance to interferon (IFN) α and β (Parrish *et al*., 2013; Iyer *et al*., 2017). It was reported that TFs are more resistant to restriction by interferon-induced transmembrane proteins (IFITMs) (Foster *et al*., 2016) than the chronic strains. This suggests that antiviral factors induced by type I IFN are an important barrier for HIV during initial stages of infection. Once the virus has established a systemic infection, the viral population diversifies. Mutations generated by the error-prone reverse transcriptase (Roberts *et al*., 1988) accumulate in the viral genome due to a selection pressure elicited by antibodies and cytotoxic T cells (Salazar-Gonzalez *et al*., 2009). During the early chronic stage, IFN levels drop and thus the evolutionary pressure to maintain high IFN resistance is reduced (Stacey *et al*., 2009; Huang *et al*., 2016). Although the immune activation decreases, it still remains abnormally high, contributing to chronic inflammation, CD4+ T cell loss, and AIDS progression (Hardy *et al*., 2013).

1.4 Innate immune recognition and restriction of HIV

HIV presence at the site of infection can be recognized by multiple pattern recognition receptors (PRRs) expressed by the immune cells. The best known PRRs are Toll-like receptors (TLRs), but also other proteins such as retinoic acid-inducible gene I (RIG-I) helicase and the cytosolic DNA-sensor cyclic guanosine monophosphate–adenosine monophosphate (GMP-AMP) synthase have been found to recognize HIV nucleic acids in the cytoplasm (Yoneyama *et al*., 2004, Gao *et al*., 2013). Presence of viral DNA is also sensed by the IFN-γ inducible protein 16 (IFI16), which promotes innate immune responses through a stimulator of interferon genes (STING)-dependent pathway (Jakobsen *et al*., 2013). Binding of viral components to these receptors commonly results in nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and IFN-induced immune activation. The main producers of type I IFN in response to HIV infection are plasmacytoid dendritic cells (pDCs) (Ghanekar *et al*., 1996). pDCs act as sentinel cells in many tissues including gut and genital mucosa (Jameson *et al*., 2002; Rodriguez-Garcia *et al*., 2017), where they exert their immune surveillance function. Upon the endocytosis of HIV-1 ssRNA it is sensed by TLR7 (Heil *et al*., 2004). The ligand-receptor complex recruits the global TLR adaptor molecule Myeloid differentiation primary response 88 (MyD88), thereby initiating a signalling cascade resulting in the activation of transcription factors such as interferon regulatory factor 7 (IRF7) and NF-κB (Medzhitov *et al*., 1998; Honda *et al*., 2005), inducing the production of type I IFN. Interferon acts as a signalling molecule, activating immune effector cells and upregulating the expression of proteins specifically targeting viral replication. More than 30 antiviral factors have been described (Kluge *et al*., 2015) and the list is growing considering the recent discovery of serine incorporator 5 (SERINC5) and guanylate binding protein 5 (GBP5) as inhibitors of viral infectivity (Rosa *et al*., 2015; Usami *et al*., 2015; Krapp *et al.*, 2016). The best-studied antiviral restriction factors include tripartite motif containing protein 5 alpha (TRIM5α) (Stremlau *et al*., 2004), SAM domain and HD domain-containing protein 1 (SAMHD1) (Laguette *et al*., 2011), Tetherin (Neil *et al*., 2008; Van Damme *et al*., 2008) and Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G protein (APOBEC3G) (Sheehy *et al*., 2002), which target viral uncoating and reverse transcription, inhibit release or introduce lethal mutations into the viral genome, respectively. To propagate efficiently despite the presence of these anti-viral proteins, HIV evolved strategies to evade or directly counteract them. As a consequence of the continuous

evolutionary arms race between the pathogen and its host, antiviral proteins and their antagonists are under strong positive selection. Thus, restriction factors are often less effective against viruses in their natural hosts but represent potent barriers for cross-species transmissions.

1.5 Tetherin and its counteraction

Tetherin possesses broad antiviral activity and targets not only HIV, but also other enveloped viruses including Ebola (Kaletsky *et al*., 2009) Hepatitis B (Lv *et al*., 2015), Herpes type 1 and 2 (Blondeau *et al*., 2013; Liu *et al*., 2015), Lassa and Marburg (Sakuma *et al*., 2009) as well as Chikungunya (Jones *et al*., 2013) viruses. Tetherin forms disulphide bridge linked homodimers and possesses an N-terminal cytoplasmic tail, followed by a transmembrane domain (TMD) as well as a glycophosphatidylinositol (GPI) at the C-terminus (Kupzig *et al*., 2003). GPI anchors tetherin homodimers to the membrane lipid rafts, which are the main sites of viral assembly (Nguyen and Hildreth, 2000). When new virions are about to bud, tetherin provides a physical link between the viral and host membrane, anchoring the virion and preventing its release (Fig.4). It has been shown that a few dozen tetherin dimers are required to tether a single virion to the plasma membrane (Venkatesh and Bieniasz, 2013). Entrapment of virions also enhances the recognition of HIV-infected cells by the immune system and their subsequent elimination by the natural killer (NK) cells (Arias *et al*., 2013; Alvarez *et al*., 2014). In addition, binding of virions by tetherin results in the induction of immune signalling in the infected cell, activating the transcription of NF-κB associated pro-inflammatory mechanisms (Galão *et al*., 2012; Tokarev *et al*., 2013; Weinelt and Neil, 2014).

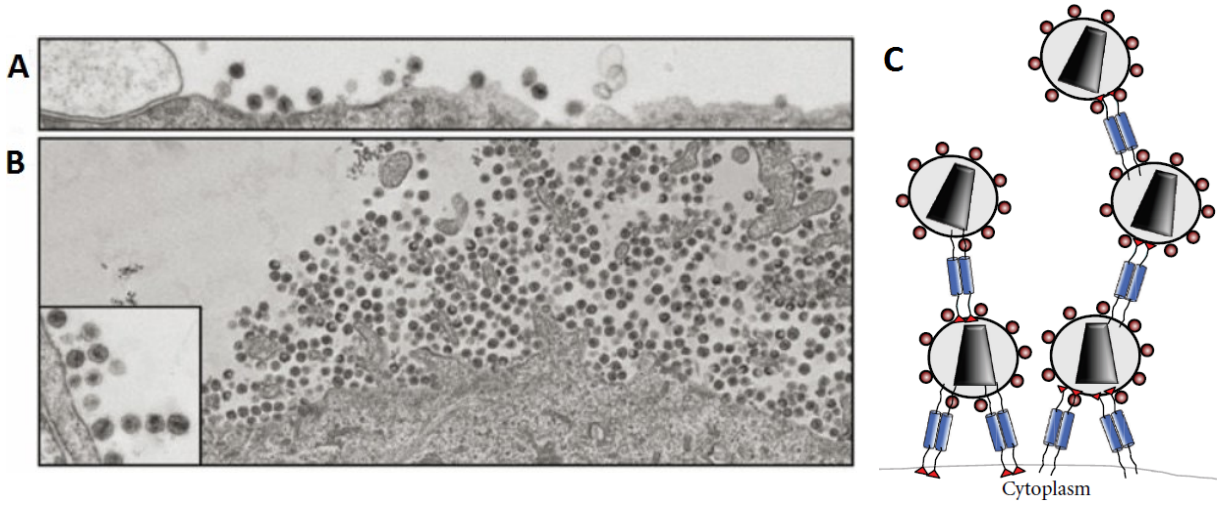


Figure 4: Tetherin-mediated restriction of HIV release. Electron micrographs showing the plasma membrane of HT1080 cells after infection with Vpu-deficient HIV-1 in the absence (A) or presence (B) of tetherin. Note the difference in the amount of cell-associated virus as well as the presence of multiple virion aggregates (lower left panel) in the presence of tetherin. Figures reproduced from Neil *et al*., 2008, page 428. (C) Schematic depiction of tetherin homodimers (blue) trapping HIV particles at the plasma membrane. Figure reproduced from Hammonds *et al*., 2012, page 3.

The importance of tetherin as an antiretroviral restriction factor is highlighted by the fact that all successfully spreading HIV strains and their precursors encode effective antagonists of this protein. Most SIVs use the accessory protein Nef to counteract tetherin of their respective host (Sauter *et al*., 2009; Zhang *et al*., 2009). Unlike its simian ortholog, human tetherin is resistant to SIV Nef as it contains a five amino acid deletion in its cytoplasmic tail targeted by the virus (Serra-Moreno *et al*., 2013; Zhang *et al*., 2009). Therefore, it was necessary for HIV to evolve alternative mechanisms to antagonise tetherin in the new human host. Pandemic HIV-1 group M and (to a lesser extent) rare group N strains adapted their accessory protein Vpu to perform this function in humans (Sauter *et al*., 2009, 2012). In case of HIV-1 group O, which has spread mainly among populations living in Cameroon, tetherin counteraction is still performed by Nef, which now targets a different motif adjacent to the missing part of the cytoplasmic tail (Kluge *et al*., 2014). HIV-1 group P, which was only found in two patients so far, has no tetherin antagonist (Sauter *et al*., 2011). Only pandemic HIV-1 group M encodes a Vpu protein that efficiently counteracts human tetherin. Thus, human tetherin represents an important barrier for cross-species viral transmission and its efficient antagonism might have contributed to successful spread of HIV-1 (Sauter *et al*., 2009).

Vpu is a classical lentiviral accessory protein, as it is dispensable for viral replication in some cell lines (Neil *et al*., 2008), but important for viral fitness and modulation of the immune

responses *in vivo* (Stephens *et al.*, 2002; Sato *et al.*, 2012; Dave *et al.*, 2013). Vpu is present in HIV-1 and its precursors but not in the majority of SIVs. This 16-18 kDa protein consists of a short N-terminal region, followed by an α-helical transmembrane domain (TMD), two cytoplasmic domains connected by a short hinge region, and a C- terminal tail (Maldarelli *et al.*, 1993; Park *et al.*, 2003; Wray *et al.*, 1995). It localizes mainly to the trans-Golgi network (TGN) (Dubé *et al.*, 2009; Fritz *et al.*, 2012). Despite its small size, it performs a variety of functions including downmodulation of tetherin, CD4, CD1d, NTB-A and HLA-C as well as inhibition of NF-κB signalling (reviewed in Soper *et al.,* 2017). As shown in Fig.5, the TMD of HIV-1 group M Vpus directly interacts with the TMD of human tetherin, inducing its clathrin-mediated endocytosis and endolysosomal degradation through the interaction with β-TrCP containing SCF (Skp-Cullin-F-box)/CRL1 (Cullin1-RING ubiquitin ligase) E3 ubiquitin ligase complex (Mitchell *et al.*, 2009; Kueck *et al.*, 2012; Kueck *et al.*, 2015) and AP-1 mediated displacement of tetherin from viral assembly sites (Pujol *et al.*, 2016). Thereby Vpu enhances virus release and protects HIV-infected cells from antibody-dependent cell-mediated cytotoxicity (Arias *et al.*, 2013).

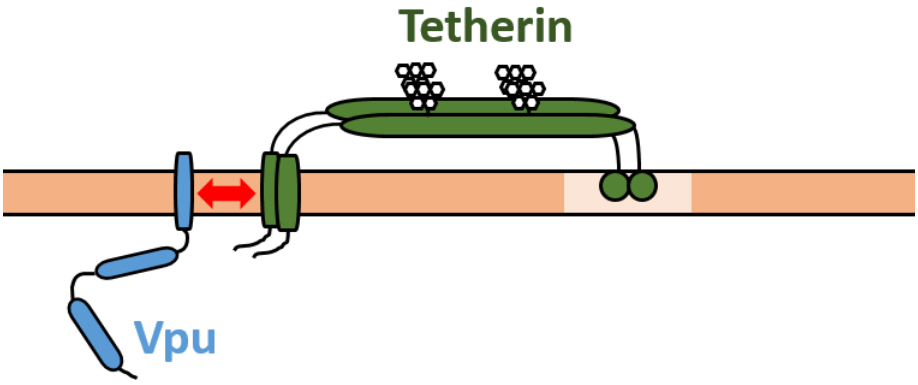


Figure 5: Tetherin antagonism by Vpu. The TMD of pandemic HIV-1 Vpu interacts with the TMD of a tetherin homodimer. This direct interaction is crucial for endolysosomal degradation of tetherin and thus exclusion from HIV-1 assembly sites.

Vpu also cooperates with Nef and Env in the downmodulation of CD4, by targeting the newly synthesized CD4 for proteasomal degradation (Willey *et al.*, 1992a; Wildum *et al.*, 2006). This prevents the interaction of immature Env with the newly synthesized CD4 in the endoplasmic reticulum (ER), which interferes with Env processing and transport to HIV assembly sites, potentially affecting viral infectivity (Dubé *et al.*, 2010). In some cases, Nef and Vpu have opposing roles. For example, Nef induces NF-κB activation early during infection to aid initiation of efficient viral transcription, whereas the late expressed Vpu inhibits NF-κB

signalling to suppress the induction of cellular antiviral factors (Sauter *et al*., 2015). Vpu is also known to downregulate natural killer, T and B cell antigen (NTB-A), polio virus receptor (PVR), CD1d and intercellular adhesion molecule 1 (ICAM-1) from the cell surface, which helps the infected cell to evade detection by natural killer (NK) and natural killer T (NKT) cells (Moll *et al*., 2010; Shah *et al.,* 2010; Matusali *et al*., 2012; Sugden *et al*., 2017). Recently, Vpu was also found to downmodulate HLA-C, a receptor involved in recognition by cytotoxic T lymphocytes (Apps *et al*., 2016). It has been established that HIV-1 deficient in Vpu replicates less efficiently, disseminates slower, and becomes more sensitive to IFN-α as shown by studies in humanized mouse model (Sato *et al*., 2012; Dave *et al.*, 2013); however the individual contribution of the different functions of Vpu to the observed effect remains unclear.

1.6 Scientific aims

Understanding which viral features allowed HIV-1 to spread efficiently and cause the AIDS pandemic might help to identify novel targets for prevention or therapy. One aspect that distinguishes pandemic HIV-1 group M strains from less prevalent and geographically limited O, N, and P viruses is the evolution of Vpu as efficient antagonist of human tetherin. It has been suggested that this adaptation may have been a prerequisite for the pandemic spread of HIV-1 in the human population. However, direct evidence for a role of efficient tetherin antagonism in viral transmission and replication fitness is missing.

The aim of this study was thus to elucidate the role of Vpu-mediated tetherin antagonism in interferon sensitivity and replication fitness of HIV-1. In contrast to most previous studies, which exclusively utilized the T-cell line adapted HIV-1 strains, I examined a variety of primary HIV-1 strains representing different groups, subtypes and infection stages, including pandemic transmitted founder viruses highly relevant for HIV spread. To selectively disrupt the anti-tetherin activity of Vpu, I mutated the amino acids that emerged in Vpu proteins of pandemic HIV-1 group M strains but are absent in non-pandemic HIV-1 or SIVcpz Vpu. Functional characterization of generated mutants was then performed to verify that these mutations disrupt anti-tetherin activity but no other properties, such as CD4, NTB-A, and CD1d downmodulation, as well as inhibition of NF-κB activity. Finally, the contribution of Vpu-mediated tetherin antagonism to viral replication fitness and IFN resistance was analysed in primary human CD4+ T cells.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell culture

Immortalized cell lines

HEK293T	Human embryonic kidney cell line expressing the simian virus 40 (SV40) large T antigen (Graham and Smiley, 1977).
HEK293T MaMTH reporter	Genetically modified HEK293T cells containing stably integrated reporter system consisting of five Gal4 upstream activating sequence repeats followed by a gene expressing *Gaussia* luciferase (Petschnigg *et al*., 2014). Gift from Prof. Igor Stagljar.
TZM-bl	Derivative of HeLa cell line expressing relatively high levels of human CD4, CCR5, and CXCR4. Contains reporter firefly luciferase and β-galactosidase genes under the control of the HIV long terminal repeat (LTR) promoter (Platt *et al*., 1998). This reagent was obtained through the NIH AIDS Reagent Program Division of AIDS, NIAID, from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

Primary human cells

PBMCs (peripheral blood mononuclear cells from human blood)	DRK
CD4+T cells (CD4+ T lymphocytes from human blood)	DRK

Cell culture reagents

Biocoll separation solution	Biochrom
Calcium dichloride: filter-sterilized 2 M CaCl2 solution	Applichem

2x HBS	1.64 % NaCl (w/v), 1.19 % HEPES (w/v) 0.04 % Na_2HPO_4 (w/v) distilled H_2O pH 7.1	Honeywell Applichem J.T.Baker
CD4+ T Cell Isolation Kit		Miltenyi
Dulbecco's modified eagle medium (DMEM)		Gibco
Foetal calf serum (FCS)		Gibco
Interferon-α2 (IFN-α2)		PBLAssay Science
Interleukin-2 (IL-2)		Sigma-Ark
L-glutamine		Gibco
Penicillin/Streptomycin		Gibco
Dulbecco's Phosphate Buffered Saline (DPBS)		Gibco
Phytohaemagglutinin (PHA)		Murex
Roswell Park Memorial Institute medium (RPMI-1640)		Gibco
T cell activation/expansion kit		Miltenyi

All plastic consumables (plates, flasks) were obtained from Sarstedt.

2.1.2 Bacterial culture

Bacterial strains

Escherichia coli (E. coli) XL2-Blue (Genotype: endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac glnV44 F' [::Tn10 proAB+ lacIq Δ (lacZ) M15 Amy CmR] hsdR17 (rK- mK+))	Agilent Technologies
Escherichia coli (E. coli) XL2-Blue MRF´ (Genotype: endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac glnV44 e14- Δ (mcrCB-hsdSMR-mrr) 171 recB recJ sbcC umuC::Tn5 uvrC F' [::Tn10 proAB+ lacIq Δ(lacZ) M15 Amy CmR])	Agilent Technologies

Bacterial culture reagents

Dimethyl sulfoxide (DMSO)		Fluka
Super Optimal Catabolite repression (SOC) medium		LifeTechnologies
Lysogeny Broth (LB)	10 g/l bacto-tryptone	BD Biosciences
	5 g/l yeast extract	BD Biosciences
	8 g/l NaCl	Honeywell
	1 g/l glucose	Roth
	distilled H_2O	
LB ampicillin	100 mg/l ampicillin	Ratiopharm
	LB medium	
LB ampicillin agar	15 g/l agar	BD Biosciences
	100 mg/l ampicillin	Ratiopharm
	LB medium	
LB kanamycin	50 µg/l kanamycin	LifeTechnologies
	LB medium	
LB kanamycin agar	15 g/l agar	BD Biosciences
	100 µg/l kanamycin	LifeTechnologies
	LB medium	

2.1.3 Plasmids

Vector types

pBR322	Expression construct containing ampicillin and tetracycline resistance genes for negative selection in bacteria. (Bolivar *et al*., 1977;Watson *et al*., 1988).
pcDNA3.1(+)	CMV-promoter based expression vector. Contains ampicillin resistance gene for negative selection in bacteria. (Invitrogen/LifeTechnologies)

pCG	CMV promoter based expression vector. Promotes eGFP expression via an IRES element. Contains ampicillin resistance gene for negative selection in bacteria. (Tanaka and Herr, 1990)
pCR XL TOPO	Expression vector containing topoisomerase covalently bound to vector ends, allowing efficient cloning of long PCR-products. Contains kanamycin resistance gene for negative selection in bacteria. (Invitrogen/LifeTechnologies)
pDONR221	Gateway® cloning adapted vector. Allows recombination with an attB expression clone or an attB PCR product for generation of entry clones. Contains kanamycin resistance gene for negative selection in bacteria. (Invitrogen/Life Technologies)
pHIT-G VSV-g	CMV promoter based vector expressing envelope glycoprotein of Vesicular Stomatitis Virus. Contains ampicillin resistance gene for negative selection in bacteria. (Fouchier *et al*., 1997)
p_human c.a. IKKβ	Plasmid expressing mutated version of human IKKβ containing two phosphomimetic changes (S177E and S181E) in the activation loop that render the protein constitutively active. Contains ampicillin resistance gene for negative selection in bacteria. Gift from Prof. Bernd Baumann.
pMaMTH Bait	Tetracycline-inducible expression vector containing in which expressed protein is fused with C-terminal portion of ubiquitin fragment and transcription factor Gal4. Contains ampicillin resistance gene for negative selection in bacteria (Petschnigg *et al*., 2014). Gift from Prof. Igor Stagljar.
pMaMTH Prey	Expression vector containing CMV promoter in which expressed protein is fused with N-terminal portion of ubiquitin. Contains ampicillin resistance gene for negative selection in bacteria (Petschnigg *et al*., 2014). Gift from Prof. Igor Stagljar.

pNF-kB — Firefly luciferase reporter construct plasmid expressing firefly luciferase under the control of three NF-κB response elements. Contains ampicillin resistance gene for negative selection in bacteria (Karl *et al*., 2009). Gift from Prof. Bernd Baumann.

pQCXIP — CMV promoter based vector with bicistronic retroviral element allowing expression of a gene together with a puromycin-resistance marker. Contains ampicillin resistance gene for negative selection in bacteria. Gift from Prof. Edward Barker.

pTAL — Reporter construct, which expresses *Gaussia* luciferase under the control of a minimal TATA-like (TAL) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter. Contains ampicillin resistance gene for negative selection in bacteria. (Thermo Scientific)

pUC57 — Expression construct containing ampicillin and tetracycline resistance genes for negative selection in bacteria. (Thermo Scientific)

Infectious molecular clones

Table 1: Infectious molecular clones (IMCs) of used HIV strains. Information includes official and shortened names of strains used throughout this study, accession number as a reference to full viral genome available in the public sequence database (NCBI Nucleotide https://www.ncbi.nlm.nih.gov/nucleotide/), expression vector in which the viral genome was cloned into and a reference publication describing generation of each IMC. Vpu-defective IMCs used contained a premature stop codon at amino acid positions 2 and 3 of the *vpu* reading frame, except for NL4-3 that contained a 120-bp deletion at the start of *vpu*.

