Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen
Departement Biomedische Wetenschappen

Expression analysis of the cellular HIV-related host factors LEDGF/p75, APOBEC3G, TRIM5alpha and tetherin in frequently HIV-exposed seronegative individuals

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen aan de Universiteit Antwerpen
te verdedigen door **Kim Mous**

Promotor: Prof. Dr. Xaveer Van Ostade
Promotor: Prof. Dr. Luc Kestens
Co-promotor/Supervisor: Dr. Ir. Wim Jennes

Antwerpen, 2012

The research described in this thesis was performed at the Laboratory for Proteinscience, Proteomics and Epigenetic Signaling (PPES), University of Antwerp; and at the Laboratory of Immunology, Institute of Tropical Medicine. The study was funded by the University of Antwerp (NOI-BOF) and by the National Fund for Scientific Research-Flanders (NFWO grant G.0660.06 and Kim Mous' doctoral fellowship).

Front cover image: Confocal image of LEDGF/p75 in PBMC
Back cover image: Confocal image of tetherin in interferon-stimulated PBMC

Cover design: Natacha Hoevenaegel, Nieuwe Media Dienst, University Antwerp

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Expressie analyse van de cellulaire HIV-geassocieerde gastheerfactoren LEDGF/p75, APOBEC3G, TRIM5alfa en tetherine bij HIV-seronegatieve, frequent blootgestelde individuen

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Abbreviations

A3G, APOBEC3G	apolipoprotein B mRNA-editing catalytic polypeptide-like 3G
AGM	African Green Monkey
AIDS	Acquired Immune Deficiency Syndrome
AME	amphotericin B methyl ester
APC	allophycocyanin
ASK	activator of S-phase kinase
at RT	at room (ambient) temperature
B2M	beta-2-Microglobulin
β-TrCP	F-box/WD repeat-containing protein 1A
BSA	bovine serum albumin
BST-2	bone marrow stromal cell antigen, tetherin
CA	capsid protein
CAF	cell antiviral factor
CAI	capsid assembly inhibitor
CBF-β	core binding factor beta
CC	coiled-coil
CCD	catalytic core domain
CCR5	CC-chemokine receptor 5
CDA	cytidine deaminase domain
cDC	conventional dendritic cells
CNRQ	Calibrated Normalized Relative Quantity
COPRIM	correlates of protective immunity
Cq	quantification cycle
CRF	circulating recombinant form
CTD	C-terminal domain
CV	coefficients of variance
CXCR4	C-X-C chemokine receptor type 4
CypA	Cyclophilin A protein

DEPC	diethylpyrocarbonate
dsDNA	double-stranded DNA
ECS	extracellular staining
ELISA	enzyme-linked immunosorbent assay
ESN	exposed seronegatives
EU	exposed uninfected
FBS	fetal bovine serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
Fw	forward
FWO	Fund for Scientific Research Flanders
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gp120	glycoprotein 120
gp41	glycoprotein 41
GPI	glycosylphosphatidylinositol
HAART	highly active anti-retroviral therapy
HBSS	Hank's balanced salt solution
HC	healthy control
HDGF	hepatoma-derived growth factor
HEPS	highly exposed persistently seronegatives
HERC5	HECT domain and RCC1-like domain-containing protein-5
HESN	HIV-1-exposed seronegatives
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HIV-ART	antiretroviral therapy treated HIV-1-infected patient
HIV-UT	untreated or therapy-naïve HIV-1-infected patient
HLA	human leukocyte antigen
HMM	high molecular mass complex

HR	HIV resistants
HRP	Hepatoma-derived growth factor related protein
HRP-2	Hepatoma-derived growth factor related protein 2
HSV-2	Herpes simplex virus type 2
IBD	integrase binding domain
ICS	intracellular staining
IgG	immunoglobulin G
IFNα	interferon-alpha
IFNβ	interferon-beta
IFNγ	interferon-gamma
IL-22	interleukin 22
ILT7	immunoglobulin-like transcript 7
IN	integrase
InSTI	integrase strand transfer inhibitor
IQR	interquartile range
ITM	Institute of Tropical Medicine
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KAP1	KRAB-associated protein-1
KD	knock-down
KIR	killer immunoglobulin-like receptor
KSHV	Kaposi's sarcoma-associated herpesvirus
LGF, LEDGF/p75	lens epithelium-derived growth factor splice variant 75
LH	gonadotropic luteinizing hormone
LMM	low molecular mass complex
LNA flow-FISH	locked nucleic acid flow-fluorescence in situ hybridization
LNA	locked nucleic acid
LTNP	long-term-non-progressors
Ly	lymphocytes
MA	matrix protein