Name	Viral sequence accession no.	Reference	Vpu deficient mutant
NL4-3	KM390026	Adachi *et al*., 1986	120bp deletion in Vpu
CH058	JN944940	Parrish *et al*., 2013	ATG 2x STOP
CH077	JN944941	Parrish *et al*., 2013	ATG 2x STOP
CH167	KC156213	Parrish *et al*., 2013	ATG 2x STOP
STCO-1 (STCO)	KC312457	Parrish *et al*., 2013	ATG 2x STOP
DJO0131 (DJO)	AY532635	Bodelle *et al*., 2004	ATG 2x STOP

DNA constructs

Table 2: Expression constructs used in this study. Plasmid type is included in the name (e.g. pCG). Internal number (column 2) and enzymes routinely used for control digestion (column 3) are also provided.

Name	Internal no.	Control digestion
pCG_NL4-3 Vpu C-AU1 IRES eGFP	DK_II_14	XbaI/MluI
pCG_ NL4-3 Vpu TMDmut C-AU1 IRES eGFP	DK_II_25	XbaI/MluI
pCG_ CH058 Vpu C-AU1 IRES eGFP	DK_II_5	XbaI/MluI
pCG_CH058 Vpu TMDmut C-AU1 IRES eGFP	DK_II_8	XbaI/MluI
pCG_CH077 Vpu C-AU1 IRES eGFP	DK_II_6	XbaI/MluI
pCG_CH077 Vpu TMDmut C-AU1 IRES eGFP	DK_II_7	XbaI/MluI
pCG_CH167 Vpu C-AU1 IRES eGFP	DK_II_104	XbaI/MluI
pCG_CH167 Vpu TMDmut C-AU1 IRES eGFP	DK_II_31	XbaI/MluI
pCG_STCO Vpu C-AU1 IRES eGFP	DK_II_28	XbaI/MluI
pCG_ STCO Vpu TMDmut C-AU1 IRES eGFP	DK_II_23	XbaI/MluI
pCG_ DJO0131 Vpu C-AU1 IRES eGFP	DK_II_20	XbaI/MluI
pCG_ DJO0131 Vpu TMDmut C-AU1 IRES eGFP	DK_II_27	XbaI/MluI
pCG_NL43 Nef 3xSTOP (empty) IRES eGFP	DK_II_15	XbaI/MluI
pCG_human tetherin ΔIRES eGFP	DK_II_17	XbaI/MluI
pCDNA3.1_human CD4	DK_II_16	HindIII/XbaI
pQCXIP_human NTB-A transcript variant 2	DK_II_21	EcoRI
pCG_human CD1d ΔIRES eGFP	DK_II_13	XbaI/MluI
pTAL-*Gaussia* Luciferase	DK_II_19	NcoI/XbaI
pNFkB(3x)-Firefly Luciferase	DK_II_22	HindIII/BamHI
p_human IKKβ c. a. mutant (S177E, S181E)	DK_II_29	NcoI/ClaI
pBR_HIV-1 M NL43 (R5) Vpu STOP	DK_II_18	HindIII
pBR_HIV-1 M NL4-3	DK_II_102	HindIII
pBR_HIV-1 M NL4-3 TMDmut Vpu	DK_II_101	HindIII
pBR_HIV-1 M NL4-3 Vpu STOP	DK_II_44	HindIII
pCR-XL-TOPO_CH058	DK_II_37	NdeI

pCR-XL-TOPO_CH058 TMDmut Vpu	DK_II_38	NdeI
pCR-XL-TOPO_CH058 Vpu STOP	DK_II_100	NdeI
pCR-XL-TOPO_CH077	DK_II_36	HindIII
pCR-XL-TOPO_CH077 TMDmut Vpu	DK_II_39	HindIII
pCR-XL-TOPO_CH077 Vpu STOP	DK_II_99	HindIII
pUC57_HIV-1 M CH167	DK_II_35	NheI
pUC57_HIV-1 M CH167 TMDmut Vpu	DK_II_47	NheI
pUC57_HIV-1 M CH167 Vpu STOP	DK_II_98	NheI
pBR_HIV-1 M STCO	DK_II_33	MfeI
pBR_HIV-1 M STCO TMDmut Vpu	DK_II_43	MfeI
pBR_HIV-1 M STCO Vpu STOP	DK_II_116	MfeI
pCR-XL-TOPO_HIV-1 N DJO0131	DK_II_32	NotI/MluI
pCR-XL-TOPO_HIV-1N DJO0131 TMDmut Vpu	DK_II_96	NotI/MluI
pCR-XL-TOPO_HIV-1N DJO0131 Vpu STOP	DK_II_97	NotI/MluI
pHIT-G_VSVg	DK_II_122	EcoRI
pMaMTH_Bait	DK_II_361	NcoI
pMaMTH_Prey	DK_II_363	NcoI
pDONR221 entry vector	DK_II_376	AflII/EcoNI
pMamTH_Bait STCO CC Vpu	DK_II_392	NcoI
pMaMTH_Bait STCO TMDmut Vpu	DK_II_393	NcoI
pMaMTH_Prey human Tetherin	DK_II_398	NcoI
pMaMTH_Prey Pex7	DK_II_460	NcoI
pMaMTH_Prey SHC1	DK_II_461	NcoI
pMaMTH_Bait EGFR	DK_II_459	NcoI

2.1.4 Cloning and DNA purification

Oligonucleotides

Table 3. Oligonucleotides used in TMDmut Vpu cloning. Name denotes internal oligonucleotide number, sequence given in 5' to 3' direction, forward (fwd) or reverse (rev) strand binding primer, purpose describes which mutant Vpu was generated using indicated primer whereas inner /outer indicates whether primer binds within

mutation region (inner) or outside of Vpu gene (outer). In all cases alanines (GCA) were mutated to leucines (CTA). Last two rows list primers used for verification of generated constructs by nucleotide sequencing. All oligonucleotides were synthesized by biomers.net (Ulm).

Name	Sequence	fwd/rev	purpose	type
SK405	caatattactaatagttgtgtggaccatagtatacatag	rev	CH077 Vpu	inner
SK404	ccacacaactattagtaatattgctagtactac	fwd	CH077 Vpu	inner
SK406	gtccacacaattattagtaatattgctagcac	fwd	CH058 Vpu	inner
SK407	caatattactaataattgtgtggaccatagtattc	rev	CH058 Vpu	inner
SK412	ctatgctccacacaattattagtatgattaatagtactac	rev	CH167 Vpu	inner
SK413	gtagcattagtagtactattaatcatactaataattgtgtg	fwd	CH167 Vpu	inner
DS961	gcattagtagtactaataataatactaatagttgtgtgg	fwd	NL4-3 Vpu	inner
DS962	ccacacaactattagtattattattagtactactaatgc	rev	NL4-3 Vpu	inner
DS963	gttgggagtgctaattataatactagtaataatc	fwd	DJO Vpu	inner
DS964	gattattactagtattataattagcactcccaac	rev	DJO Vpu	inner
DS965	ggattagtagtactagcaatactactaatagttgtgtg	fw	STCO Vpu	inner
DS966	cacacaactattagtagtattgctagtactactaatcc	rev	STCO Vpu	inner
pDKII1	caaaattatggggatacttgggcagg	fwd	CH058 Vpu	outer
pDKII2	ccattgaaagtcttattattacactttagaatcg	rev	CH058 Vpu	outer
pDKII3	cagagggagccatacaatgaatgg	fwd	CH077 Vpu	outer
pDKII5	gcagagggaaccatacaatgaatgg	fwd	CH167 Vpu	outer
pDKII6	cttccttgtattattgccgggcc	rev	CH167 Vpu	outer
pDKII7	gaaatggcagacaagctaatacatc	fwd	DJO Vpu	outer
pDKII8	gttccctctgctactacaatagctattg	rev	DJO Vpu	outer
pDKII11	caaaactgacagaggacagatggaac	fwd	STCO Vpu	outer
pDKII12	cctccttcctctatgctactgttactac	rev	STCO Vpu	outer
pDKII21	ggagtgtgatatttcctgtagtaccagtcc	rev	CH077 Vpu	outer
pDKII_58	cggtgggaggtctatataagc	fwd	MaMTH seq	outer
pDKII_19	ggagacgccatccacgctgttttgacctcc	fwd	pCG seq	outer

Reagents

Agarose	Roth
DNA Ligation Kit Ver.2.1	Takara Bio Inc.
dNTPs	Life Technologies
DreamTaq™ DNA Polymerase	Fisher Scientific GmbH
Ethanol (99.9 %)	Sigma-Aldrich
Ethidium bromide	Sigma-Aldrich
Gateway LR Clonase Enzyme mix	Invitrogen/ThermoFischer
Gateway BP Clonase II Enzyme mix	Invitrogen/ThermoFischer
Isopropanol	Merck
Loading Dye	Roth
Miniprep Kit	Qiagen
Molecular weight size marker “1 kb plus ladder”	Life Technologies
Phire™ Hot Start II DNA polymerase Kit	Fisher Scientific GmbH
Restriction endonucleases:	
AfeI	New England Biolabs
AflII	New England Biolabs
AleI	New England Biolabs
AvrII	New England Biolabs
BamHI	New England Biolabs
ClaI	New England Biolabs
DraIII	New England Biolabs
EcoNI	New England Biolabs
EcoRI	New England Biolabs
HindIII	New England Biolabs
MfeI	New England Biolabs
MluI	New England Biolabs
NcoI	New England Biolabs
NdeI	New England Biolabs
NheI	New England Biolabs
NotI	New England Biolabs

XbaI	New England Biolabs
TAE buffer, 50x	5Prime GmbH
UltraClean® DNA Purification Kit	MoBio Laboratories Inc.
Wizard™ Plus Midiprep Kit	Promega

2.1.5 Western blot

Antibodies

Mouse anti-AU1 (NB600-453)	Novus Biologicals
Mouse anti-FLAG (F1804)	Sigma Aldrich
Rabbit anti-actin (ab8227)	Abcam
Rabbit anti-GFP (ab290)	Abcam
Rabbit anti-V5 (13202)	Cell Signaling
Goat anti-mouse IRDye Odyssey 680RD	LI-COR
Goat anti-rabbit IRDye Odyssey 800CW	LI-COR

Reagents

Antibody buffer: 0.2 % Tween-20 (v/v)	Sigma-Aldrich
1 % milk powder (w/v)	Sigma-Aldrich
DPBS	Gibco
β-mercaptoethanol	Sigma-Aldrich
Blocking buffer: 5 % milk powder (w/v)	Sigma-Aldrich
DPBS	Gibco
Filter paper	BioRad
Co-IP cell lysis buffer: 150 mM NaCl	Honeywell
50 mM HEPES	Applichem
5 mM EDTA	Sigma-Aldrich
0.5 mM sodium orthovanadate	Sigma-Aldrich
0.5 mM NaF	Sigma-Aldrich
0.1 % NP40 (v/v)	Abcam
Distilled H_2O	
pH 7.5	

Complete ULTRA Tablets, Mini EDTA-free	Roche
Immobilon-FL polyvinylidene difluoride (PVDF) membrane	Merck Millipore
Protein Sample Loading Buffer x4	LI-COR
Milk powder	Sigma-Aldrich
NuPAGE Antioxidant	LifeTechnologies
NuPAGE MES SDS Running Buffer	LifeTechnologies
NuPAGE Novex Bis-Tris 4-12 % gradient precast gels	LifeTechnologies
DPBS	Gibco
Precision PlusProtein Standard	Bio-Rad
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Transfer buffer: 47.9 mM Tris	AppliChem
38.6 mM Glycine	Sigma-Aldrich
1.3 mM SDS	Sigma-Aldrich
20 % Methanol (v/v)	Honeywell
distilled H_2O	
pH 8.3	
Wash buffer: 0.2 % Tween-20 (v/v)	Sigma-Aldrich
DPBS	Gibco

2.1.6 Confocal microscopy

Antibodies and stains

Hoest nucleic acid stain (33342)	ThermoFischer
Rabbit anti-V5 (13202)	Cell Signaling
Sheep anti-TGN46 (AHP500GT)	Serotec

Reagents

Microslides 8-well	IBIDI
Fixation buffer: 4 % Paraformaldehyde (v/v)	Merck
DPBS	Gibco
Blocking buffer: BSA diluent solution 10 % (final conc. 5 %)	KPL

0.5 % Triton X	Sigma Aldrich
DPBS	Gibco
Staining buffer: BSA diluent solution 10 % (final conc.1 %)	KPL
DPBS	Gibco
Mounting medium	IBIDI
DPBS	Gibco

2.1.7 Flow cytometry

Antibodies

anti-CD4/APC antibody	Invitrogen
anti-CD1d7/PE antibody	BD Biosciences
anti-CD11c/ FITC antibody	Abcam
anti-SLAM6 (NTB-A)/APC antibody	R&D
anti-tetherin/APC antibody	BioLegend
anti-p24/FITC antibody	Beckman Coulter
IgG1 Isotype Ctrl/APC antibody	BioLegend
IgG2a Isotype Ctrl/APC antibody	Invitrogen
IgG2a Isotype Ctrl/ PE antibody	Ancell

Reagents

FACS buffer: 1 % FCS (v/v)	Gibco
DPBS	Gibco
FIX&PERM® Kit	MuBio
Paraformaldehyde (PFA)	Merck
DPBS	Gibco

2.1.8 β-galactosidase assay

DEAE-Dextran	Sigma
Gal-screen® substrate	Applied Biosystems

2.1.9 AlphaLisa p24 detection assay

AlphaLisa p24 detection kit	PerkinElmer
Glycine 1.5 M, pH 1.85	Sigma-Aldrich
Tris 1.5 M, pH 11.0	AppliChem
Triton X-100, 5 %	Sigma-Aldrich

2.1.10 Dual luciferase assay

Coelenterazine	PJK
Luciferase Assay Systems	Promega
Luciferase Cell Culture Lysis 5x Reagent	Promega
Poly-L-lysine	Sigma Aldrich

2.1.11 Mammalian Membrane Two-Hybrid (MaMTH) assay

Polyethylenimine transfection reagent (PEI) 1 mg/ml	Polysciences
Tetracycline hydrochloride	Sigma Aldrich
Coelenterazine	PJK
White plate, 96-well, flat bottom	Sarstedt

2.1.12 Software and databases

BD FACSDiva ™	BD Biosciences
CLC Main Workbench 7	Qiagen Bioinformatics
CorelDRAW™ X4	Corel Corporation
GraphPad Prism 5.0	GraphPad Software
HIV Sequence Database (https://www.hiv.lanl.gov)	Los Alamos Laboratory
ImageJ	NIH
Microsoft Office Package	Microsoft Corporation
Multiple sequence alignment with hierarchical clustering (http://multalin.toulouse.inra.fr)	(Corpet, 1988)

Odyssey Image Studio Lite	LI-COR
Sequence logo generator (http://weblogo.berkeley.edu/)	(Schneider *et al*., 1990)
Simplicity 4.02	Berthold systems
SoftMax® Pro	Molecular Devices
ZEN 2010	Carl Zeiss

2.1.13 Laboratory equipment

BD FACS Canto II	BD Biosciences
Blotting Chamber Trans-Blot SD Cell	Bio-Rad
Microtube centrifuge	Eppendorf
Refrigerator with freezer	Liebherr
Swing-bucket centrifuge	Eppendorf
Electrophoresis power supply	Bio-Rad
Gel reader	Bio-Rad
Confocal laser-scanning microscope	Carl Zeiss
Incubator for bacterial culture	Binder
Shaking incubator	Sartorius
Scale	OHaus
Thermal Cycler	Analytik Jena
Biological safety cabinet	Heraeus
UV Trans-illuminator	Syngene
Incubator for cell culture with CO2 module	Thermo Fisher Scientific
Kinetic microplate reader	MDS analytical technology
Odyssey fluorescence scanner	LI-COR
Inverted light microscope	Carl Zeiss
Nano Drop 2000 spectrophotometer	peQlab
Microplate Luminometer	Berthold Systems
Thermomixer	Eppendorf
Vortexer	VWR International

2.2 METHODS

2.2.1 Isolation and cell culture

Cell culture

All used immortalized cell lines were cultured in DMEM medium supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and 10 % (v/v) heat inactivated foetal calf serum (FCS). Cells were split 1:10 upon reaching 90 % confluence. Primary human cells were cultured in RPMI1640 medium supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin, 10 % (v/v) heat inactivated FCS.

PBMC isolation

Lymphocyte concentrate (buffy coat) from 500 ml whole blood of healthy donors was diluted 1:4 with DPBS. The same volume of Biocoll lymphocyte separation medium was overlaid with the blood/lymphocyte solution and centrifuged at 1200 RCF for 20 min with the brake switched off. The white interface (buffy coat) containing PBMCs was transferred to a fresh tube. Cells were washed twice with DPBS and resuspended in RPMI1640 supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin, 10 % (v/v) heat inactivated FCS. Cells were stimulated 3 days prior to virus infection with 1 µg/ml PHA and 10 ng/ml IL-2.

CD4+ T cell isolation

Human CD4+ T cells were isolated from blood samples of three healthy donors using the CD4 cell isolation kit according to manufacturer's protocol. Cells were activated using anti-CD2/CD3/CD28 beads and cultured in RPMI1640 supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and 15 % FCS (v/v) for 4 days at 37 °C and 5 % CO2. Cells were pooled and either pre-treated with 500 U/ml of IFN-α2 or left untreated 24 h prior to infection.

2.2.2 Cloning

Gateway cloning

Primers introducing AttB1 and AttB2 at sites flanking the genes of interest (Vpu and tetherin) were designed using CLC Main Bench software. Following PCR reaction performed using Phire Hot Start Polymerase kit according to manufacturer's protocol, gene products were run on 1 % agarose and purified using UltraClean DNA purification Kit. BP recombination reaction between PCR product and pDONR221 entry vector was performed using BP Clonase Reaction kit according to manufacturer's recommendations. Recombination sample was transformed into XL2 blue *E.coli* and grown overnight on kanamycin agar plates at 37 °C. Colonies of entry clones were used to inoculate 5ml of kanamycin LB medium, followed by mini DNA preparation according to manufacturer's recommendations. Clones were sent to Eurofins genomics for insert verification (M13 vector site sequencing). Correct entry clones were used for a recombination reaction with pMaMTH Bait (Vpu) or Prey (tetherin) vectors using LR Clonase Reaction kit, following manufacturer's recommendations. Recombination sample was transformed into XL2 blue *E.coli* and grown overnight on ampicillin agar plates at 37 °C. Colonies were used to inoculate 5ml of ampicillin LB medium, followed by mini DNA preparation according to manufacturer's recommendations. Clones were sent to Eurofins genomics for insert verification with pDKII_58 primer. Confirmed clones were used to inoculate 150ml LB ampicillin medium and 16-24 h later DNA was prepared with Wizard Plus Midiprep Kit according to manufacturer's protocol. The purified DNA concentration and quality was determined using Nanodrop spectrophotometer.

SOE-PCR

Mutations in the *vpu* alleles of viral IMCs were introduced by splice overlap extension PCR using the Phire Hot Start DNA Polymerase Kit according to the manufacturer's protocol.
DreamTaq polymerase was used for cloning of WT and TMDmut Vpu genes into pCG IRES eGFP vector according to the manufacturer's protocol.

Restriction digestion

DNA digestion was performed using NEB enzymes according to the manufacturer's instructions and DNA fragments were separated by gel electrophoresis on agarose gel (1 % w/v, containing ethidium bromide) together with 1 kb plus ladder.

Isolation of DNA from an agarose gel

DNA is an agarose gel was visualized on a UV screen (366 nm). The bands of the desired size were cut out and the DNA was purified from the gel using the UltraClean 15 DNA purification Kit according to the manufacturer's instructions.

Ligation

Vector and insert DNA were mixed at a ratio of 1:4 and ligated overnight at 16 °C using the DNA Ligation Kit Ver.2.1 according to the manufacturer's instructions.

Transformation

Ligation mix was incubated on ice with 10 µl of XL2-Blue *E. coli* for 20 min. After a heat-shock at 42 °C for 30 s the bacteria were incubated on ice for 2 min. Following the addition of 200 µl S.O.C. medium the transformed cells were incubated at 37 °C on a shaker for 40 min and plated on LB agar plates containing the appropriate antibiotic (ampicillin or kanamycin).

DNA preparation

Plasmid DNA for cloning and sequencing was prepared with the Qiagen Miniprep Kit according to the manufacturer's protocol. To confirm the correctness of obtained clones, 5 µl of Mini-prep DNA (filled up to 15 µl with dist. H_2O) and appropriate primer (10 pmol/µl) were sent to Eurofins MWG GmbH for nucleotide sequencing service. Confirmed clones were used to inoculate 150ml LB culture containing appropriate antibiotic and 16-24 h later DNA was prepared with Wizard Plus Midiprep Kit according to manufacturer's protocol. The purified DNA concentration and quality was determined using Nanodrop spectrophotometer.