MAPK	mitogen-activated protein kinase
memTeth	membrane tetherin
MFI	median fluorescence intensity
MHC	major histocompatibility class
MIP1α	macrophage inflammatory protein 1 apha
MIP1β	macrophase inflammatory protein 1 beta
Mo	monocytes
MoAb	monoclonal antibody
mRNA	messenger RNA
NC	nucleocapsid protein
ncRNA	non-coding RNA
ND	not determined
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NLS	nuclear localization signal
N-MLV	N-tropic murine leukemia virus
NNRTI	non-nucleoside reverse transcriptase inhibitors
NRTI	nucleoside and nucleotide reverse transcriptase inhibitors
NT	non-transmitter
NTD	N-terminal domain
P/I	phorbol myristate acetate in combination with ionomycin
P/S	penicillin/streptomycin
PAF1	peroxisome assembly factor-1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PE	phycoërythrin
PerCP	peridinin-chlorophyl protein
PI	propidium iodide

PIC	pre-integration complex
PKA	Protein kinase A
PMA	phorbol myristate acetate
PoAb	polyclonal antibody
pogZ	pogo transposable element with ZNF domain
PPES	Proteinscience, Proteomics and Epigenetic Signalling
PR	protease
PRDX2	peroxiredoxin 2 gene
PSIP1	PC4- and SFRS-interacting protein 1 gene
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBCC motif	Ring domain – B box domain – Coiled-coil domain
Re	reverse
RING	really interesting new gene
RPL13A	60S ribosomal protein L13a
RT	reverse transcriptase
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAMHD1	sterile alpha motif- and metal-dependent phosphohydrolase domain and HD domain-containing protein-1
SDF-1	stromal cell-derived factor-1
SEB	staphylococcus enterotoxin B
SILAC	Stable Isotopes Labeling by Amino Acids in Cell Culture
siRNA	small inhibitory RNA
SIV	Simian Immunodeficiency Virus
SNP	single nucleotide polymorphism
SSC	standard sodium citrate
ssRNA	singe-stranded RNA
STI	sexually transmitted infections
T	transmitter

T5α, TRIM5α	tripartite motif 5alpha
TAB2	TAK1 binding protein 2
TBP	TATA-binding protein
TGF-β	transforming growth factor beta
TGN	trans-Golgi network
Th1	T helper cells
TLR	Toll-like receptor
TM	transmembrane
TNF-α	tumor necrosis factor-alpha
totTeth	total tetherin
UBA-2	ubiquitin-associated domain 2
UBC	ubiquitin C
US FDA	United States Food and Drug Administration
UZA	University Hospital of Antwerp
Vif	viral infectivity factor
VL	viral load
Vpu	viral protein U
WT	wild-type

Samenvatting

Tot op heden is het onduidelijk waarom bepaalde personen HIV-1 seronegatief blijven ondanks frequente blootstelling aan het HIV-1 virus. Verschillende onderzoeksgroepen bestudeerden mucosale, CD4+, CD8+ en antilichaam immuunreacties alsook genetische factoren in HIV-1 resistente personen. De eerste en tot hiertoe enige met zekerheid bepaalde correlaat van bescherming tegen HIV-1 infectie betreft de interne 32 basepaar deletie in het *CCR5* gen. Er wordt tevens gesuggereerd dat intrinsieke cel-resistentie bij zou dragen tot HIV resistentie. In deze doctoraatsthesis wordt het fenomeen van HIV-1 resistentie bestudeerd aan de hand van vier cellulaire gastheerfactoren zijnde de co-factor LEDGF/p75 en drie restrictiefactoren APOBEC3G, TRIM5α, en tetherine. Elk van deze factoren beïnvloedt de replicatiecyclus van HIV-1 aan de hand van een eigen werkingsmechanisme via dewelke deze gastheerfactoren van belang kunnen zijn bij de ontwikkeling van nieuwe antivirale therapieën. Wij onderzochten het expressieprofiel van de gastheerfactoren LEDGF/p75, APOBEC3G, TRIM5α, en tetherine in verschillende studiepopluaties zoals frequent HIV-1-blootgestelde seronegatieven (HESN) en HIV-1-geïnfecteerde individuen.