2.2.3 Transfection

Calcium-phosphate transfection

One day prior to transfection, 0.8 mln HEK293T were seeded in 6-well plates. Appropriate amount of DNA and 13 µl 2 M CaCl2 were mixed and filled up to a volume of 100 µl with distilled water. 100 µl 2x HBS was added dropwise to the DNA mixture. After mixing via pipetting and the mixture was added dropwise to the cells (200 µl for 6-well, 10 µl for 96-well). Cells were incubated for 8-16 h before medium was replaced by fresh medium. Cells were used for analysis 48 h post-transfection.

PEI transfection

MaMTH HEK293T reporter cells (~25.000/well) were seeded into F-well 96-well plates and incubated for 6 h. MaMTH Prey and Bait construct DNA (100ng of each) were mixed with 20 µl DPBS and 0.6 µl PEI transfection regent and incubated for 15 min at RT. Transfection mix (10 µl) was dropped onto the HEK293T MaMTH reporter cells seeded in the 96-well plates in triplicates and 24 h later medium was changed to a fresh one containing 100 ng/ml tetracycline.

To confirm MaMTH construct expression by a Western blot, MaMTH HEK293T reporter cells (~0.5 mln/well) were seeded into 12-well plate and incubated for 6h. DNA (2 µg) was mixed with 200 µl DPBS and 5 µl PEI transfection regent and incubated for 15 min at RT. Transfection mix was dropped onto the cells and 24 h later medium was changed to DMEM containing 100 ng/ml tetracycline. Cells were used for a Western blot analysis 40 h post-transfection.

2.2.4 Western blot

To compare WT and TMDmut Vpu expression, HEK293T cells were transfected with 5 µg of vector DNA expressing AU1 tagged Vpus and IRES eGFP. Untransfected cells (mock) and cells transfected with an empty vector (pCG_NL43 Nef 3xSTOP IRES eGFP) served as negative controls. Two days post transfection, cells were harvested and lysed in CO-IP buffer containing protease inhibitor cocktail. Cell lysates were separated in gradient 4-12 % Bis-Tris gel. After gel electrophoresis (120 V, 90 min), proteins were transferred onto PVDF membrane and probed

with anti-AU1 antibody. For loading and transfection controls, membrane was incubated with antibodies specific for actin and GFP respectively. Subsequently, the blots were probed with anti-mouse or anti-rabbit secondary antibodies. Protein bands were detected using LI-COR fluorescence scanner.

To verify the expression of Vpu and tetherin MaMTH expression vectors, WT and TMDmut of STCO Vpu V5-tagged Baits as well as human tetherin FLAG-tagged Prey constructs were transfected into MaMTH reporter HEK293T cells using PEI reagent. After 2 days cells were lysed in Co-IP buffer containing 1 % SDS and a protease inhibitor cocktail. Cell lysates were separated in gradient 4-12 % Bis-Tris gel. After gel electrophoresis (120 V, 90 min), proteins were transferred onto PVDF membrane and probed with anti-FLAG and anti-V5. For loading control, blot was incubated with antibodies specific for actin. Subsequently, the blot was probed with anti-mouse or anti-rabbit IRDye Odyssey antibodies and protein bands were detected using LI-COR fluorescence scanner.

2.2.5 Flow cytometry

Receptor downmodulation in HEK293T cells

To determine the effects of Vpu on CD4, CD1d, NTB-A, and tetherin cell surface expression, HEK293T cells were transfected by the calcium phosphate method with 1 µg of CD4, CD1d, NTB-A, or tetherin expression vector and 5 µg of pCG eGFP/Vpu constructs expressing eGFP alone or together with Vpu. Two days post transfection, CD4, CD1d, NTB-A, or tetherin present on cell surface were stained with either allophycocyanin (APC)-conjugated anti-human tetherin antibody, APC conjugated anti-human CD4 antibody, a phycoerythrin-conjugated anti-CD1d antibody, or an APC-conjugated anti-SLAM6 antibody. Cells were analysed by flow cytometry using BD FACS Canto II. Mean fluorescence intensity (MFI) obtained for cells transfected with the control construct expressing only eGFP was compared to the MFI obtained for cells expressing Vpu and eGFP to determine the efficiency of downregulation.

Receptor downmodulation in PBMCs

VSV-g pseudotyped virus stocks (VS) were prepared by calcium phosphate transfection of HEK293T cells with 5 µg proviral construct and 0.5 µg VSV-g expression vector, followed by supernatant harvest 48 h later. To determine the effect of Vpu on surface receptor expression levels in primary cells, PHA-stimulated PBMCs were transduced with VS by spinoculation (2 h at 37 °C, 1,300 x *g*). Three days after transduction, PBMCs were dual stained for surface tetherin (APC) and CD4 (PE) using fluorochrome conjugated antibodies, and stained intracellularly using FIX&PERM® Kit and p24 fluorescein isothiocyanate (FITC)-conjugated antibody. The fluorescence of stained cells was detected by flow cytometry BD FACS CantoII and Vpu- mediated downmodulation of cell surface receptors was calculated by comparing APC or PE MFI of infected cell population (FITC+) against MFI of cells infected with vpu-deficient strains (BD FACSDiva software).

2.2.6 Mammalian Membrane Two-Hybrid (MaMTH) assay

To determine the level of interaction between tetherin and WT or TMDmut Vpu of STCO strain, 20,000 HEK293T cells were co-transfected in a 96-well format with 1:1 ratio of Vpu Bait and Tetherin N- Prey expression vectors. The following day, bait expression was induced by adding 0.1 mg/ml tetracycline to the culture medium and the cells were incubated overnight. Supernatant was harvested around 40 h post transfection and *Gaussia* luciferase measurement was performed using 0.1 s measurement following 50 mM coelenterazine injection by microplate luminometer. The enzyme activity was measured as relative light units/second (RLU/s) using the Software Simplicity 4.02.

2.2.7 β-galactosidase assay

TZM-bl cells were seeded in F-bottom 96-well plates (6000 cells/well). The following day, cells were infected with supernatants of transfected HEK293T cells. Three days post-infection, the β-galactosidase activity was measured in the cells after addition of 1:4 diluted Gal-screen

substrate using a microplate luminometer. The enzyme activity was measured as relative light units/second (RLU/s) using the Software Simplicity 4.02.

2.2.8 Vpu antagonism of tetherin-mediated viral restriction

To determine the capability of Vpu and Nef to antagonize tetherin, HEK293T were seeded in 6-well plates and transfected with 2 µg of NL4-3 vpu-deficient provirus, 0.5 µg of Vpu, and dilutions of tetherin expression plasmid (6.25, 12.5, 25, 100, and 250 ng). pCG vector expressing eGFP was used to equalize the DNA input. Two days later, cell supernatants were harvested and the infectious virus yield was determined by the β-galactosidase assay.

2.2.9 AlphaLisa p24 detection assay

Virus-containing supernatants and cells were lysed by 5 % Triton X-100. Commercially available p24 AlphaLisa detection kit (PerkinElmer) was used to quantify the level of HIV capsid p24 antigen according to manufacturer's protocol. Efficiency of release was quantified by comparing the level of supernatant p24 to total p24 detected in the cells and supernatants. This assay was performed by Dr Shilpa Iyer (University of Pennsylvania).

2.2.10 Dual luciferase assay

HEK293T were seeded into flat-bottom 96-well plates (25.000 /well) pre-coated with poly-L-lysine and incubated overnight. Cells were transfected in triplicates using calcium phosphate method with 0.1 µg of Firefly luciferase reporter construct under the control of three NF-κB binding sites, 0.025 µg of *Gaussia* luciferase construct under the control of a minimal pTAL promoter for normalization, and 0.04 µg of expression vectors for c.a. IKKβ or increasing concentration of tetherin, as well as 0.025 µg pCG Vpu IRES eGFP. Two days later Firfely luciferase activity was measured in the cells and *Gaussia* luciferase activity was measured in the supernatant using microplate luminometer. The enzyme activity was measured as relative

light units/second (RLU/s) using the Software Simplicity 4.02. Firefly activity readout was normalized to *Gaussia* value for each sample.

2.2.11 Confocal microscopy

HEK293T MaMTH reporter cells were seeded into 8-well microslides (~20.000 cells/well) and after 6 h transfected with 100 ng of MaMTH Bait construct using PEI. Protein expression was induced by adding 100 ng/ml tetracycline. After 40 h post-transfection cells were washed in DPBS and fixed with 4 % PFA for 20 min. Fixing buffer was removed and cells were washed three times, followed by 60 min blocking with DPBS containing 5 % BSA and 0.5 % Triton X. Antibodies against V5 (raised in rabbit) and trans-Golgi network marker TGN46 (raised in sheep) were added at 1/1000 dilution in staining buffer and incubated for 120 min at 4 °C. Cells were washed three times in DPBS and secondary anti-rabbit Alexa Fluor 647 and anti-sheep Alexa Fluor 488 antibodies were added (1/5000 dilution) followed by 60 min incubation at 4 °C. Cells were washed three times in DPBS. Mounting medium was added immediately and the samples were stored in the dark at 4 °C to prevent bleaching and degradation. Slides were analysed using confocal laser scanning microscope and the images were processed using ZEN 2010 software.

2.2.12 Viral replication in CD4+ T cells

Replication kinetic

To assess the contribution of tetherin antagonism to the IFN-α resistance of full-length infectious molecular clones (IMCs), we generated virus stocks of wild-type (WT), TMD mutant (TMDmut), and Vpu-defective IMCs by calcium phosphate transfection of HEK293T cells with 5 µg of proviral DNA, followed by supernatant harvest 48 h later. Activated, IFN-α treated or untreated human CD4+ T cells were infected with normalized amounts of virus and incubated overnight (12-15h). Cells were washed with DPBS and resuspended in RPMI1640 medium supplemented with 15 % FCS, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin. Every 48 h, supernatants were sampled for cell-free p24 measurements, and medium

(containing 500 u/ml IFN-α or not containing IFN) was replenished. At days 7 and 9 cells were harvested to determine viral release efficiency. Each virus was tested in duplicate per experiment, and experiments were repeated twice in two separate pools of primary CD4+T cells. This assay was performed by Dr Shilpa Iyer (University of Pennsylvania).

Infectivity of virions released from CD4+ T cells

A total of 8.000 TZM-bl cells were seeded per well in a 96-well flat bottom plate. At a confluence of ~40 %, the cells were infected with 100 µl of cell-free supernatant of infected CD4+ T cells obtained 7 days post infection. DEAE-dextran (final concentration, 40 µg/ml) was used to enhance infection. Two days later, the cells were lysed with Cell Culture Lysis Reagent, lysates were frozen at -80 °C for 2 h, and relative light units (RLU) were determined using the luciferase assay system (Promega). The RLUs obtained were normalized to the capsid antigen p24 levels to obtain RLUs per pg of p24 capsid antigen. Each measurement was performed in duplicate. This assay was performed by Shilpa Iyer (University of Pennsylvania).

2.2.13 Gene sequence analysis

Vpu sequences were obtained from the HIV Sequence Database (www.hiv.lanl.gov).
Vpu sequences were aligned using multiple sequence alignment with hierarchical clustering (http://multalin.toulouse.inra.fr/multalin).
Sequence logos were produced using Weblogo online tool (http://weblogo.berkeley.edu).

2.2.14 Calculations and statistical analysis

The activities of different *vpu* alleles and their mutants were compared using two-tailed Student's t test. All statistical calculations were performed using Prism package version 5.0 software.

2.2.15 Ethics statement

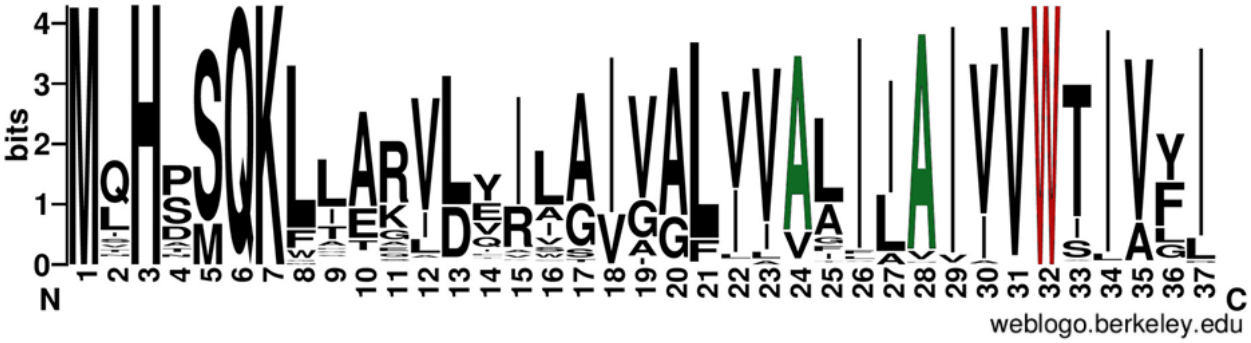
Ethical approval for the utilization of human-derived cells was obtained from the Ethics Committee of Ulm University Medical Center (Ethics vote 50/16).

3 RESULTS

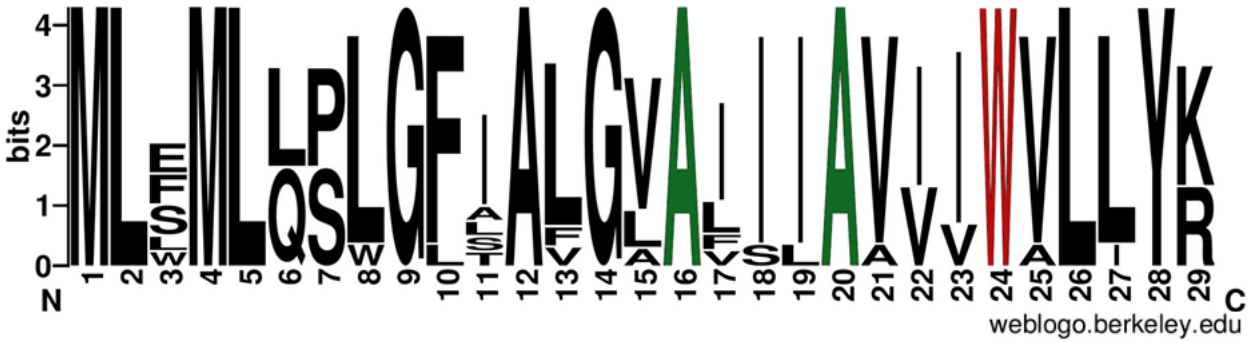
3.1 AxxxA TMD Vpu motif represents an adaptation to human tetherin

Although a *vpu* gene is present in both SIVcpz and SIVgor (Takeuchi *et al*., 2015), it does not serve as a tetherin antagonist in these simian viruses, as this function is efficiently performed by Nef (Yang *et al*., 2010). It was shown that substitution of the TMD of SIVcpz and SIVgor Vpu with that of HIV-1 group M is sufficient to confer anti-tetherin activity (Kluge *et al*., 2013). Moreover, a specific hydrophobic motif (AxxxAxxxW) in the transmembrane domain (TMD) of the lab-adapted NL4-3 Vpu was reported to be crucial for counteraction of tetherin (Vigan and Neil, 2010). These residues form a face of a single α-helix important for Vpu function (Skasko *et al*., 2012) with the alanines (A) directly interacting with the TMD of tetherin, and the tryptophan (W) being involved in CD4 counteraction (Pickering *et al*., 2014; Skasko *et al*., 2012). In order to test whether this AxxxA motif represents an adaptation to human tetherin, Vpu sequences from various viral groups were obtained from the HIV Sequence Database and the TMD domains of these accessory proteins were compared (Fig.6).

HIV-1 group M (n=225)



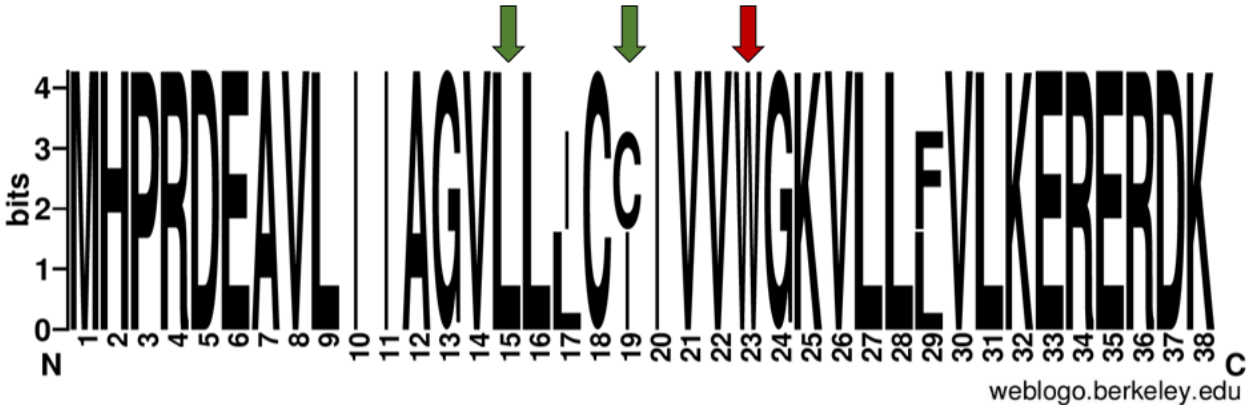
HIV-1 group N (n=10)



SIVcpz (n=10)

HIV-1 group O (n=41)

HIV-1 group P (n=2)



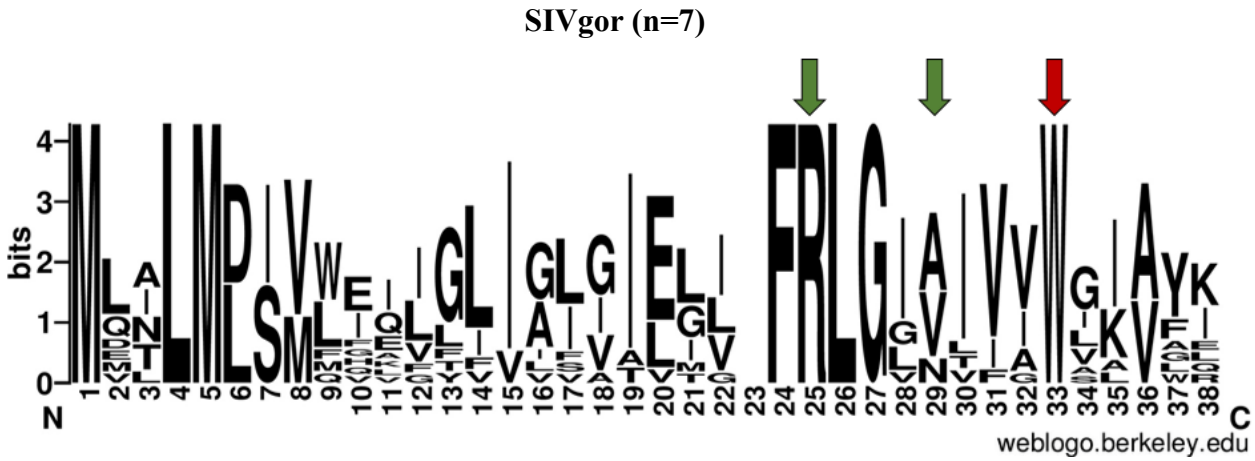


Figure 6: Logo alignments of the Vpu TMDs of HIV-1 groups M, N, O and P and their simian precursors. Group M comprises a randomly selected mix of primary isolates representing different subtypes. In case of HIV-1 groups O, N and P as well as SIVgor, all available complete Vpu sequences were used. SIV naturally infecting *Pan troglodytes troglodytes* were used to create the SIVcpz alignment. Alanines reported to be important for tetherin counteraction are highlighted in green, whereas the tryptophan residue important for CD4 downmodulation is shown in red. Red and green arrows show respective positions of this TMD motif in viral species which in general do not counteract tetherin using Vpu. Sequences were obtained from the HIV Sequence Database (www.hiv.lanl.gov) and logo alignments were generated using WebLogo sequence logo generator (www.weblogo.berkeley.edu).

Indeed, HIV-1 groups and M and N, which use their Vpu to counteract human tetherin, show a high degree of conservation in the AxxxA motif. The most common naturally found deviation from this motif is the substitution of alanine to valine (V), which has similar properties (nonpolar, neutral charge). In contrast, viruses commonly known to use their Nef protein to downmodulate tetherin (SIVs and HIV-1 group O) or lacking this function (group P) harbour no conserved AxxxA motif in their TMD domain, even though they all have a conserved tryptophan residue at position 23 or 33. This resembles the conserved tryptophan found in group M Vpu important for CD4 entry receptor downmodulation. Indeed, SIV and HIV-1 group O and P Vpus are known to efficiently downmodulate CD4 from the cells surface (Sauter *et al*., 2009; Sauter *et al.*, 2011).

Conservation of the AxxxA motif among Vpus which are able counteract human tetherin provided basis for the generation of tetherin inactive HIV-1 group M and N Vpu mutants, which would be used to determine the contribution of Vpu-mediated tetherin antagonism to viral fitness.

3.2 Generation of Vpu mutants inactive in tetherin counteraction

Based on findings that the AxxxA motif important for tetherin counteraction is highly conserved in primary HIV-1 group M and N strains, I decided to mutate the respective two alanines to leucines (L), which are also neutral and nonpolar but more bulky. Leucines are not usually present at these positions in HIV-1 M and N, but are often found instead of the first A in group O and P strains that do not use Vpu to downmodulate tetherin (Fig.6). Besides, it has been shown that single A to L mutations in the TMD of NL4-3 Vpu partially decrease its ability to enhance viral release in the presence of this restriction factor (Vigan and Neil, 2010).Therefore, AxxxA to LxxxL mutations were introduced in Vpus of several HIV-1 group M and N primary isolates (Table 4; Fig.7). Among group M, these included the most prevalent subtypes found in the US and Europe (B and C), as well as transmitted founder (TF) and chronic (CC) strains, representing viral isolates found during the acute and chronic stages of HIV infection, respectively. The previously characterized T cell line adapted (LAB) HIV-1 group M NL4-3 strain was used as a control (Vigan and Neil, 2010).

Table 4: Panel of HIV strains used in this study. Panel includes HIV-1 group M primary isolates of different subtypes (B, C), co-receptor tropism (CXCR4 – X4; CCR5 – R5) and stages of infection (TF – transmitted founder, CC-chronic) as well as one primary group N virus (DJO) and the lab-adapted NL4-3 strain. Isolates were obtained mainly from men who have sex with men (MSM) except for CH167, which came from a heterosexual (HTSX) female. No information about tropism or infection stage of the group N patient is available. Last column shows the position of the mutated AxxxA motif relative to the start codon of Vpu.

Strain	Group	Subtype	Tropism	Patient	Infection stage	Vpu length	TMD mutant
NL4-3	M	B	X4	MSM	CC; LAB	81 aa	A14L, A18L
CH058	M	B	R5	MSM	TF	80 aa	A14L, A18L
CH077	M	B	X4/R5	MSM	TF	81 aa	A15L, A19L
CH167	M	C	R5	HTSX woman	CC	84 aa	A20L, A24L
STCO	M	B	R5	MSM	CC	81 aa	A15L, A19L
DJO	N	-	unknown	MSM	primary isolate	74 aa	A12L, A16L

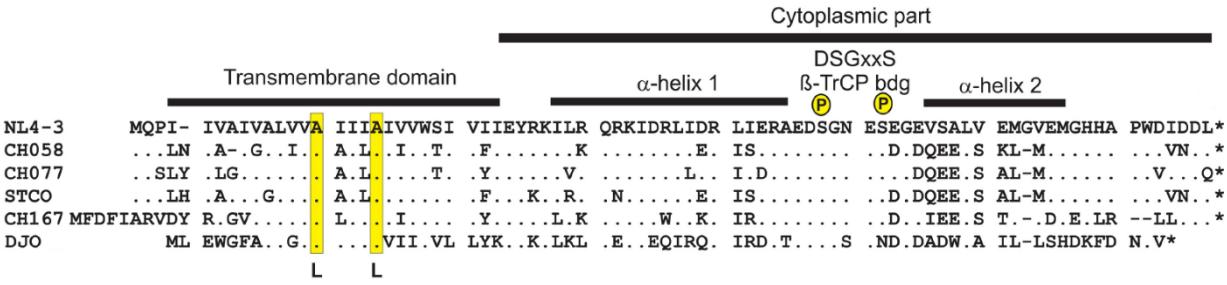


Figure 7: Alignment of TMD Vpu domains of strains chosen for this study. Positions of highly conserved alanines involved in tetherin counteraction are highlighted in yellow. These residues were mutated to leucines in all strains. Dots specify amino acid identity, and dashes represent gaps. A conserved DSGxxS motif containing two phosphorylated serines, found in the cytoplasmic part of Vpu is responsible for β-TrCP binding (bdg) and subsequent endolysosomal or proteasomal degradation of tetherin and CD4, respectively (Schubert *et al*., 1994; Mitchell *et al*., 2009). Figure previously published in Kmiec *et al.,* 2016, page 2.

Mutations were introduced into wild-type (WT) infectious molecular clones (IMC) and Vpu protein expression constructs using SOE-PCR and confirmed by nucleotide sequencing. All HIV-1 group M Vpus were well expressed (Fig.8), in contrast to the group N, which had very weak expression. This is in agreement with previously published data (Sauter *et al*., 2012). Introduced TMD mutations had no detectable effects on protein stability or expression levels. In agreement with leucine being larger (131 Da) than alanine (89 Da), TMDmut Vpus had slightly higher molecular weights than WT proteins.

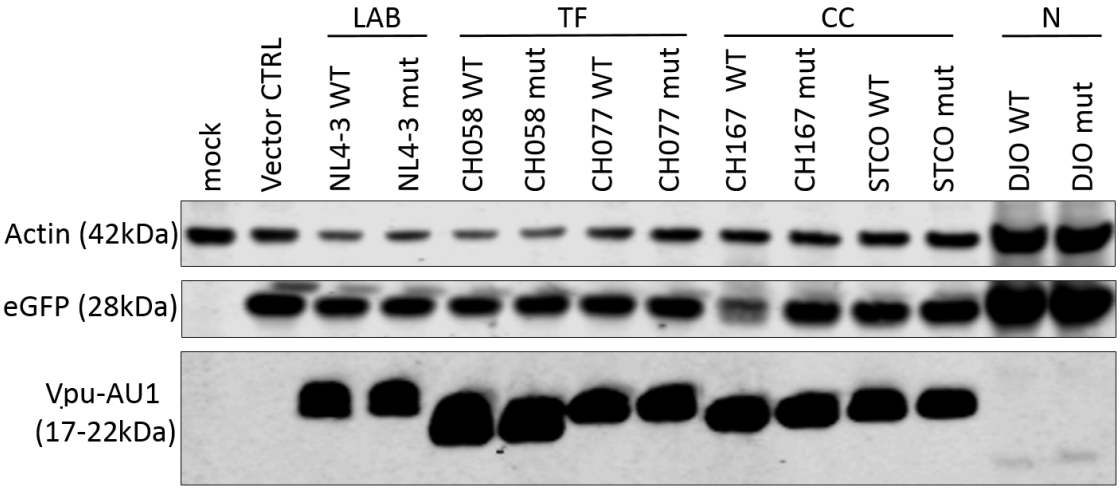


Figure 8: Expression of WT and TMDmut Vpu variants. HEK293T cells were transfected with 5 µg pCG_Vpu-AU1 IRES eGFP constructs, and the Vpu expression was assessed by Western blot. Actin served as a loading control, whereas eGFP was used to control for transfection rates. Untransfected cells (mock) and pCG_IRES eGFP empty vector transfected cells served a negative controls. Due to poor expression/stability of group N Vpus, 4x more cell lysate was loaded in case of the DJO samples. Expression results were confirmed in an independent Western blot experiment (not shown). Data previously published in Kmiec *et al.,* 2016.

3.3 Functional characterization of TMD mutants of HIV-1 Vpu

3.3.1 Specific TMD mutations in Vpu disrupt interaction with tetherin

Vigan and Neil (2010) have previously shown that mutation of alanine 14 and to a lesser extent 18 partially decrease the ability of NL4-3 Vpu to promote virus release in the presence of human tetherin. To determine whether a double A to L substitution is sufficient to disrupt the interaction between Vpu from a primary viral isolate and human tetherin, a Mammalian Membrane Two Hybrid (MaMTH) assay was performed. Membrane proteins known to directly interact with each other (EGFR and SHC1; Zheng *et al*., 2013) as well as the transcription factor Gal4 were used as positive controls, whereas negative controls included WT and TMDmut Vpu constructs together with Peroxisomal Biogenesis Factor 7 (Pex7) naturally found in peroxisomes and cell cytoplasm. The assay confirmed an interaction between primary HIV-1 group M STCO Vpu and human tetherin, which was 1.6-fold weaker upon TMD mutation (13.5 % vs. 8.5 % of Gal4 positive control) (Fig.9A). Again, the alanine to leucine mutations had no detectable effects on Vpu protein stability (Fig.9B). In agreement with published studies (Vigan and Neil, 2010; Vigan and Neil, 2011), WT and TMDmut Vpus were found in intracellular compartments positive for the trans-Golgi network marker TGN46 (Fig.9C).

This confirms that the published observations regarding lab-adapted NL4-3 are also relevant for Vpus of primary isolates and shows that a double A to L mutation significantly disrupts the interaction between Vpu and human tetherin.

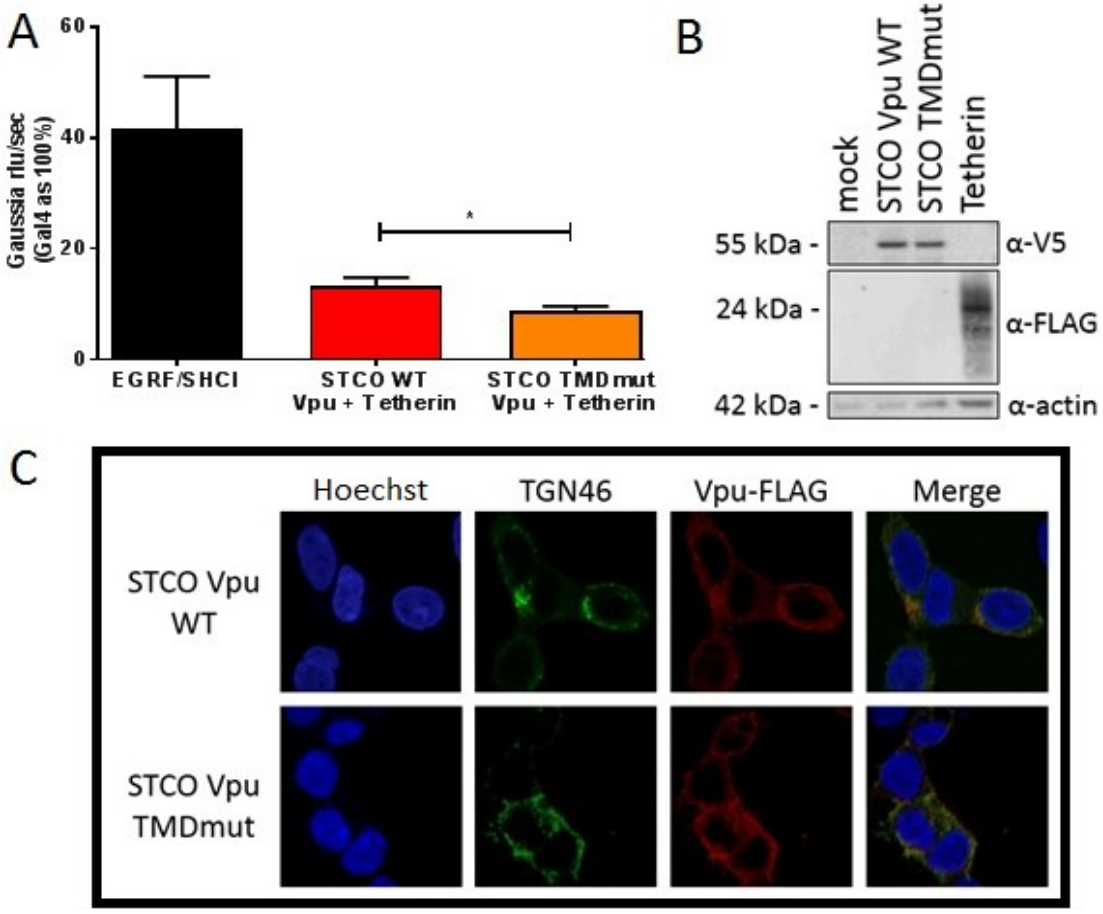


Figure 9: Effect of A15L A19L TMD mutation on the interaction between HIV-1 group M STCO Vpu and human tetherin. (A) MaMTH assay showing Vpu interaction with tetherin. Shown is the mean of 3 independent experiments performed in triplicates ± SEM. Significant difference is indicated by * ($p>0.05$) calculated using Student's t-test. Data was normalized to the positive control (Gal4), and the signal of negative control was subtracted from the means. (B) Western blot showing expression levels of tetherin and Vpu in cells used in the MaMTH assay. Vpu was detected by anti-V5-tag and tetherin was detected by anti-FLAG-tag antibody. (C) Confocal microscopy images showing localization of WT and TMDmut STCO Vpu (red) in MaMTH reporter cells. Trans-Golgi network marker TGN46 is shown in green, whereas blue indicates nuclei stained with Hoechst.

3.3.2 Vpu mutants fail to downmodulate tetherin from the cell surface

To shed more light on the consequences of the impaired interaction between mutant Vpu and tetherin, the downmodulation of this receptor was measured. FACS analysis (Fig.10C) of HEK293T cells co-transfected with indicated viral protein and human tetherin (Fig.10A) revealed that all HIV-1 group M Vpus with mutated AxxxA motif were significantly less efficient at downmodulating tetherin from the cell surface than the corresponding WT proteins. Similarly, HIV-1 group N (DJO) lost all of its modest activity upon AxxxA mutation. Wild type HIV-1 group M Vpus had comparable activities in this assay and decreased surface expression

of tetherin by 60-70 %, whereas group N achieved only 30 % (Fig.10B), which is consistent with its poor expression (Fig.8B).

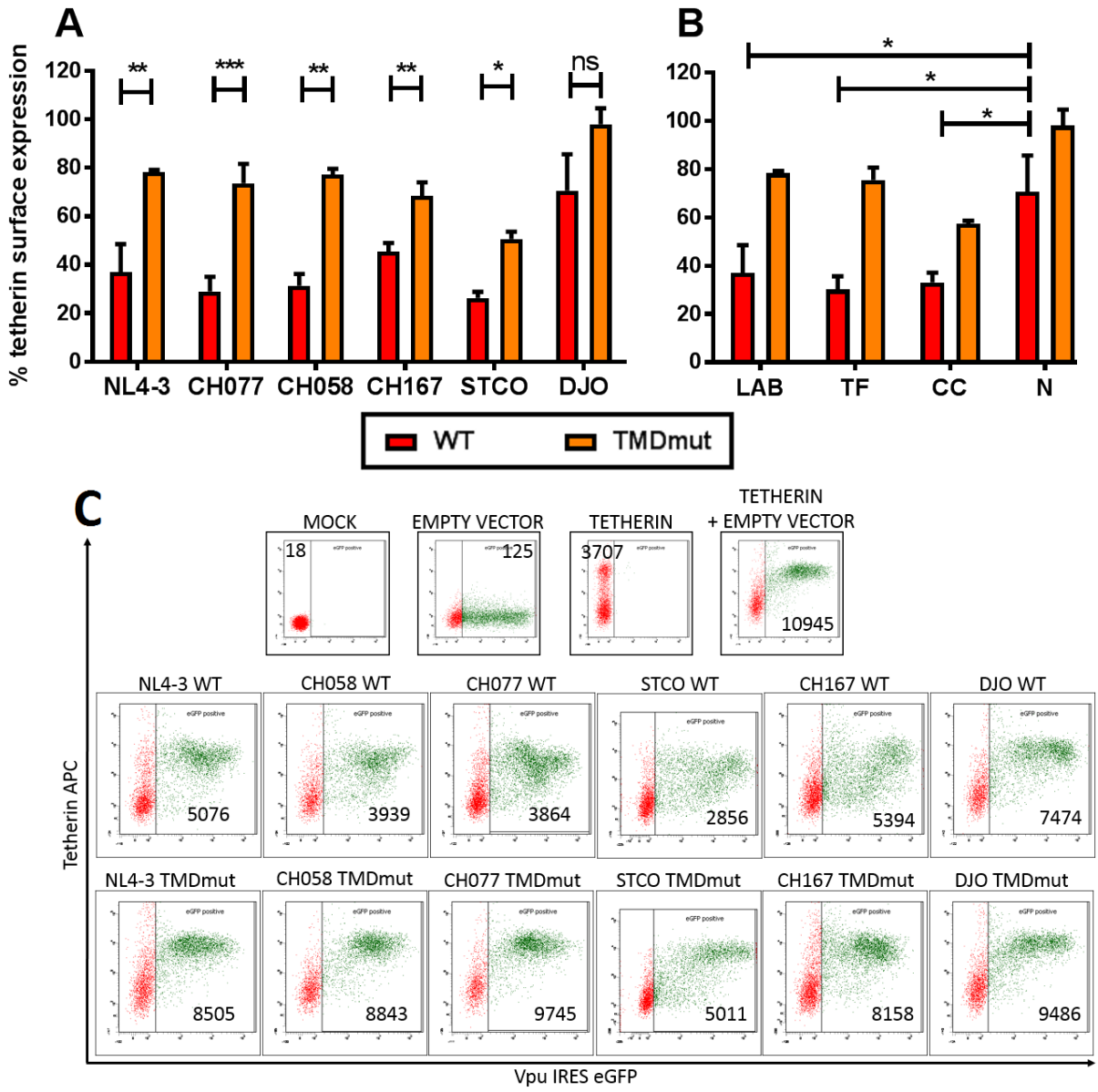


Figure 10: Vpu-mediated tetherin downmodulation in transfected HEK293T cells. (A) Downmodulation of tetherin in HEK293T cells co-transfected with individual pCG Vpu IRES eGFP and pCG human tetherin protein expression constructs. (B) Group analysis of tested viral genes LAB – laboratory adapted HIV-1 M (NL4-3); TF – transmitted founder HIV-1 group M (CH058 and CH077); CC – chronic HIV-1 group M (CH167 and STCO); N- HIV-1 group N (DJO) with comparison of relative potency of surface tetherin downmodulation of WT Vpu proteins. Values are mean of three independent experiments (+ SEM). Statistically significantly differences are indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns= not significant) and were calculated using unpaired Student's t-test. (C) Representative primary FACS data. Levels of mean fluorescence intensity (MFI, values indicated on the graphs) of tetherin APC in Vpu expressing (eGFP+ shown in green) cells was calculated relative to cells transfected with tetherin and control empty vector expressing eGFP (upper right panel). Untransfected (mock) and empty vector transfected cells stained with anti-tetherin APC antibody served as negative controls. Data previously published in Kmiec *et al.,* 2016.

In order to test whether the effects observed during overexpression of both proteins are also relevant for infected primary cells expressing endogenous levels of tetherin, human PBMCs were transduced with viruses expressing WT, TMDmut or no Vpu. FACS analysis of tetherin surface levels (Fig.11C) revealed that WT Vpus removed ~50 % of tetherin from the surface of infected cells whereas all TMD mutants lost this function (Fig.11A). Even though no significant differences in the anti-tetherin activity of wild type HIV-1 group M viruses were observed, NL4-3 and CH167 strains were slightly less efficient, which was consistent with the effects observed in HEK293T (Fig.10). Surprisingly, WT group N (DJO) Vpu which was almost inactive against overexpressed receptor (Fig.10A), was able to downmodulate endogenous tetherin with almost the same efficiency as HIV-1 group M strains (down to 65 % as shown on Fig.11B).

Thus, all tested strains were able to efficiently downmodulate human tetherin from the cell surface and this effect was abrogated upon the double A to L mutation in the TMD of Vpu.

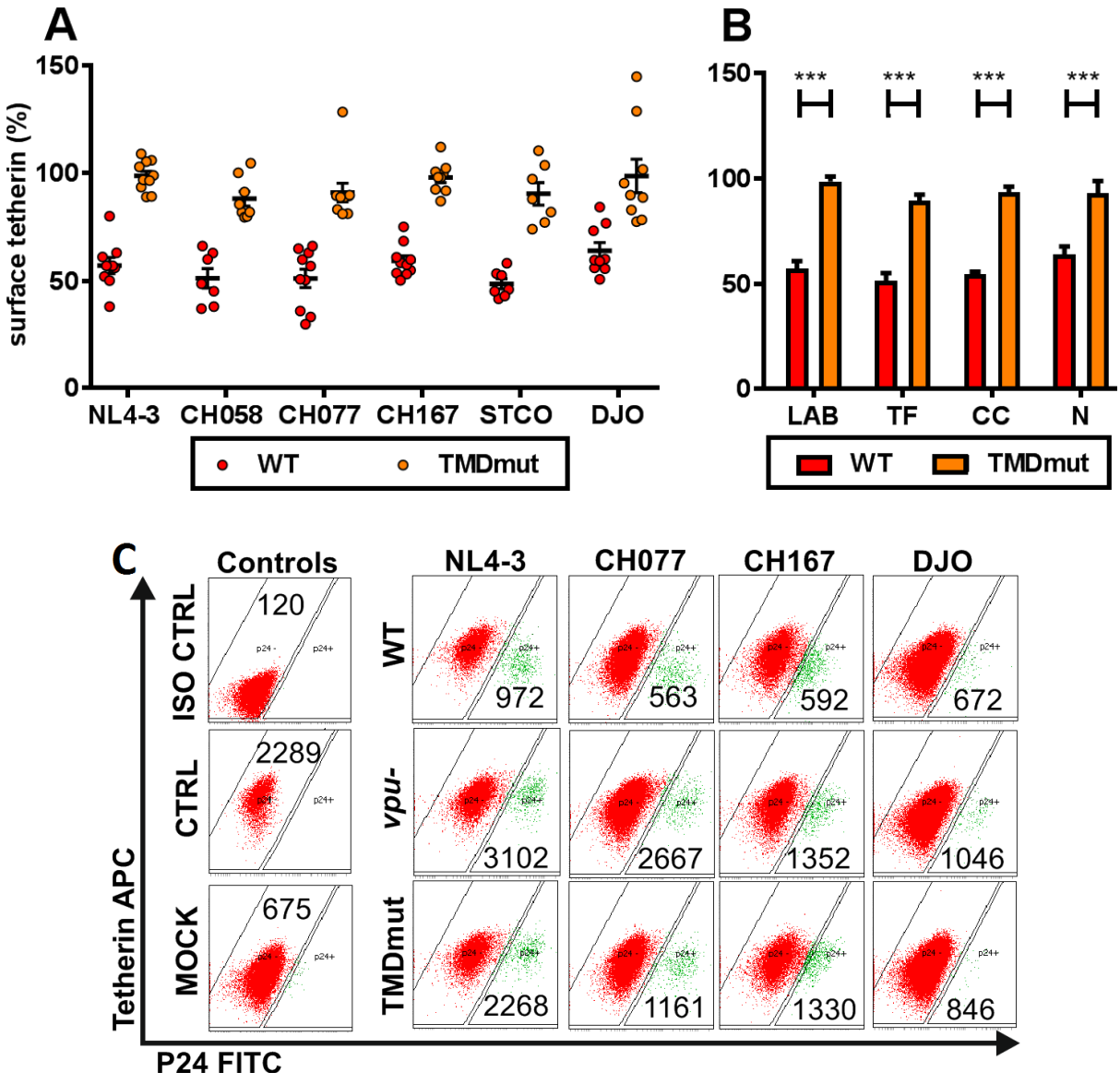


Figure 11: Vpu-mediated tetherin downmodulation in infected PBMCs. Downmodulation of tetherin in PBMCs infected with HIV-1 IMCs encoding WT, TMDmut or no *vpu*. PHA-activated PBMCs were transduced with the indicated VSV-g-pseudotyped HIV-1 IMC and examined for tetherin surface expression 3 days later by flow cytometry. (A) Levels of surface expression in virally infected cells was calculated relative to cells infected with corresponding control virus deficient in Vpu (100 %). Each symbol represents one donor (7-9 donors). The mean + SEM is also indicated. (B) Group analysis of tested virus types. LAB – laboratory adapted HIV-1 M (NL4-3); TF – transmitted founder HIV-1 group M (CH058 and CH077); CC – chronic HIV-1 group M (CH167 and STCO); N- HIV-1 group N (DJO). Values that are significantly different are indicated by asterisks ***, $p<0.001$; and were calculated using paired Student's t-test. (C) Representative primary FACS data. Levels of mean fluorescence intensity (MFI) are shown. Uninfected (mock) and isotype antibody stained cells (ISO CTRL) served as negative controls. Parts of figure previously published in Kmiec *et al.,* 2016, page 3.