In het eerste deel van deze thesis optimalizeerden we de methodologie waarmee de expressie van HIV-1-geassocieerde gastheereiwitten in immuuncellen bestudeerd wordt. Een indirecte intracellulaire eiwitkleuringsmethode gevolgd door flow cytometrie werd op punt gesteld om eiwitlevels van LEDGF/p75, APOBEC3G, en TRIM5α te bepalen. Fluorescent gelabelde antilichamen werden gebruikt om de specifieke gastheereiwitten te kwantificeren, alsook om tegelijkertijd meerdere celtypes van elkaar te kunnen onderscheiden. Met de flow cytometer konden we de eiwitexpressie simultaan op celniveau bestuderen in specifieke celtypes zoals CD4+ lymfocyten en CD14+ monocyten. Eens geoptimaliseerd werd de methode toegepast op een Belgische studiepopulatie van zowel gezonde als HIV-1-geïnfecteerde personen al dan niet behandeld met antiretrovirale therapie. Met behulp van deze methode slaagden we erin om differentiële expressiepatronen waar te nemen tussen celtypes alsook studiepopulaties. Daarna werd deze eiwitkleuringsmethode verder geoptimaliseerd om ook intracellulaire en extracellulaire expressie-analyses van respectie-

velijk totaal en membranair tetherine uit te kunnen voeren. Zowel de intracellulaire als extracellulaire eiwitkleuringsmethode liet de detectie van intrinsieke alsook gestimuleerde tetherine levels toe. Vervolgens werd de eiwitkleuringsprocedure aangepast ter ontwikkeling van een intracellulaire mRNA-kwantificeringsmethode. Verschillende in situ hybridizatie procedures werden gecombineerd met flow cytometrie. Gezien het grote aantal inconsistente resultaten zagen wij ons genoodzaakt om deze experimenten stop te zetten en de vaak gebruikte “real-time quantitative polymerase chain reaction (RT-qPCR) methode” voor mRNA kwantificering toe te passen.

In het tweede deel van deze thesis bepaalden we zowel de eiwit als mRNA expressie van LEDGF/p75, APOBEC3G, TRIM5α, en tetherine in een Senegalese studiepopulatie van HIV-1-discordante koppels om hun impact op HIV-1 resistentie en/of gebrek aan HIV-1 transmissie na te gaan. Naast de discordante koppels werden ook seronegative en HIV-1-geïnfecteerde concordante koppels opgenomen in de studie. We vergeleken de expressie van de vier HIV-1-geassocieerde gastheerfactoren tussen HIV-1-blootgestelde seronegatieve individuen en gezonde personen, tussen onbehandelde HIV-1-geïnfecteerde en gezonde personen, en tussen HIV-1 transmitters en niet-transmitters. In CD4+ lymfocyten van HIV-1-blootgestelde seronegatieve individuen zagen we dat een lagere LEDGF/p75 expressie een rol zou kunnen spelen bij HIV-1 resistentie. Verder zagen we een verhoogde mRNA en eiwitexpressie van de bestudeerde gastheerfactoren in onbehandelde HIV-1-geïnfecteerde individuen ten opzichte van gezonde personen. Specifiek voor tetherine zagen we verhoogde levels die positief correleerden met virale lading en T cell activatiemerkers, en negatief correleerden met het aantal CD4 cellen. Aangezien virale lading, T cell activatie en CD4 aantal merkers van HIV-1 progressie zijn, lijkt het dat de verhoogde tetherine expressie een merker voor geavanceerde HIV-1 ziekteprogressie zou kunnen zijn. Daarnaast werden HIV-1 transmitters en niet-transmitters met elkaar vergeleken om te zien of de expressie van de gastheerfactoren LEDGF/p75, APOBEC3G, TRIM5α, en tetherine mogelijks betrokken is bij HIV-1 transmissie. Onze gegevens tonen aan dat de expressie van de bestudeerde HIV-1-geassocieerde gastheerfactoren LEDGF/p75, APOBEC3G, TRIM5α, and tetherine niet betrokken zou zijn bij HIV-1 transmissie.