3.3.3 Vpu mutants fail to enhance virus release in the presence of tetherin

Even though it is generally accepted that the Vpu-mediated sequestration of tetherin from the cell surface is an important steps in Vpu-mediated enhancement of viral release (McNatt *et al*., 2013), it has been suggested that in certain cases tetherin downmodulation might be dispensable for successful counteraction (Miyagi *et al.*, 2009; Kuhl *et al*., 2011). Therefore, to demonstrate the full effect of TMD mutations, it was important to test to what degree mutant Vpus are able to rescue viral release in the presence of tetherin as compared to the wild-type counterparts. To test this, HEK293T cells were transfected with vpu-deficient NL4-3 proviral constructs, Vpu expression vectors provided in *trans* and increasing amounts of tetherin. All HIV-1 group M Vpus were able to efficiently increase virus release in the presence of tetherin, while Vpu of HIV-1 group N (DJO) was barely able to do so, which is in agreement with its weaker effect on tetherin surface expression (Fig.10) and published data (Sauter *et al*., 2012). Notably, the Vpu of CH167, which belongs to HIV-1 group M subtype C and was isolated from a female patient, antagonized tetherin more efficiently than the remaining strains belonging to subtype B, and efficiently enhanced virion release even at the highest tetherin concentration. Most importantly, all of the tested Vpus lost the ability to overcome the inhibitory effect of tetherin and rescue virus release upon alanine motif disruption (Fig.12). These results demonstrate that double A to L mutations in the TMD of HIV-1 group M and N Vpus are sufficient to completely abrogate their ability to enhance viral release in the presence of human tetherin.

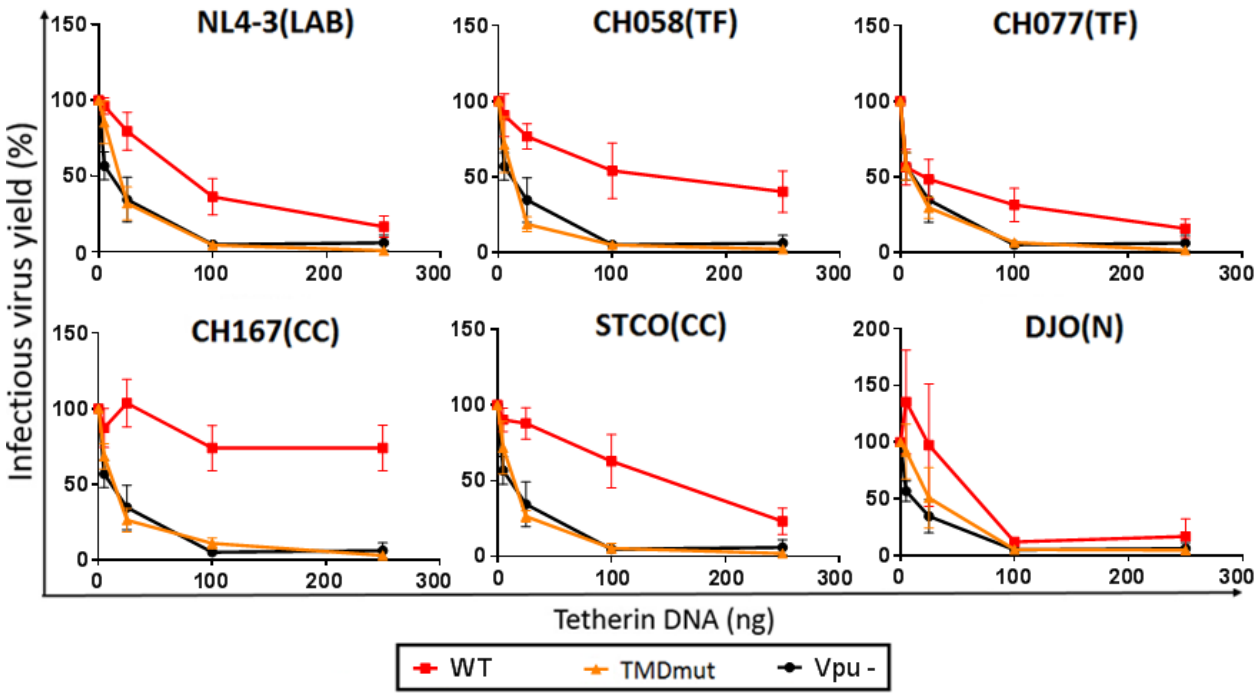


Figure 12: Infectious virus production in the presence of tetherin and WT/TMD mutant Vpu expression. Virus release from HEK293T cells following co-transfection with *vpu*-defective HIV-1 NL4-3, pCG expression constructs for the indicated Vpu proteins or empty vector expressing eGFP, and increasing amounts (5-250 ng) of pCG plasmid expressing human tetherin. Infectious virus yield was determined by infection of TZM-bl indicator cells and β-galactosidase assay 3 days later. Shown is the percentage of detected infectious virus yield normalized to that in the absence of tetherin (100 %). Results show n=3 ± SEM. Data previously published in Kmiec *et al.*, 2016.

3.3.4 TMD Vpu mutations do not affect CD4 downmodulation

Surface downmodulation of the viral entry receptor CD4 is another well-described activity of Vpu. It is achieved in cooperation with Nef and Env, preventing superinfection, immune recognition of the infected cells and aiding the assembly of fully-infectious viruses. As it has already been reported that TMD residue W20 is crucial for CD4 downmodulation by NL4-3 Vpu (Vigan and Neil, 2010), it was important to determine whether the TMD mutations affect this function in any of the primary Vpus. Overexpression of CD4 together with WT or TMDmut Vpu in HEK293T cells followed by surface staining of this receptor and flow cytometry, revealed no significant effects on CD4 downmodulation by TMD mutants (Fig.13A). Group M Vpus were very active in targeting CD4, while the group N Vpu had only a weak effect on this receptor. This was in agreement with its poor expression (Fig.8) and findings of Sauter *et al.*, 2012. The ability to downmodulate CD4 was also tested in the presence of other CD4

antagonists Nef and Env in a full viral context (Fig.13B). As expected, the TMD mutations had no effect on CD4 downmodulation and the differences between groups M and N were almost unnoticeable. These findings support the notion that Vpu uses differential mechanisms to downmodulate CD4 and tetherin.

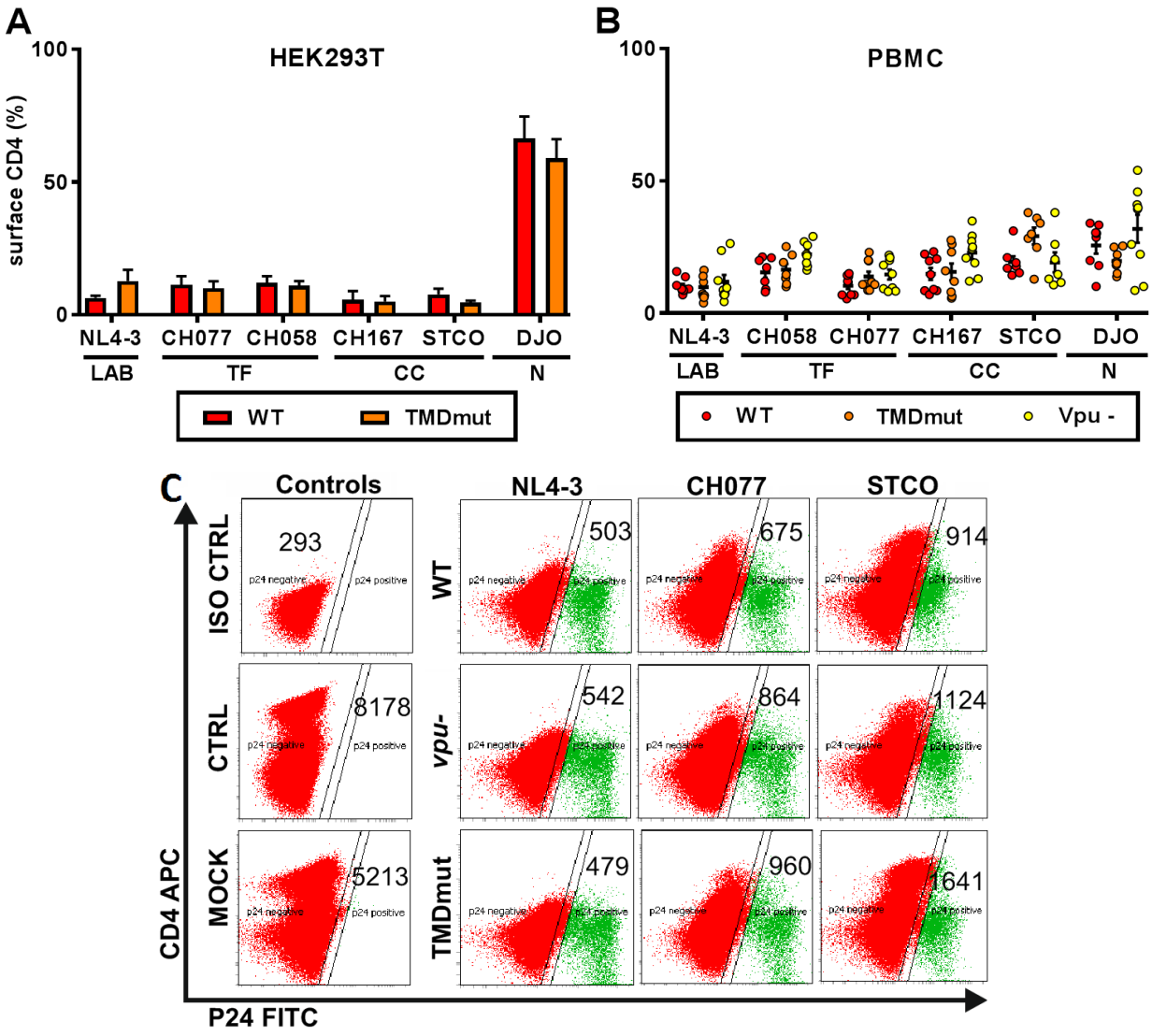


Figure 13: Surface CD4 downmodulation in the presence of Vpu variants in transfected HEK293T cells and infected PBMCs. (A) FACS analysis of HEK293T cells co-transfected with CD4 expression vectors and pCG plasmids expressing Vpu and eGFP via an IRES element. Shown are the levels of CD4 surface expression relative to those measured in cells transfected with the eGFP control vector as mean values of n=3 ±SEM. Wild-type *vpu* alleles are shown in red, whereas TMDmut Vpu proteins are indicated by orange. (B) Downmodulation of CD4 in PBMCs infected with HIV-1 IMCs encoding WT, TMDmut or no *vpu*. PBMCs were transduced with the indicated VSV-g-pseudotyped HIV-1 IMCs and examined for CD4 surface expression 3 days later. Levels of surface receptor expression in virally infected (p24+) cells are shown relative to uninfected cells (100 %). Each symbol represents one PBMC donor (total of 6-7 donor tested). (C) Representative primary FACS data. Levels of CD4 APC MFI (values indicated on graph) in infected (FITC p24+ population shown in green) cells was calculated relative to cells infected with *vpu*- control (middle panel). Uninfected (mock) and isotype antibody stained cells (ISO CTRL) served as negative controls. Data previously published in Kmiec *et al.,* 2016.

3.3.5 TMD Vpu mutations have no effect on CD1d and NTB-A downmodulation

It has been reported that HIV-1 group M Vpus can downmodulate the surface expression of immune receptors such as CD1d expressed mainly on pDCs, and NTB-A expressed on NK, T and B cells (Moll *et al.*, 2010; Shah *et al.*, 2010), however the exact mechanism of these activities is not so well characterized. In order to test whether the AxxxA motif in the TMD of Vpu was involved in any of these functions, HEK293T were co-transfected with constructs encoding WT or TMDmut Vpu together with eGFP and constructs expressing CD1d or NTB-A. Receptor expression was compared 2 days later by flow cytometric analysis (Fig.14). Analysis of surface CD1d and NTB-A levels revealed that only some of the primary Vpu downmodulate these receptors, with mostly modest effects. Only Vpus from the two chronic HIV-1 M strains STCO and CH167 potently reduced NTB-A and CD1d cell surface expression and in their case these activities were not affected by the TMD mutations.

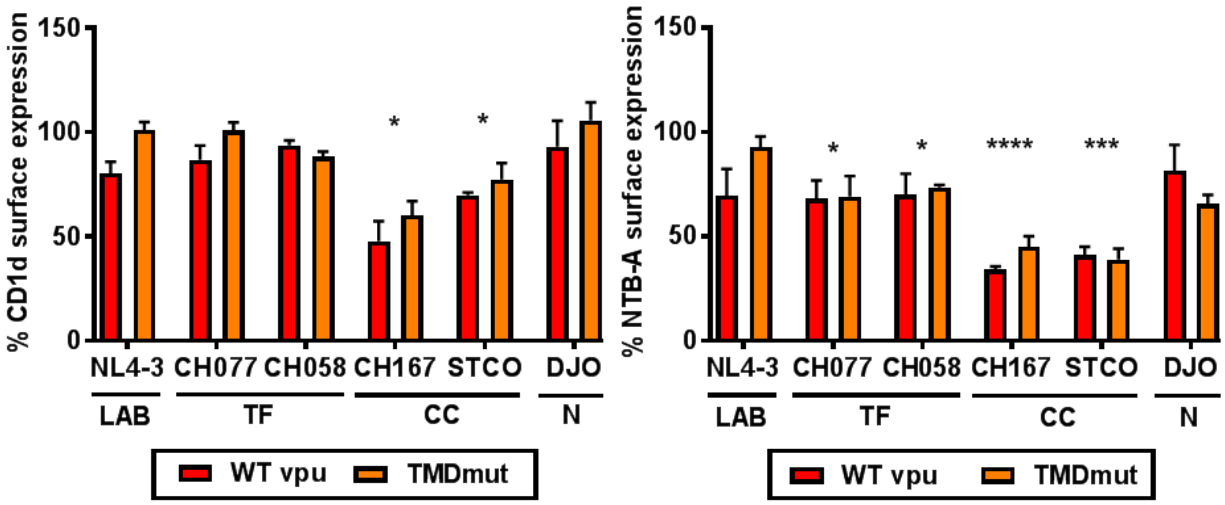


Figure 14: Vpu-mediated CD1d and NTB-A surface downmodulation in transfected HEK293T cells.

HEK293T cells were co-transfected with pCG Vpu IRES eGFP and CD1d (left panel) or NTB-A (right panel) protein expression constructs and analysed 2 days later for surface receptor expression. Data was analysed in the same manner as in case of tetherin (Fig.10). Shown are mean values of n=3 ±SEM. Statistically significant downmodulation of the receptors is indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ and were calculated using unpaired Student's t-test. Data previously published in Kmiec *et al.*, 2016.

3.3.6 TMD mutations do not affect Vpu-mediated inhibition of NF-κB

Most HIV-1 strains use their late-expressed Vpu proteins to inhibit NF-κB activation in order to suppress antiviral gene expression and immune activation (Bour *et al*., 2001; Sauter *et al*., 2015). This inhibitory effect involves stabilization of the NF-κB inhibitor IκB and prevention of nuclear translocation of p65 (Hotter *et al*., 2017). Co-transfection of HEK293T cells with vectors co-expressing Vpu together with an NF-κB-dependent firefly luciferase reporter construct and a constitutively active mutant of IκB kinase β (IKKβ) showed that the primary HIV Vpus suppressed IKKβ-induced NF-κB activation by about 5-fold, whereas the NL4-3 Vpu achieved only 2-fold inhibition (Fig.15). Among primary HIV-1 group M Vpus, subtype C (CH167) was the least efficient one in NF-κB inhibition, achieving a 3-fold decrease. In contrast, HIV-1 N Vpu did not inhibit NF-κB at all, which is consistent with published observations (Sauter *et al*., 2015). No major differences between WT and TMDmut Vpus were observed.

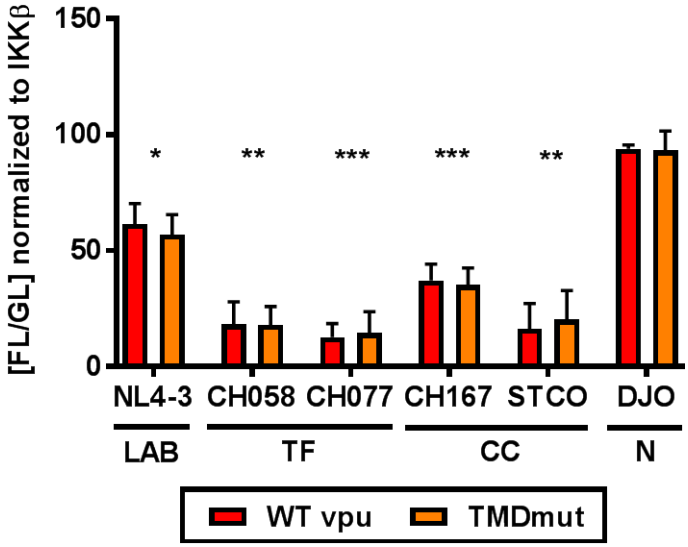


Figure 15: Vpu-mediated inhibition of IKKβ-induced NF-κB activation. HEK293T cells were co-transfected in triplicates with the indicated *vpu* alleles, a firefly luciferase reporter construct under the control of three NF-κB binding sites, a *Gaussia* luciferase construct for normalization, and expression vectors for a constitutively active mutant of IKKβ as an inducer of NF-κB. Luciferase activities were determined 2 days later. Shown are mean values of n=3 ±SEM. Statistically significantly downmodulation of the receptor are indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ and were calculated using unpaired Student's t-test. Data previously published in Kmiec *et al.,* 2016.

Vpu can suppress NF-κB activation by another mechanism linked to antagonism of tetherin, as the cytoplasmic tail of the long isoform of this restriction factor can transduce immune activation signals (Galão *et al*., 2012; Tokarev *et al*., 2013). It was therefore important to investigate

whether the TMD Vpu mutations affect the ability of Vpu to inhibit tetherin-mediated NF-κB stimulation. Tetherin overexpression in HEK293T cells induced NF-κB activation in a dose-dependent manner (Fig.16). During co-expression of WT and TMDmut Vpu proteins, tetherin-mediated NF-κB activation was suppressed to similar extents. This indicates that HIV-1 Vpu proteins efficiently prevent NF-κB activation independently of their anti-tetherin activity, as has already been suggested by Sauter *et al*., 2015.

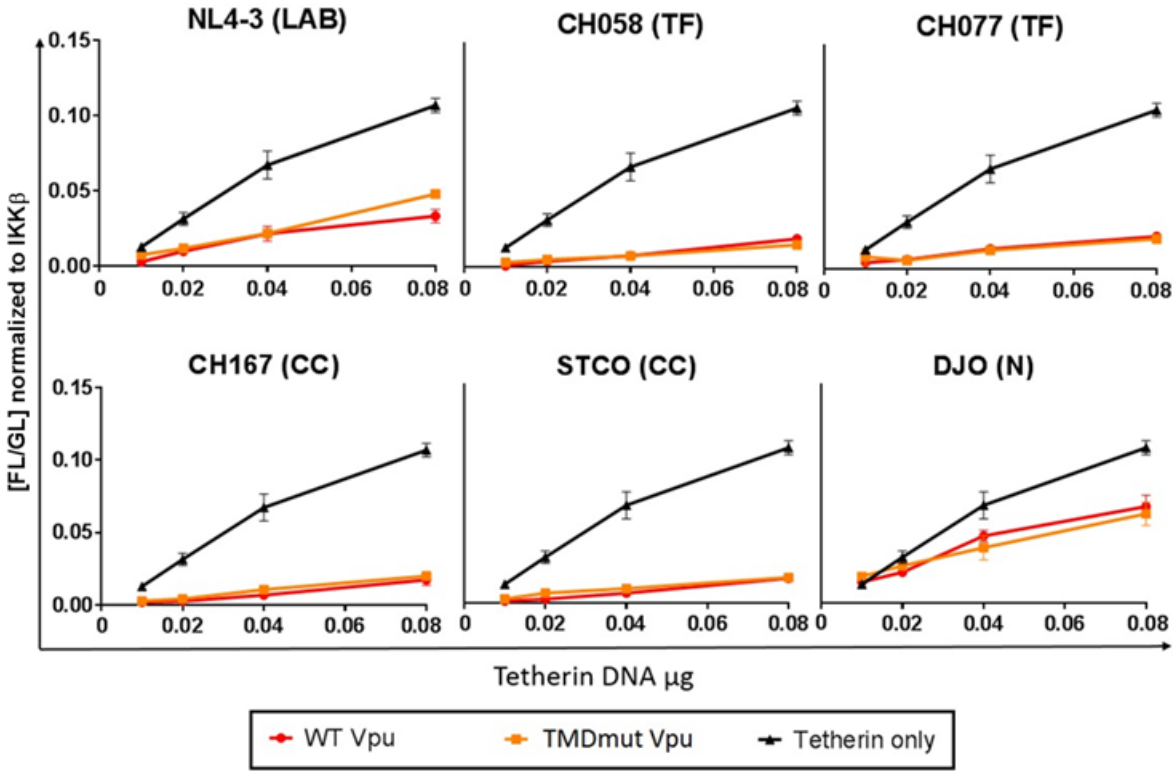


Figure 16: Vpu-mediated inhibition of tetherin-induced NF-κB activation. HEK293T cells were co-transfected in triplicates with the indicated *vpu* alleles, a firefly luciferase reporter construct under the control of three NF-κB binding sites, a *Gaussia* luciferase construct for normalization, and increasing amounts of tetherin expression vector as an inducer of NF-κB. Luciferase activities were determined 2 days later. Values are mean values (±SEM) derived from triplicates. Results were confirmed in an independent experiment (not shown). Data previously published in Kmiec *et al.,* 2016.

3.3.7 Vpu-mediated tetherin antagonism has no major effect on viral infectivity

In order to examine whether Vpu-mediated tetherin antagonism has any effect on the infectiousness of viral particles produced in the main natural target cells of HIV, the CD4+ T-cells, relative particle infectivity was calculated for each virus. Virions produced by transmitter

founder (TF) strains CH058 and CH077 were at least 5 times more infectious than those of chronic isolates (CC) CH167 and STCO or DJO representing HIV-1 group N (Fig.17A). In general, viruses carrying mutations abrogating tetherin antagonism or completely lacking Vpu expression showed no reduction in virion infectivity (Fig.17B). The only exception was the highly infectious TF CH077 strain, which showed a partial loss of particle infectivity upon TMD Vpu mutation (Fig.17A).

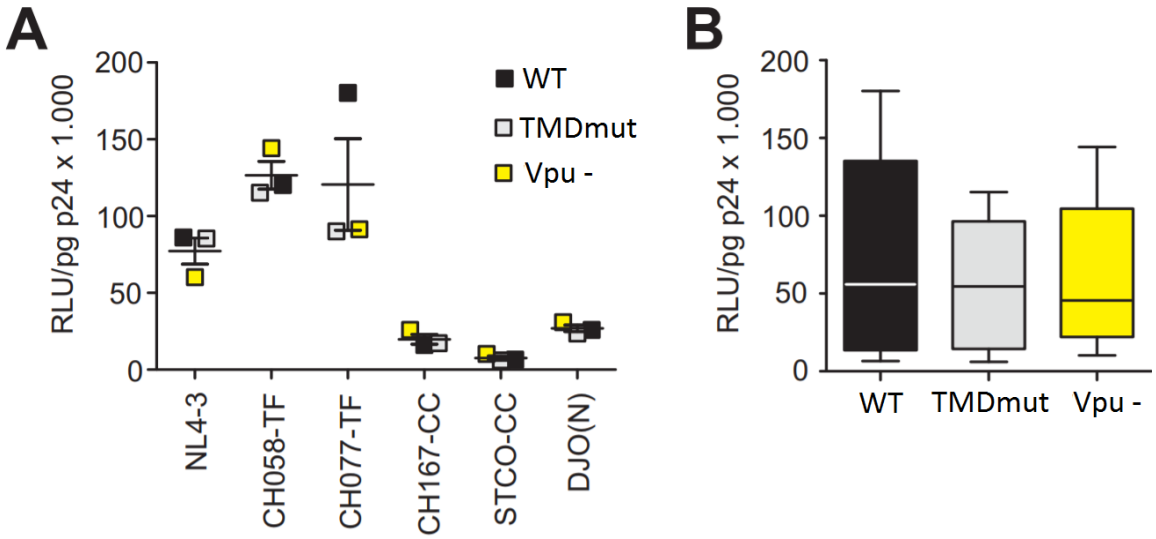


Figure 17: Infectivity of HIV-1 strains and their mutants produced in infected CD4+ T cells.
(A) Infectivity of HIV-1 IMCs expressing WT, TMDmut, or no (−) Vpu proteins obtained from infected CD4+ T cells 7 days after infection. Values represent mean of duplicate infection ±SEM. (B) Infectivity of the HIV-1 IMCs shown in panel A grouped according to their Vpu function. Shown are median values with the minimum and maximum values, 25 % and 75 % percentiles. Particle infectivity was calculated as a ratio of infectious virus yield (RLU/s) and the concentration of viral capsid protein p24 released from CD4+ T cells. Data kindly provided by Dr Shilpa Iyer. Figure previously published in Kmiec *et al.,* 2016, Fig.S7.

3.4 Contribution of tetherin antagonism to HIV replication and IFN sensitivity

3.4.1 IFN-α upregulates surface tetherin expression in CD4+ T cells

It has been reported that expression of tetherin is strongly induced by type I IFN due to the presence of IFN response elements and a binding site for STAT3 in its promoter (Blasius *et al*., 2006). It is known that not all cells react equally to IFN stimulation (Holmgren *et al*., 2015). For that reason, it was important to determine the extent of IFN-α-mediated endogenous tetherin upregulation in CD4+ T cells, the primary target cells of HIV-1. Flow cytometric analysis of these cells following 24 h stimulation with 500 U/ml of exogenous IFN-α2 revealed ~2-fold increase in surface tetherin levels as compared to unstimulated CD4+ T cells (Fig.18). This supports the notion that tetherin is constitutively expressed in main target cells of HIV and its surface levels are upmodulated soon after IFN-α2 treatment.

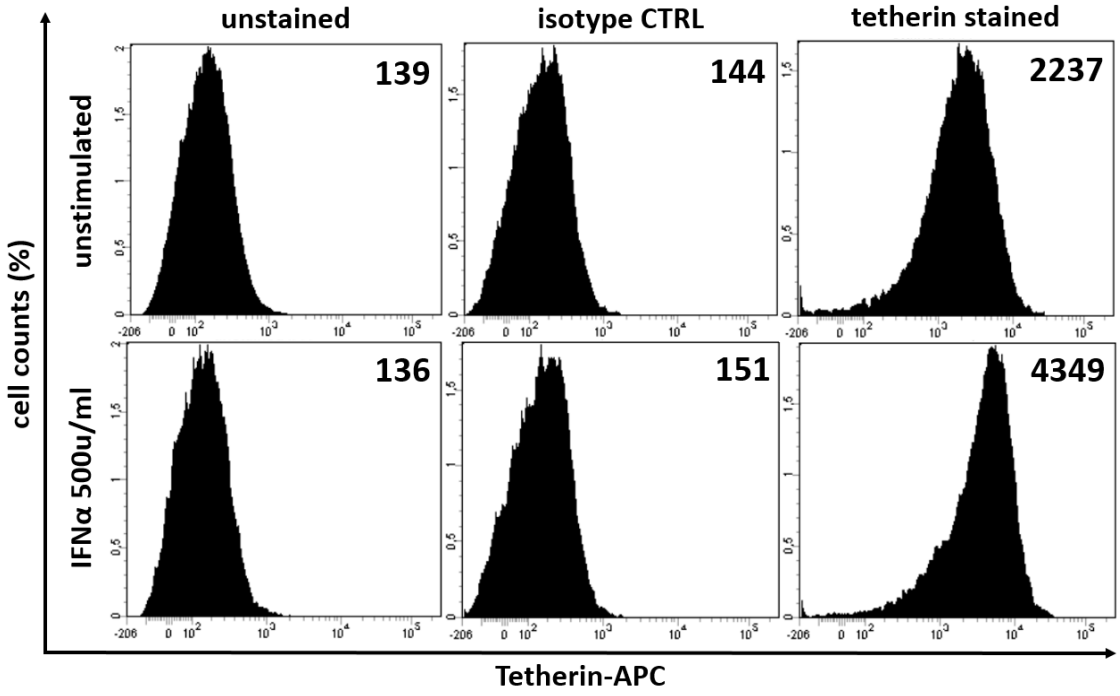


Figure 18: Induction of surface tetherin expression in IFN-α2 stimulated CD4+ T cells. CD4+ T cells isolated from human blood were stimulated for 3 days with T cell activator CD3/CD28 beads and IL-2. Cells were either treated with 500 U/ml IFN-α2 for 24 h or left untreated. Surface tetherin was stained with an anti-tetherin APC conjugated antibody or non-specific isotype control antibody and the expression of this receptor was measured using flow cytometry. Numbers in the top right corner indicate the MFI value of each sample. Isolated CD4+ T cell purity was determined by CD4+/CD11d – surface marker staining and FACS and found to be >95 % (data not shown).

3.4.2 Efficient HIV-1 production in CD4+ T cells requires tetherin antagonism

It is known that TF strains establishing infection in a newly infected individual, are less sensitive to inhibition by type I interferons than strains found in the chronic phase (Parrish *et al*., 2013; Iyer *et al*., 2017). In order to investigate the role of Vpu-mediated tetherin antagonism in TF virus production and sensitivity to IFN, activated human CD4+ T cells were infected with equal amounts of virus in the presence or absence of IFN-α2. Lab-adapted NL4-3 and the primary chronic CH167 strain served as controls. Seven days post-infection viral production was assessed by quantifying the level of viral capsid antigen (p24) in the cell culture supernatant. In agreement with published studies (Fenton-May *et al.,* 2013; Parrish *et al*., 2013; Iyer *et al*., 2017), TF viruses (CH058 and CH077) produced substantially more cell-free virus than the control strains upon IFN-α2 treatment (Fig.19B). In the absence of such treatment lab adapted NL4-3 and chronic strains were much more comparable to TF viruses (Fig.19A). IFN stimulation reduced cell-free virus production of two WT TF viruses by ~9-fold, NL4-3 by 58-fold and the chronic CH167 by 44-fold (Fig.19C). In the untreated cells, WT TF Vpus enhanced viral production by 85 % and 190 %, which was much lower for the tetherin defective Vpu mutants that resulted in only 38 % and 121 % increase respectively. The extent of enhancement of cell-free p24 levels by Vpu was higher in the presence of IFN-α. Fully competent TF and lab-adapted Vpu proteins increased cell-free viral production by 4- to 5-fold, whereas the chronic Vpu achieved a high 9-fold enhancement (Fig.19D), in agreement with its high anti-tetherin activity demonstrated in transient overexpression experiments (Fig.12). Tetherin-defective TMDmut Vpus increased cell-free virus production in the supernatants of IFN-α treated cells only minimally (Fig.19D). The observation that WT Vpu proteins enhanced the levels of cell-free HIV-1 TF viruses in the presence of IFN-α much more efficiently than their selectively tetherin defective counterparts supports the relevant role of tetherin antagonism in viral fitness.

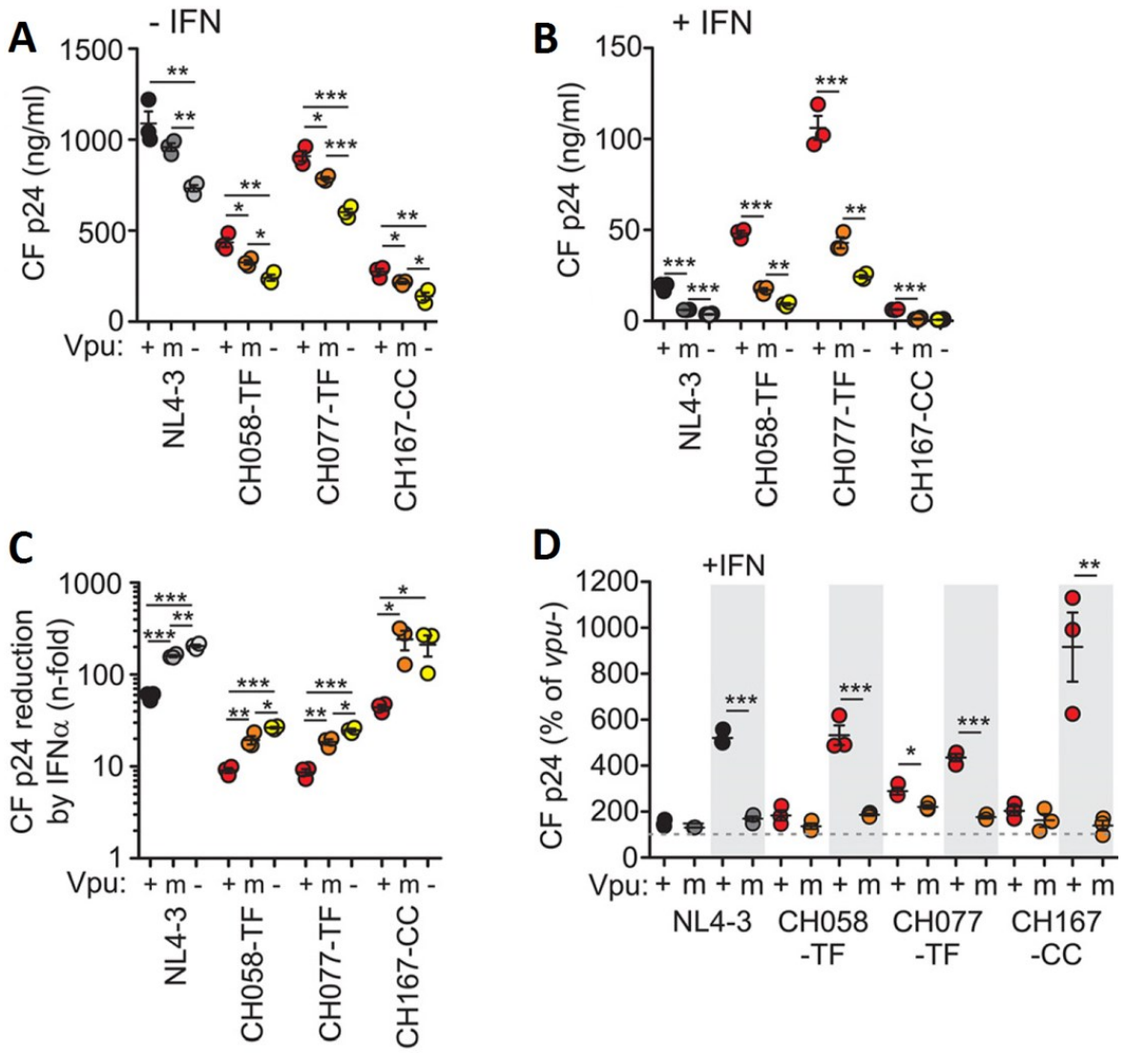


Figure 19: Production of cell-free (CF) virus in the absence and presence of IFN-α. (A,B) Cell-free (CF) p24 capsid antigen levels in the supernatant of CD4+ T cells 7 days after infection with HIV-1 expressing WT (+), TMDmut (m), or no (-) Vpu. Capsid levels were determined using triplicate infection in the absence (A) or (B) presence of 500 U/ml IFN-α2. (C) N-fold reduction of cell-free virus yield by IFN-α. Levels of p24 antigen in the absence of IFN were divided by those detected under IFN-α treatment. (D) Enhancement of cell-free p24 production by WT (+) and TMDmut (m) Vpus in the presence (grey background) or absence of 500 U/ml IFN-α2 normalized to viruses lacking Vpu expression (*vpu*-). Results show median values of p24 antigen production (n = 3 ±SEM) in cells of two donors. Statistically significant differences (paired Student's t-test) are marked as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data was kindly provided by Dr Shilpa Iyer and the figure was previously published in Kmiec *et al.,* 2016, page 4.

3.4.3 IFN-α impairs HIV-1 release in the absence of fully functional Vpu

In order to determine the effects of Vpu-mediated tetherin antagonism on total virus production and the efficiency of virion release in HIV-1-infected CD4+ T cells, the cell-associated (CA) p24 antigen was measured (Fig.20). This was then used to calculate the total p24 as the sum of

cell-free (CF) and cell-associated p24. The presence of Vpu affected CA p24 levels to variable extents (Fig.20A), probably because enhancing viral production by functional *vpu* genes may also increase the number of infected cells. On average, WT Vpus increased the total amount of p24 antigen produced in IFN-α treated cultures by 3-fold, whereas TMDmut Vpus achieved less than 2-fold. Quantification of released p24 calculated as the ratio of CF p24 to total p24 (Fig.20C) showed that IFN treatment generally decreased the efficiency of virus release in the absence of tetherin antagonism (Fig.20B). TMD mutations (m) or lack of Vpu (-) reduced virion release efficiency by ~20 % in the absence of IFN-α treatment, which is consistent with constitutive tetherin expression in these cells (Fig.18). Stimulation with exogenous IFN-α substantially decreased the viral release levels by an average of 50 % supporting the earlier observations that TMDmut Vpus lost their ability to efficiently promote viral release (Fig.20B).

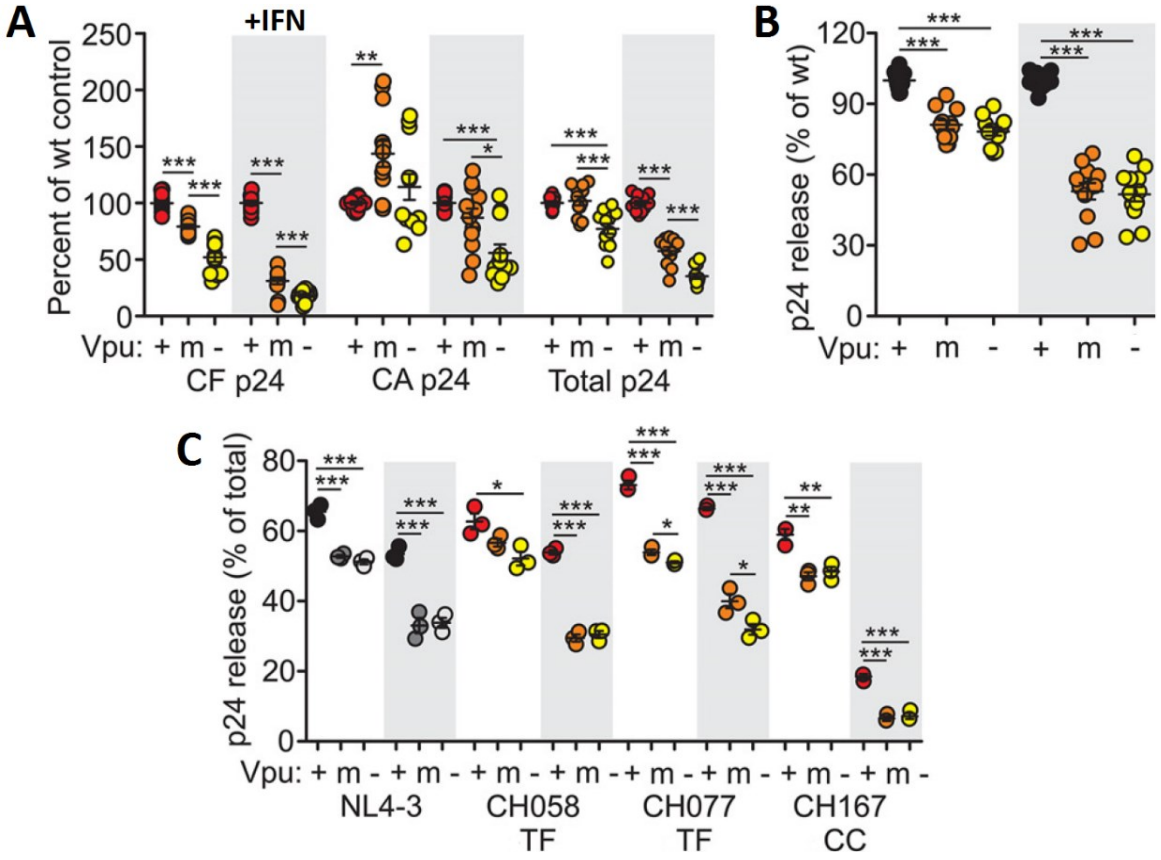


Figure 20: Effects of Vpu on HIV-1 production and release in the presence or absence of IFN.
(A) Cell-free (CF), cell-associated (CA), and total p24 yield in CD4+ T cells infected with HIV-1 IMC expressing WT (+), TMDmut (m), or no Vpu (-) detected in the presence (shaded) and absence of IFN-α2. (B) Efficiency of p24 release from infected CD4+ T cells. Values were normalized to those obtained for the respective WT IMCs (100 %) and averaged or (C) normalized to the total produced p24. Results show median values of p24 antigen production (n = 3 ± SEM) in cells of two donors. Values that are significantly different (paired Student's t-test) are indicated by asterisks as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data was kindly provided by Dr Shilpa Iyer. Figure previously published in Kmiec *et al.,* 2016, page 4.