Veralgemenend kunnen we stellen dat we een expressie-analyse studie uitgevoerd hebben waarbij de restrictiefactoren (APOBEC3G, TRIM5α, en tetherine) geen rol bleken te spelen in HIV-1 resistentie, daar waar het tegenovergestelde bleek voor de co-factor LEDGF/p75. Bekend is dat het virus interageert met vele gastheerfactoren tijdens de virale replicatiecyclus. Technieken zoals micro-array en kwantitatieve massa spectrometrie gecombineerd met bv. SILAC of iTRAQ laten toe om tegelijkertijd de expressieprofielen van vele potentiële HIV-1-geassocieerde gastheerfactoren te bestuderen. Gedetailleerde expressieprofielen in specifieke celtypes kunnen vervolgens bestudeerd worden met de in deze thesis beschreven intracellulaire flow cytometrische eiwitkleuringsmethode. Naast expressie-analyses blijft fundamenteel onderzoek met betrekking tot HIV-1 transmissie, replicatie, latentie, en progressie naar AIDS nodig met als uiteindelijk doel een therapie te ontwikkelen die HIV-1 infectie kan voorkomen of uitroeien.

Summary

Until today, it is unclear why certain subjects remain HIV-1 seronegative even though they are frequently exposed to the HIV-1 virus. Several research groups have investigated mucosal, CD4+, CD8+, and antibody immune responses as well as genetic factors in HIV-1 resistant subjects. The first and thus far only correlate of protection defined against HIV-1 infection is the internal 32 base pair deletion in the *CCR5* gene. It is suggested that intrinsic cell resistance would also contribute to HIV-1 restriction. In this PhD-thesis, the phenomenon of resistance to HIV-1 infection is studied thereby focusing on the potential interest of four specific cellular HIV-1-related host factors being the co-factor LEDGF/p75 and three putative host restriction factors being APOBEC3G, TRIM5α, and tetherin. Each of these host factors uses specific working mechanisms to assist or to counteract the HIV-1 replication cycle which may be of relevance for the development of new antiviral therapies. We focussed specifically on the expression profile of the host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in distinct study populations including HIV-1-exposed seronegative (HESN) and HIV-1-infected individuals.

In the first part of this thesis, we optimized the methodology to study HIV-1-related host factor expression in immune cells. An intracellular protein staining method was optimized to study LEDGF/p75, APOBEC3G, and TRIM5α protein levels thereby using an indirect protein staining set-up followed by flow cytometry. Fluorescently labelled antibodies were used to quantify the host protein of interest, as well as to distinguish between different cell-types. The flow cytometer allowed us to study expression levels at the single cell level simultaneously in particular cell-types like CD4+ lymphocytes and CD14+ monocytes. In first instance, the method was applied to a Belgian study population of healthy controls and HIV-1-infected subjects (therapy-treated and untreated). The method allowed us to demonstrate differential host factor expression levels between different cell-types and study populations at single-cell level. Then, the flow cytometry-based protein staining method was further optimized to allow for intracellular as well as extracellular staining of total and membrane tetherin. Both staining procedures appeared useful in the detection of constitutively

expressed as well as stimulation-induced tetherin levels. Finally, the protein staining procedure was used as a template to develop a flow cytometry-based method to quantify intracellular mRNA instead of protein levels. Several in situ hybridization protocols were combined with flow cytometry. Due to the high number of inconsistent data, we decided to cease our efforts and to use the real-time quantitative polymerase chain reaction (RT-qPCR) method for mRNA quantification instead.