3.4.4 Enhanced production and release of TF virus is only partially dependent on Vpu

Initial results obtained from group M viruses tested at a single time point suggested that other Vpu functions aside from anti-tetherin activity might contribute to viral replication in primary CD4+ T cells because viruses carrying TMDmut Vpus could still enhance release and CF virus production. To gain more insight into the importance of Vpu-mediated tetherin antagonism throughout HIV-1 group M and N replication, virus production in primary CD4+ T cells infected with WT and Vpu mutant HIV-1 viruses was monitored every two days at 5 subsequent time points (Fig.21). In all cases, IFN-α stimulation profoundly inhibited cell-free (CF) virus production, although with varying efficiencies. As anticipated, viral production increased exponentially during the time-course of the experiment and CH058 and CH077 TF strains were much more resistant to IFN-α-mediated viral inhibition than the chronic and group N IMCs. In case of the TF CH077 and the lab adapted NL4-3 strain, the peak level of viral production was reached sooner (days 5 and 7 respectively) which is consistent with efficient utilization of CXCR4 entry co-receptor by these two strains (Table 4). This can be explained by higher expression levels of CXCR4 than CCR5 in activated lymphocytes (Bleul *et al.*, 1997). The remaining CCR5-tropic strains reached the peak plateau level slightly later (CH058) or showed a consistent increasing trend until the last tested time point (CH167, STCO, DJO).

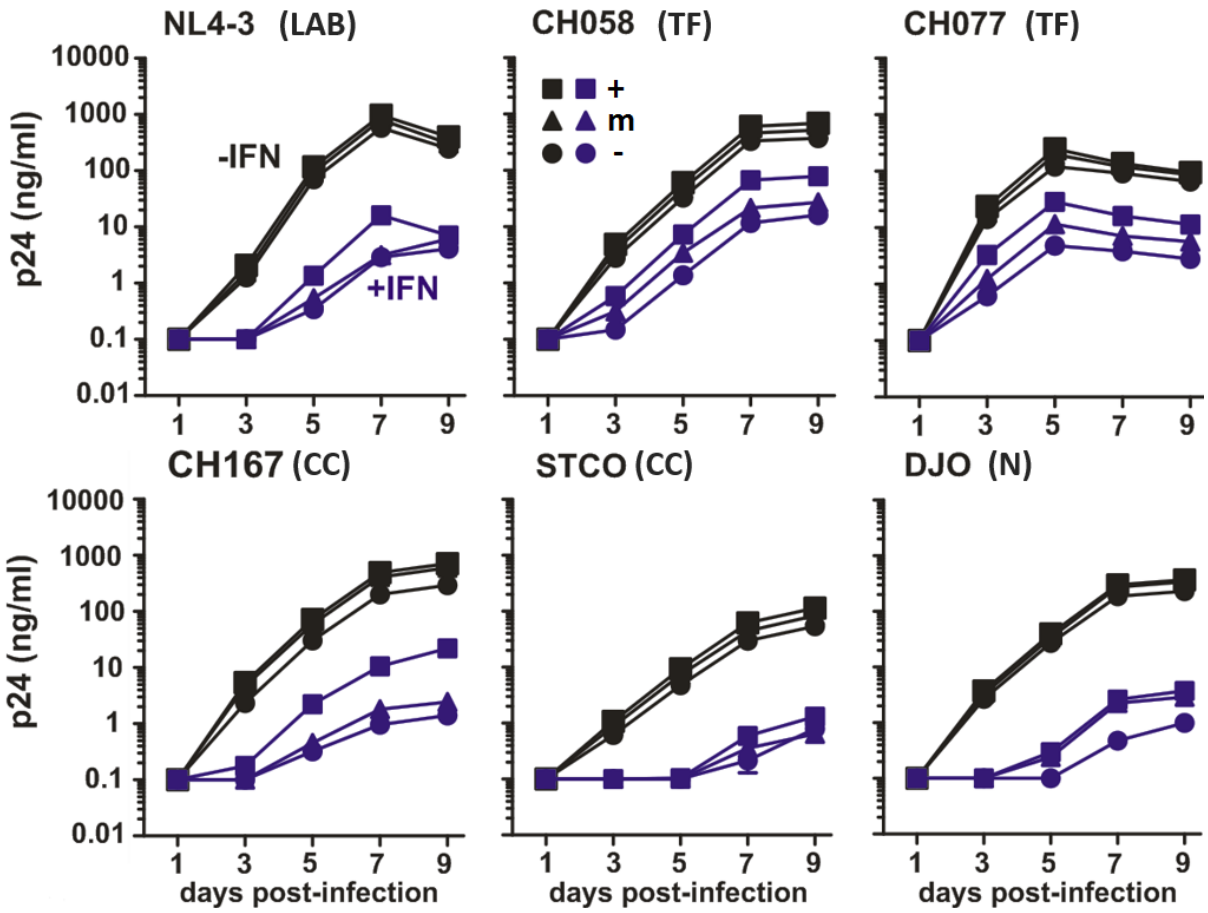


Figure 21: Replication kinetics of HIV-1 IMCs expressing WT, TMD mutant, or no Vpu proteins in CD4+ T cells in the presence (blue lines) or absence of 500 U/ml IFN-α2 (black lines). CD4+ T cells were infected with group M lab-adapted (NL4-3), transmitter founder (subtype B CH058 and CH077), chronic (subtype C CH167 and subtype B STCO) or HIV-1 group N (DJO) IMCs expressing WT (+; square), TMD mutant (m; triangle) or no Vpu (-; circle) and tested for cell-free p24 production at days 1, 3, 5, 7 and 9 post-infection. Results show median values ± SEM tested in triplicates in cells of two different donors. Data was kindly provided by Dr Shilpa Iyer. Figure previously published in Kmiec *et al.,* 2016, page 5.

In the absence of IFN-α treatment, loss of Vpu-mediated anti-tetherin activity resulted in virus yields that were intermediate between WT and Vpu-deficient HIV-1 group M strains. Upon treatment with IFN-α, the TMDmut Vpus could no longer enhance the CF p24 levels in CD4+ T cells infected with lab-adapted or the chronic strains and had lost most of their enhancing effect in case of TF viruses (Fig.22). In case of group N, which possessed weaker anti-tetherin activity (Fig.12), only a very modest enhancing effect was seen in the presence of IFN-α2 (Fig.22) and there was no difference between WT and TMDmut Vpu.

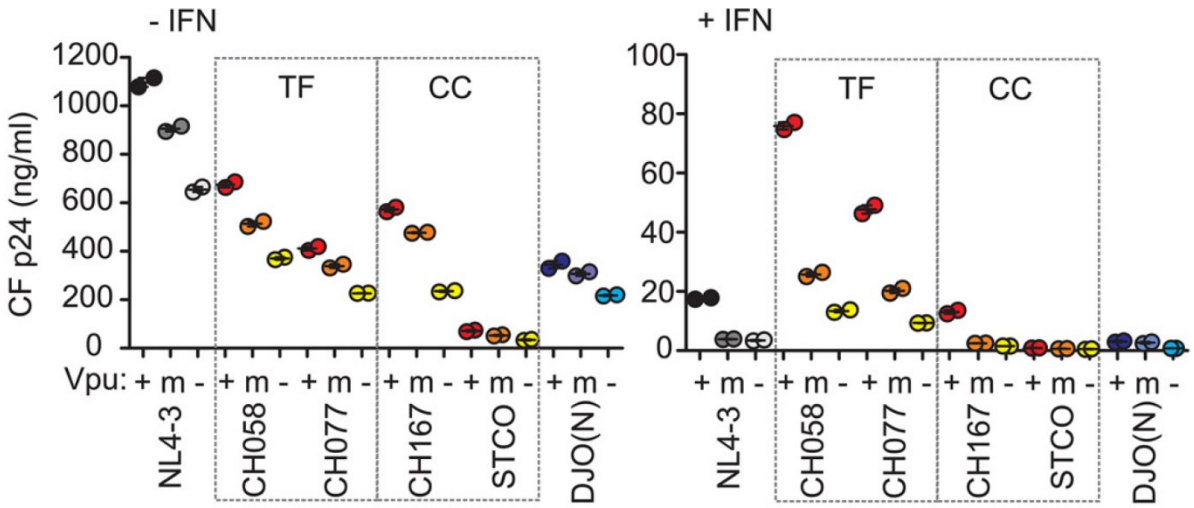


Figure 22: Effect of IFN-α treatment on cumulative viral production. Cumulative cell-free (CF) capsid antigen levels produced in the absence (left) or presence (right) of exogenous IFN-α2 measured at 1, 3, 5, 7, and 9 days post-infection shown in Fig.21. The lab-adapted NL4-3 strain is shown in black/grey/white, group M strains in red/orange yellow and group N in shades of blue. Dark colors denote WT (+), medium colors TMD mutant (m) and light colors no Vpu (-). Note the differences in maximum values shown on the Y axis in the absence (- IFN) and presence (+ IFN) of IFN-α treatment. Each dot represents data obtained from one donor together with median ±SEM. Figure previously published in Kmiec *et al.,* 2016, page 5.

IFN-α treatment decreased virus yield of TF viruses by ~9-fold, whereas chronic HIV-1 strains (CC) were affected to a much higher extent (47- and 75-fold). Group N had the highest IFN sensitivity and treatment with 500 U/ml caused more than a 100-fold decrease in CF virus (Fig.23). Lab adapted NL4-3 which represents a chronic-type virus showed similar sensitivity as the strain isolates from chronic-stage HIV patients (CH167, STCO).

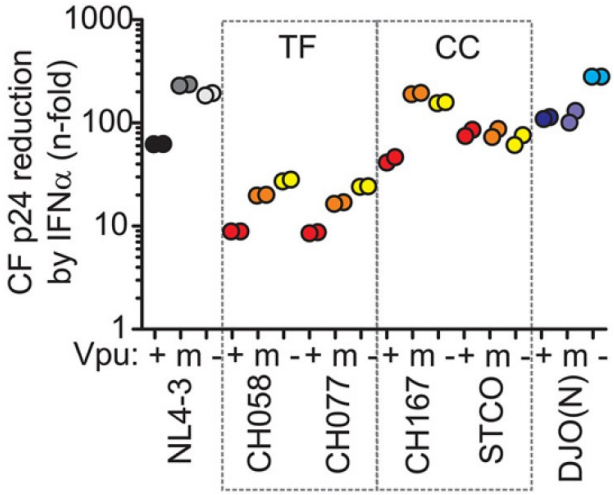


Figure 23: IFN-mediated reduction of virus production. Values of cumulative cell-free (CF) capsid antigen levels produced in the absence of exogenous IFN-α were divided by levels produced in the presence of IFN-α (shown in Fig.22) to calculate n-fold reduction. The lab-adapted NL4-3 strain is shown in black/grey/white, group M strains in red/orange yellow and group N in shades of blue. Dark colors denote WT (+), medium colors TMD mutant (m) and light colors no Vpu (-). Each dot represents data obtained from one donor. Figure previously published in Kmiec *et al.,* 2016, page 5.

Inability to counteract tetherin or complete lack of Vpu enhanced IFN-α sensitivity of most HIV-1 M IMCs by ~3-4 fold (Fig.21). The exception was the chronic STCO strain, which replicated to relatively low levels (Fig.21 and 22) and was highly susceptible to IFN inhibition independently of Vpu expression (Fig.23). In contrast, the ~5-fold enhancement by TF Vpus was severely decreased by the TMD mutations abrogating tetherin counteraction (Fig.24). In the case of the TMDmut of chronic CH167 strain, which counteracted tetherin very efficiently in transient assays (Fig.12), the loss of enhancement of viral production during IFN-α treatment was even more pronounced (~9 fold). Surprisingly, the ~4 fold enhancement of p24 production by HIV-1 N Vpu in the presence of IFN-α was not impaired by the TMD mutations abrogating anti-tetherin function (Fig.21). Therefore, HIV-1 group N appears to enhance viral production independently of its modest Vpu-mediated tetherin antagonism.

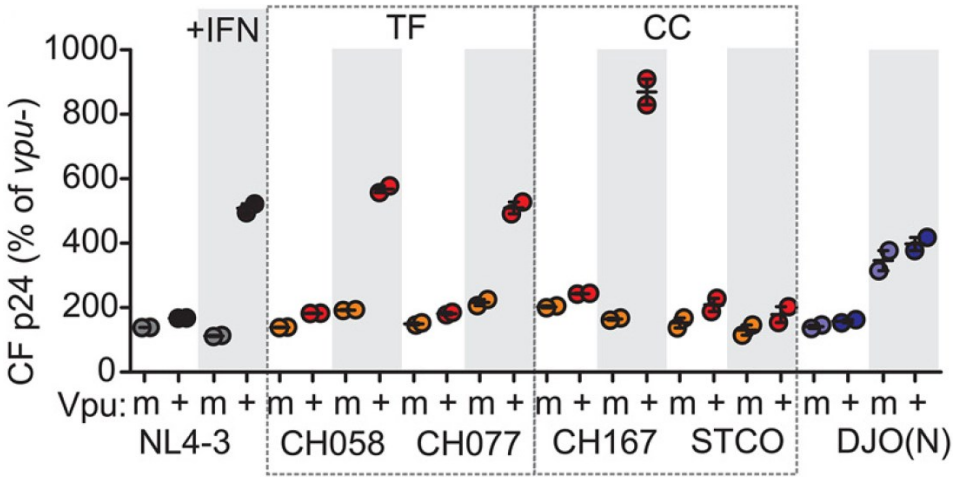


Figure 24: Enhancement of cumulative p24 yield by WT and TMDmut Vpu proteins. Cumulative cell-free (CF) capsid antigen levels produced in the absence (white background) or presence (shaded background) of exogenous IFN-α. Data were derived from the experiment shown in Fig.20. Values present total cell-free virus yield relative to the respective *vpu*-defective HIV-1 IMC (100 %). Each dot represents data obtained from one donor with mean ±SEM. Figure previously published in Kmiec *et al.,* 2016, page 5.

To quantify the level of viral release, amounts of cell-associated and total p24 antigen in the infected CD4+ T cell cultures were measured and compared (Fig.25A). Surprisingly, the Vpu TMD mutant strains of HIV-1 M produced equal amounts or even slightly more p24 antigen than the corresponding WT viruses (Fig.25B), which could be due to effective tetherin-independent cell-to-cell spread in the infected CD4+ T cell cultures (Jolly *et al*., 2010).

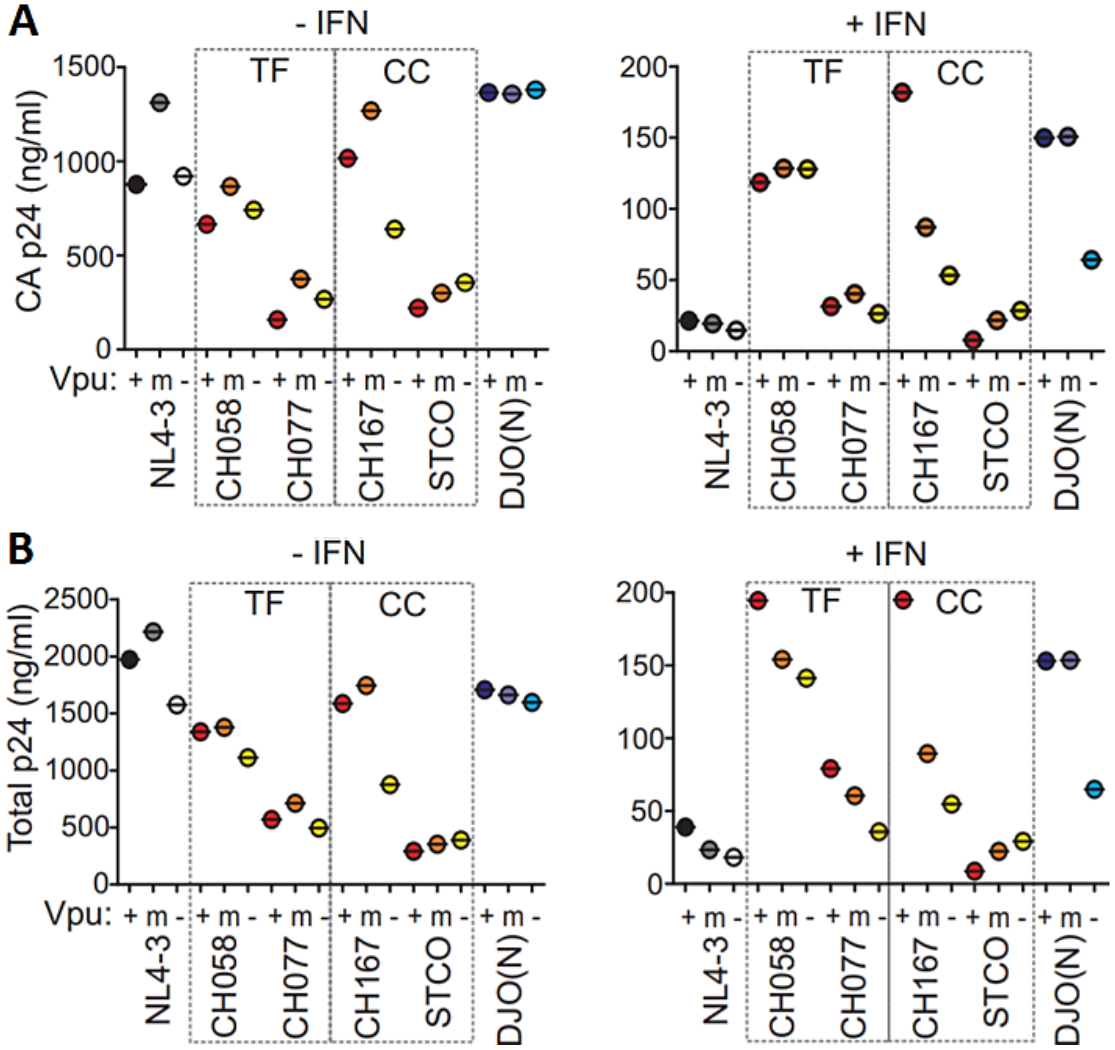


Figure 25: Effects of Vpu on cumulative cell-associated and total p24 production in the presence and absence of IFN. Cumulative cell-associated (A) or total (B) p24 antigen levels in CD4+ T cells at 5, 7, and 9 days post-infection with HIV-1 IMCs expressing WT (+), TMDmut (m), or no (−) Vpu proteins. Mean viral capsid protein p24 levels from 2 donors were determined in the absence (left) or presence (right) of 500 U/ml IFN-α. Data was kindly provided by Dr Shilpa Iyer. Figure previously published in Kmiec *et al.,* 2016, Fig.S4.

In agreement with earlier data (Fig.20), treatment with 500 U/ml IFN-α markedly reduced the efficiency of virus release from infected CD4+ T cells, especially in the absence of Vpu mediated tetherin counteraction (Fig.26). The release of the TF virus was significantly more efficient than that of CC viruses both in the absence and presence of IFN (Fig.25B), while the release of HIV-1 group N DJO was the lowest among the viruses tested (Fig.25A). Surprisingly, TF HIV-1 strains partially (m) or completely (*vpu*-) lacking Vpu function showed significantly higher efficiencies of virion release than the WT chronic strains in the presence of IFN-α. Strain-dependent differences in virion release capacity were much more pronounced in IFN-α -treated than in untreated CD4+ T-cell cultures. Chronic HIV strains containing disrupted or mutated

vpu genes and HIV-1 group N exhibited very low efficiencies of virion release (Fig.26A) with almost 95 % inhibition upon IFN-α treatment.

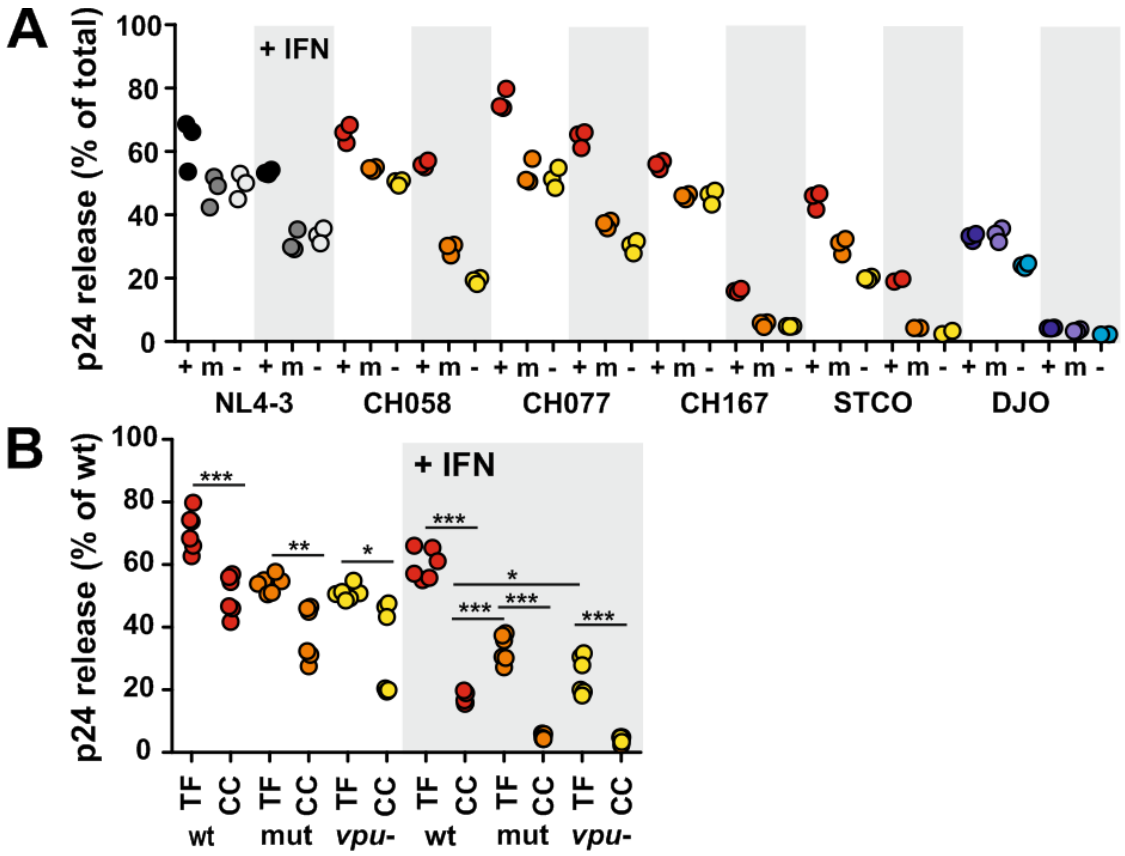


Figure 26: Efficiency of viral release from CD4+T cells in the presence or absence of IFN stimulation.