In the second part of the thesis, protein and mRNA levels of the four host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin were quantified to investigate their role in resistance to HIV-1 infection and/or lack of HIV-1 transmission in a Senegalese study population of HIV-1-discordant couples. Next to the HIV-1-discordant couples, seronegative as well as HIV-1-infected concordant couples were included in the comparative analyses. Expression patterns were investigated between HIV-1-exposed seronegative subjects and healthy controls, between untreated HIV-1-infected subjects and healthy controls, and between HIV-1 transmitters and non-transmitters. We observed significantly reduced LEDGF/p75 levels in CD4+ lymphocytes of HIV-1-exposed seronegative subjects (HESN) compared to those of healthy controls, suggesting that reduced LEDGF/p75 levels may play a role in resistance to HIV-1 infection. Overall, we observed increased host factor levels both at mRNA and protein level in untreated HIV-1-infected subjects when compared to healthy controls. Increased tetherin levels, in particular, were shown to correlate directly with viral load and T cell activation markers, and inversely with the CD4 count, suggesting that increased tetherin levels could be a marker of advanced HIV disease. Finally, we compared LEDGF/p75, APOBEC3G, TRIM5α, and tetherin expression levels between HIV-1 transmitters and non-transmitters. Our data suggest that expression levels of the HIV-1-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin are not involved in HIV-1 transmission.

In conclusion, we performed a study on the expression profile of four HIV-1-related host factors and found that the expression pattern of none of the restriction factors (APOBEC3G, TRIM5α, or tetherin) is involved in resistance to HIV-1 infection whereas the co-factor LEDGF/p75 may play a role. The virus is described to interact with many host factors during

viral replication. Today, techniques like microarray and quantitative mass spectrometry in combination with e.g. SILAC or iTRAQ can be used to investigate expression profiles of a large array of potentially involved host factors. Once “new” host factors of interest are selected, the here-discussed flow cytometry-based intracellular staining method can be applied to study detailed expression profiles in specific cell-types. In addition to expression studies, further research is required to better understand HIV-1 transmission, replication, latency, and eventual progression towards AIDS with as ultimate goal to develop a therapy that prevents or eradicates HIV-1 infection.

Chapter 1 – Introduction: Working mechanisms and therapeutic relevance of the HIV-related host factors LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin

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Reprinted from:

Mous, K., Jennes, W., Kestens, L. and Van Ostade, X. Working mechanisms and therapeutic relevance of the HIV-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and Tetherin. Review submitted for publication in *Current HIV research* 2012

1. HIV infection

Although the oldest verified case of Human Immunodeficiency Virus (HIV) infection dates back to 1959 in Congo, the Acquired Immune Deficiency Syndrome (AIDS) was first reported in the United States in 1981. The virus has caused a worldwide epidemic since then. The virus attacks the human immune system that is required to fight off bacterial and viral infections. Upon HIV infection, human immune cells get destroyed, rendering HIV seropositive people vulnerable to opportunistic infections and finally death. Despite almost 3 decades of research, an effective vaccine to prevent or to eradicate HIV infection remains elusive, making further research required.

1.1. Epidemiology

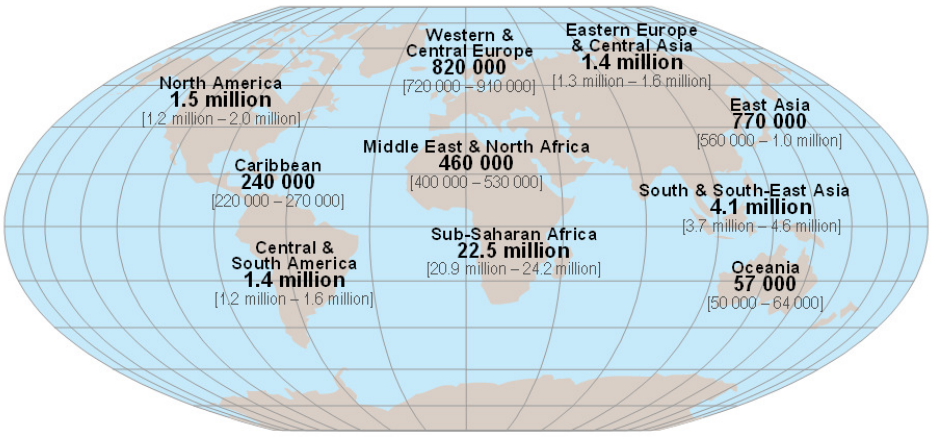

Figure 1 – Schematic overview of people living with HIV in 2009. The ranges around the estimates as depicted between the brackets define the boundaries within which the actual numbers lie, based on the best available information (UNAIDS, 2010).

Worldwide, the HIV virus has infected almost 60 million people since the beginning of the epidemic. Thus far, about 25 million people have died of HIV-related causes. In 2010, an estimated 34 million people were living with HIV, 2.7 million were newly infected, and 1.8 million died because of AIDS and/or opportunistic infections (UNAIDS, 2011; and Figure 1). Sixty-eight percent of the HIV-infected persons and about 70 % of the newly infected were living in sub-Saharan Africa.

1.2. The Human Immunodeficiency Virus

1.2.1. Classification

The RNA-virus HIV has been classified as a member of the family *Retroviridae*, genus *Lentiviruses*. At least twelve cross-species transmissions of Simian Immunodeficiency Virus (SIV) from non-human African primates to humans have been described to lead to the existence of the Human Immunodeficiency Virus (HIV). Three transmissions from the central – mainly western equatorial – African chimpanzee subspecies (*Pan troglodytes troglodytes*) led to the HIV-1 groups M, N and O (Figure 2) [1]. Recently, HIV-1 group P was added and identified to be related to the gorilla SIV [2]. For HIV-2, 8 transmissions from western African sooty mangabeys (*Cercocebus torquatus atys*) gave rise to HIV-2 groups A through H [3,4]. Both HIV-1 and HIV-2 can cause AIDS upon transmission by sexual contact, through blood, and from mother to child. HIV-2 differs from HIV-1 by the lower incidence of HIV transmission and the prolonged period prior to disease progression [5].

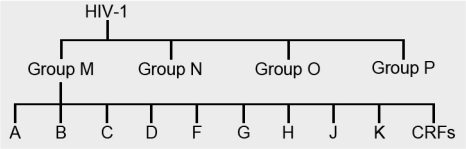

Figure 2 - HIV-1 classification.

The HIV-1 group M has been shown to account for about 90% of the infections in the HIV pandemic [3,6]. This group M can be subdivided in 9 HIV-1 subtypes labeled A, B, C, D, F, G, H, J, and K (described in Los Alamos database, http://www.hiv.lanl.gov/). Upon super-infection of one individual, two distinct subtypes may recombine. When this newly formed recombinant virus becomes a circulating strain, it is classified as a circulating recombinant form (CRF) as thus far described for at least 15 cases [7,8]. In the period 2004-2007, the subtypes mostly observed world-wide were subtype C (48%), subtype A (12%), subtype B (11%), CRF02_AG (8%), CRF01_AE (5%), subtype G (5%) and D (2%)[9]. In general, subtypes and CRFs are observed in distinct geographic regions [10]. In West and Central Africa, the most common form of HIV-1 includes the recombinant form CRF02_AG [11]. Subtype C is predominantly present in eastern and southern Africa, India, and Nepal [12], whereas subtype B occurs mainly in the western world [13].

1.2.2. HIV-1 genome

The genome of the HIV-1 virus (Figure 3) consists of two identical copies of single-stranded (+)RNA (ssRNA) molecules of about 9 kb [14]. Retrovirus synthesis occurs in multiple steps, from ssRNA to the formation and then integration of double-stranded DNA (dsDNA) in the genome of the host. Once integrated, the term provirus is used. The genes of the provirus consist of three structural (*Gag, Pol, Env*), two regulatory (*Tat, Rev*), and four accessory genes (*Vpu, Vpr, Vif, Nef*) [15].