(A) Values present percentages of cell-free p24 antigen (shown previously on Fig. 24) out of the total p24 detected in the presence (shaded) and absence of IFN-α at day 5 post-infection. (B) The efficiency of TF and CC virus release from infected CD4+ T cells. Results show values obtained from triplicates tested in cells of two different donors. Values that are significantly different (paired Student's t-test) are indicated by asterisks as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Figure previously published in Kmiec *et al.,* 2016, page 6.

4 DISCUSSION

Although SIVs have been transmitted to humans at least a dozen times, only one transmission event resulted in the emergence of highly pathogenic HIV-1 group M that is responsible for the AIDS pandemic. Viral adaptation to a new host requires the acquisition of efficient counteraction of antiviral restriction factors that are often species-specific. Shortly after the identification of inhibitory function of tetherin and its counteraction by Vpu (Neil *et al.*, 2008; Van Damme *et al*., 2008), it has been suggested that the evolution of potent tetherin antagonism by Vpu promoted efficient spread of the M (major) group of HIV-1 (Sauter *et al.*, 2010). Nevertheless, direct experimental evidence supporting this hypothesis was limited. Most studies investigating this subject used immortalized cell lines, which lack many relevant immune mechanisms present in primary target cells of HIV. Furthermore, previous studies employed laboratory-adapted viruses completely lacking Vpu expression, which undermines the specificity and relevance of reported observations. Here I show that a reversal of the recent human-specific adaptation of pandemic HIV-1 resulted in selective abrogation of tetherin counteraction and significantly lower production of cell-free virus in primary CD4+ T cells. This suggests that the gain of this specific adaptation allowed effective viral antagonism of tetherin in humans, which promoted viral particle release from the infected cells and possibly also early HIV-1 spread.

It has been shown that the AxxxA motif in the transmembrane domain of Vpu mediates the anti-tetherin activity of the lab adapted HIV strain NL4-3 (Vigan and Neil, 2010). This motif represents a conserved adaptation crucial for tetherin counteraction in primary group M and N strains, which is absent from their simian precursor SIVcpz or non-pandemic HIV-1 groups O and P. Identification of this human-specific adaptation provided basis for generation of tetherin-defective Vpu mutants of patient-derived HIV-1 clones, including those representing transmitted founder phenotype important for spread between individuals. Indeed, selective mutation of the relevant alanines to leucines rendered all tested Vpu proteins unable to counteract human tetherin. This loss of function was associated with a decreased interaction between Vpu and tetherin, as well as inefficient surface downmodulation of the latter in primary human lymphocytes. At the same time, the AxxxA mutation of Vpu had no effect on localization, stability or other immunomodulatory functions. Viral constructs carrying these selectively defective Vpu mutants were then used to dissect the relevance of tetherin

counteraction for viral replication in primary human CD4+ T cells. These cells represent the primary target cells of HIV-1 *in vivo* and express high levels of tetherin upon IFN-α stimulation, which is one of the most important cytokines released in response to the acute HIV-1 infection. My results demonstrate that Vpu-mediated tetherin antagonism plays a major role in replication capacity, virion release and viral resistance to type I IFN, which suggests that this adaptation was important for the spread of pandemic HIV.

Although it is still not clear, whether HIV can use infected donor cells as a transmission vector (Sagar *et al*., 2014), there is a good correlation between the transmission rates and the level of cell-free virus in blood or genital secretions (Butler *et al*., 2008; Stürmer *et al*., 2008). This strongly suggests that cell-free virus production and release are important for HIV-1 transmission. Unfortunately, so far studies aiming to prove that hypothesis have been held back by the lack of a suitable experimental model. The complexity of the sexual transmission event are extremely difficult to recreate in *in vitro* systems. Moreover, HIV shows high host specificity and can only infect humans and some closely related simian species (reviewed in Hatziioannou and Evans, 2012). Experimental infections of non- human primates are ethically controversial, tightly regulated and associated with significant costs. These aspects have been partially addressed by the development of a humanized mouse model for HIV (Marsden and Zack, 2017). The fact that humanized mice express human tetherin on their CD4+ T cells was of great importance for a recent follow-up study performed by our collaborator. By infecting these mice with virus expressing selectively tetherin-inactive Vpu mutant (TMDmut) characterized in this thesis (HIV-1 M STCO), Yamada *et al.*, 2018 demonstrated that Vpu-mediated counteraction of tetherin confers a selective advantage for viral spread and is critical for efficient viral replication during the initial phase of HIV-1 replication. These findings highlight the relevance of *in vitro* results presented in my thesis and support the idea that human-specific adaptations in the TMD of Vpu that mediate potent tetherin counteraction play a major role in HIV-1 fitness and spread.

Sequence alignment of primary HIV-1 group M Vpus (Fig.7) shows that despite large variability in the N and C termini of Vpus, their trans-membrane domains are well conserved. This is because TMD of Vpu together with the cytoplasmic DSCxxS motif are crucial for tetherin, CD4 and NTB-A downmodulation (Neil *et al*., 2008; Shah *et al*., 2010; Pickering *et al*., 2014). Consistent with previous data showing that CD4 and NTB-A downmodulation are conserved activities of human and simian lentiviruses (Bolduan *et al*., 2013), all primary HIV-

1 group M Vpus tested in this study were able to perform these functions. HIV-1 group M Vpu causes ubiquitination of CD4 and targets it for endoplasmic reticulum (ER)-associated degradation (Magadán and Bonifacino, 2012), which prevents superinfection (Benson *et al.*, 1993; Wildum *et al.*, 2006) and reduces binding of CD4 to immature envelope proteins (Willey *et al.*, 1992b; Argañaraz *et al*., 2003; Levesque *et al*., 2003). In contrast, HIV-1 group N DJO Vpu lacked CD4 degradation activity, similar to other members of this group (Sauter *et al*., 2012). Notably, all HIV-1 strains tested in this study efficiently downmodulated cell surface CD4 even in absence of functional Vpu protein due to Nef and Env proteins (Chen *et al.*, 1996; Wildum *et al.*, 2006). This does not argue against the importance of Vpu-mediated CD4 downmodulation as the early expressed Nef removes CD4 already present at the cell surface whilst the late expressed Vpu targets the newly synthesized receptor for degradation before it reaches the plasma membrane (Lindwasser *et al*., 2007). Similarly to CD4 downmodulation, the inhibitory effect of Vpu on NF-κB was also conserved among tested HIV-1 group M strains and independent of the AxxxA motif. This is in agreement with results showing that both CD4 downmodulation as well as NF-κB inhibition represent conserved features of HIV-1 group M and its simian precursor, SIVcpz (Gomez et al., 2005; Sauter et al., 2015) and most likely evolved earlier than Vpu-mediated tetherin counteraction.

Although this study focused primarily on tetherin counteraction by Vpu, it is apparent that other functions of this accessory protein also play a role in the fitness of HIV-1. All tested HIV-1 M strains showed significant differences in replication and IFN sensitivity between variants lacking *vpu* expression and these that expressed tetherin-defective allele. It would be interesting to investigate and compare the importance of other known Vpu-mediated functions to viral replication. This has been recently done for Vpu-mediated NF-κB inhibition, which seems to be dispensable for efficient viral replication during the initial phase of HIV-1 replication (Yamada *et al.,* 2018). Another highly conserved and well-described function of Vpu is the CD4 downmodulation. So far, similar studies investigating the importance of this function has not been undertaken as the multiple domains and motifs involved in CD4 downmodulation by Vpu are also important for other functions of this accessory protein (Pickering *et al*., 2014).

In agreement with published data showing that the main inhibitory mechanism of tetherin is the restriction of viral release (Neil *et al*., 2008; Perez-Caballero *et al*., 2009), selective abolishment of anti-tetherin activity significantly decreased the release rates of all

HIV-1 group M viruses. Furthermore, upon IFN-α2 stimulation, the lack of tetherin counteraction decreased viral production of even the most resistant transmitted founder strains by a factor of 10, and viruses specifically lacking this Vpu function produced less progeny and were released at least 50 % less efficiently than the WT controls. This can be attributed to the IFN-inducibility of tetherin, as the IFN-α concentration used in the present study upmodulated CD4+ T cell surface expression of this restriction factor by about two-fold. Although the peak IFN levels in acutely infected patients are poorly defined, some studies reported IFN-α levels reaching 100-400 U/ml (Gaines *et al*., 1990; Von Sydow *et al*., 1991; Homann *et al*., 2011), which is similar to the concentration used here (500 U/ml). In addition, a study analysing tetherin levels in HIV-1 infected patients (Homann *et al.*, 2011) showed that infection with HIV-1 upmodulates tetherin expression by 3-fold, which is slightly higher than that observed here upon exogenous IFN treatment *in vitro*. Therefore, IFN-α concentrations used in this study are probably within or close to physiological range.

Even though all tested viruses were affected by IFN treatment, intrinsic differences in sensitivities of tested strains were observed. Cell-free TF virus production was restricted by about 10-fold, for chronic and lab-adapted strains the effect was even stronger (50-fold), whereas the poorly adapted group N was almost completely inhibited by IFN (100-fold reduction). In addition, TF strains were also found to be on average ~3.5 times more infectious than other strains. This is in agreement with significantly higher IFN resistance and infectivity of HIV-1 group M TFs over chronic isolates (Parrish *et al.*, 2013; Iyer *et al.*, 2017). These different viral properties can be explained by the fact that following the initial appearance of HIV virions in the healthy host and their recognition by the innate immune system, potent, IFN-mediated immune response takes place. This activates the expression of multiple IFN-inducible proteins that specifically target viral replication. Only the fittest virions that are able to survive and replicate in these hostile conditions are able to establish an infection (TF strains). With progression to a chronic HIV-1 infection phase, IFN levels also decrease, changing the selective pressure elicited on the virus. My results show that low sensitivity to IFN-α, high particle infectivity and efficient virion release from CD4+ T cells are characteristic features of transmitted founder strains, which is consistent with previous findings (Parrish *et al.*, 2013; Iyer *et al.*, 2017).

I have shown that the transmitted founder viruses expressing tetherin-deficient Vpus were released about 3-fold more efficiently than chronic viruses carrying these mutations, and

>10-fold more efficiently than the mutant of rare group N virus in IFN-α-treated human CD4+T cells. The remaining differences between viruses expressing TMDmut or no Vpu are unlikely to be due to residual anti-tetherin activity since these variants did not show any significant rescue of virus release. Observed tetherin counteraction-independent enhancement of TF virus release is likely a result of other, possibly Vpu-independent viral properties (Jafari *et al.,* 2014). There are factors other than tetherin that have been implicated in the restriction of viral release, such as CD4 (Ross *et al*., 1999), T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins (Li *et al*., 2014). In addition, BCA2, a RING-finger E3 ubiquitin ligase that aids tetherin in restriction of viral release (Miyakawa *et al*., 2009), was recently shown to also possess tetherin-independent inhibitory effect on virus production (Nityanandam and Serra-Moreno, 2014; Colomer-Lluch and Serra-Moreno, 2017). Another IFN-induced protein called viperin was shown to inhibit viral release by disruption of membrane lipid rafts (Wang *et al.*, 2007; Nasr *et al*., 2012). So far, it is not known what determines viral sensitivity to most of these factors. Thus, it is difficult to assess whether the increased resistance of TF viruses towards mentioned factors contributes to the observed tetherin-independent release enhancement and high IFN resistance. Nevertheless, the strong differences between all wild type and tetherin-defective mutant HIV-1 group M viruses highlight the important role of Vpu-mediated tetherin antagonism in viral release and resistance to IFN.

It is known that IFN responses vary between individuals and certain characteristics, such as the sex influence the amount of IFN-α produced by stimulated pDCs (Griesbeck *et al*., 2015). Viruses used in this study were mainly obtained from men who have sex with men (MSM), with the exception of subtype C CH167, which was isolated from a heterosexual female. Vpu of this strain was the most efficient one at tetherin counteraction. It is known that HIV-1 infected women have increased rates of immune activation and are at higher risk of developing AIDS than men (Anastos *et al*., 2000., Farzadegan *et al*., 1998) which appears to be linked to higher estrogen levels (Griesbeck *et al.*, 2016). So far, it is unknown whether HIV-1 infection of females selects for higher IFN resistance, therefore it would be interesting to determine whether any gender-associated patterns in IFN sensitivity of viral species develop among related groups, for example in infected donor and recipient pairs.

The notion that IFN-resistance is one of the hallmarks of viral adaptation to its host is supported by the phenotype of HIV-1 group N strain tested in this study. Molecular clone of this rare group (HIV-1 N DJO) was highly sensitive to IFN inhibition and produced very little cell-

free virus in its presence, which was caused by the inefficient release from CD4+ T cells. This observation was consistent with the poor expression of HIV-1 group N Vpu and inefficient tetherin counteraction, which is in agreement with a previous study (Sauter *et al*., 2012) showing that functional deficiencies of group N map to disrupted putative adaptor protein binding sites and β TrCP-binding motif. Despite the low activity of group N in comparison with HIV-1 group M Vpus, in the presence of IFN stimulation, both WT and TMD mutated group N Vpus increased cell-free virus production by about four fold. Thus, it seems that the HIV-1 group N DJO Vpu promotes virus production by yet-to-be-defined tetherin-independent mechanisms. It seems that this group of HIV is still in the process of adaptation to humans (Sauter *et al*., 2012), which might help to explain why it infected a very limited number of people. There have only been around 20 HIV-1 group N infection cases reported so far (Pere *et al*., 2015). It is also noteworthy that despite its very limited spread, HIV-1 N strains can cause significant CD4+ T cell depletion and AIDS (Simon *et al*., 1998). It is interesting that of all tested Vpu functions, group N Vpu was able to moderately downmodulate tetherin, but lacked other tested functions like CD1d and NTB-A downmodulation and NF-κB inhibition and was also very poor at CD4 downmodulation. This suggests that among different Vpu functions, tetherin antagonism was one of the main prerequisites that mediated early HIV transmission among humans.

Taken together, my results show that the efficiency of virus release from infected CD4+ T cells correlates with the efficiency of HIV-1 spread in humans, and furthermore support the notion that tetherin counteraction significantly contributes to the high fitness of the pandemic HIV-1 strains.

5 SUMMARY

HIV-1 groups M, N, O, and P resulted from four independent zoonotic transmission events of simian immunodeficiency viruses (SIV) infecting chimpanzees and gorillas to humans. Studying the differences between pandemic HIV-1 group M and closely related but less successful pathogens helps to expose viral vulnerabilities, which can be exploited as targets of new treatment or preventive strategies. One characteristic that distinguishes HIV-1 group M strains from the geographically-limited and less prevalent groups N, O and P is the acquisition of adaptive mutations in the accessory protein Vpu that mediate potent activity against human tetherin. This adaptation is thought to be critical for the effective spread of HIV-1 M strains within the human population because human tetherin harbours a deletion that renders it resistant to the counteraction by its SIV precursor. However, direct evidence supporting this hypothesis has been very limited. To address this issue and determine the contribution of Vpu-mediated tetherin counteraction to viral fitness, I mutated the AxxxA transmembrane domain Vpu motif of primary HIV-1 group M and N strains to specifically disrupt their ability to antagonize tetherin, but not other Vpu functions, such as downmodulation of CD4, CD1d and NTB-A, and suppression of NF-κB activity. Reversion of this particular human-specific adaptation significantly reduced the ability of HIV-1 group M Vpu proteins to enhance virus production and release from primary CD4+ T cells in the presence of type I interferon (IFN) by about 2- to 5-fold. These consistent differences between wild type and tetherin-defective mutant HIV-1 group M viruses highlight the important role of Vpu-mediated tetherin antagonism in viral release and resistance to IFN. Surprisingly, transmitted founder (TF) HIV-1 strains exhibited higher virion release capacity than chronic control HIV-1 strains irrespective of Vpu function. In addition, all pandemic group M viruses produced higher levels of cell-free virions than the poorly-adapted HIV-1 N group strain. In agreement with these *in vitro* data showing that Vpu-mediated tetherin antagonism plays an important role in HIV-1 fitness, the anti-tetherin activity was found to be critical for an efficient early spread of HIV-1 in humanized mice as shown in a study performed by our collaborator. Thus, efficient virus release from infected cells seems to play an important role in the spread of pandemic HIV-1 and requires a Vpu protein that efficiently counteracts human tetherin.

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7 ACKNOWLEDGEMENTS

This project would not have been possible without the help of many people. I am very grateful for their contribution and want to sincerely thank them:

First of all, I would like to thank Prof. Dr Frank Kirchhoff for giving me the opportunity to perform my Ph.D. thesis in his group and all the guidance and support during these years. I appreciate his advice and constructive criticism that motivated me to constantly improve my research. Working under Frank‘s supervision was and continues to be a great learning experience fuelled by a true passion for science.

A big thank you also goes to Jun.-Prof. Dr Daniel Sauter for all the time, guidance and support he offered me during the last 3 years. His great patience and positive attitude made dealing with everyday work issues so much easier.

I would also like to thank our project collaborators from the Center for AIDS Research at the University of Pennsylvania: Dr Shilpa Iyer for her excellent experimental work and Prof. Beatrice Hahn for her valuable input in creating our publication and WT primary IMCs. In addition, I thank Prof. Igor Stagljar and his group the University of Toronto for hosting me in their lab, teaching me how to work with the MaMTH system and providing necessary reagents.

Special thanks to all the members of the Institute of Molecular Virology who spent their time to teach me new methods and were ready to help when I needed it: Silvia Kluge, Ali Gawanbacht, Dominik Hotter, Christian Krapp, Konstantin Sparrer, Elena Heusinger, Katharina Mack, Simon Langer, Christina Stürzel and all the others that my poor memory might have missed.

I also want to thank Kerstin Regensburger for the great technical assistance and other members of her lab for their everyday support and friendly working atmosphere.

Last but not least, I need to thank the most important people in my life – my family and Tomek, who gifted me with love and provided all the care and support they could before and during my PhD. Dziękuję wam z całego serca.

8 CURRICULUM VITAE

Personal Information

Name:	Dorota Janina Kmieć
Year of Birth:	1989
Nationality:	Polish

Education

Oct 2014 – Jun 2018	PhD studies at the Institute of Molecular Virology, Medical Faculty of Ulm University, Germany Dissertation: **„Role of Vpu-mediated tetherin antagonism in HIV-1 fitness“** Supervisor: Prof. Dr. Frank Kirchhoff
Sept 2008 – Jun 2013	Master in Science, upper second class Microbiology integrated degree at the University of Glasgow, Scotland **„Characterization of Protein Coated Micro-Crystals for Vaccine Formulations“** supervisor: Dr. Dorothy Xing **„Investigating the Expression of Putative Vaccine Candidates in Pathogenic Strains of *E.coli*“** supervisor: Dr. Andrew Roe
Sept 2005 – Jun 2008	High School (Liceum Ogolnoksztalcace) no. 3 in Poznan, Poland

Work and internships

Aug 2017 – Sept 2017	Internship in Prof. Dr. Igor Stagljar's group at the Department of Molecular Genetics, University of Toronto, Canada
Nov 2013 – Sept 2014	Junior Scientist position, Bacteriology division at the National Institute for Biological Standards and Control, England
May 2012 – Sept 2012	Summer internship in the Bacteriology division at the National Institute for Biological Standards and Control, England

Conferences

March 2017	Annual Meeting of the Society for Virology, Marburg, Germany Poster presentation **„Relevance and molecular determinants of SERINC5 antagonism by primate lentiviral Nef proteins"**
September 2016	Frontiers of Retrovirology conference, Erlangen, Germany Oral presentation **„Vpu-mediated counteraction of tetherin is a major determinant of HIV-1 interferon resistance“**
July 2016	Molecular Medicine Padua-Ulm Retreat, Ulm, Germany Oral presentation **„The importance of Vpu mediated tetherin antagonism for HIV-1 spread and replication“**

Publications

Li P, Asokanathan C, Liu F, Khaing KK, Kmiec D, Wei X, Song B, Xing D, Kong D. **PLGA nano/micro particles encapsulated with pertussis toxoid (PTd) enhance Th1/Th17 immune response in a murine model**. Int. J. Pharm. 2016 Nov 20;513:183-190

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