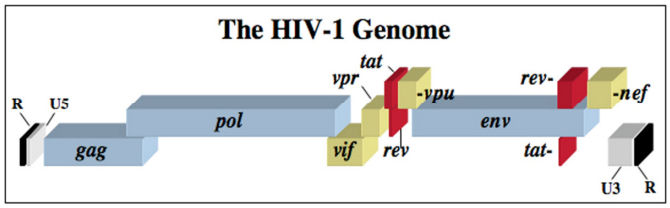

Figure 3 - HIV-1 Genome. (http:// www.mimo.unige.ch/ luban_lab/).

1.2.3. HIV-1 particle

The HIV-1 particle is structurally composed of the following components (Figure 4) [16–20]. The envelope consists of a host cell derived lipid bilayer. In the envelope, trimeric viral surface glycoproteins gp120 anchored by transmembrane gp41 are embedded next to several host membrane proteins [21]. The inner surface of the viral membrane is composed of matrix proteins (MA, p17). A core of a high number of capsid proteins (CA, p24) is located in the centre of the viral particle. This conical core enfolds the 2 viral ssRNA strains stabilized by many nucleocapsid proteins (NC, p7), as well as the enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). The virus also encapsulates the accessory proteins Nef, Vif, and Vpr; whereas Rev, Tat and Vpu appear to be expressed only in the host cell.

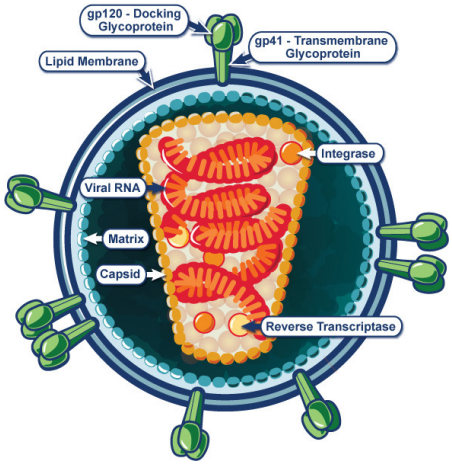

Figure 4 - HIV-1 virion. Ø100nm (NIAID).

1.2.4. Co-receptor usage

HIV preferentially targets CD4+ T lymphocytes, and cells of myeloid lineage including monocytes, macrophages and bone marrow derived dendritic cells [22]. For cell entry, HIV viruses use the CD4 receptor as well as a chemokine co-receptor. Twelve G protein-coupled seven-transmembrane chemokine receptors have been identified to function as co-receptor for HIV cell entry [23–26]; of which only two have been demonstrated to actually operate as such *in vivo* [27]. Macrophage (M)-tropic HIV-1 (R5) strains use the β-chemokine receptor CCR5 to infect macrophages, dendritic cells, brain microglial cells, and memory T cells. The α-chemokine receptor CXCR4 is used by the T-tropic HIV-1 (X4) strains to infect naïve and memory T cells, monocytes, and at lower levels mature macrophages (summarized in [28]). Dual-tropic viruses use both CCR5 and CXCR4 [29]. During transmission and early infection, most infections are predominated by R5 viruses, whereas X4 viruses are reported to emerge later in about 50% of all infections [30–33]. Recent data suggest that the molecular mechanism behind R5/X4 switches is strain specific, and that many mechanisms exist [34].

1.3. The HIV-1 replication cycle

Both viral and host cell factors contribute to the multi-step process described for the HIV-1 replication cycle (Figure 5). Fusion of the virus with the host cell is initiated by binding of viral gp120 to the host membrane protein CD4. Subsequently, structural changes make that gp120 will also interact with a co-receptor like CCR5 or CXCR4. This interaction results in membrane fusion and entry of the viral core into the host's cell cytoplasm [27,35]. Next, the uncoating process [36] of the viral core follows and the viral reverse transcriptase enzyme (RT) copies the virus' ssRNA into dsDNA [37]. Then, the viral dsDNA and integrase (IN) are transported to the nucleus as part of a pre-integration complex (PIC) [38]. Here, the IN enzyme catalyzes the integration of the viral dsDNA in the host cell genome [39]. Next, the human cell machinery transcribes viral DNA into RNA and translates the newly formed RNA into proteins [40]. After initial synthesis of the proteins Tat, Rev, and Nef; the precursor polyproteins Env, Gag, and Gag-Pol are formed.

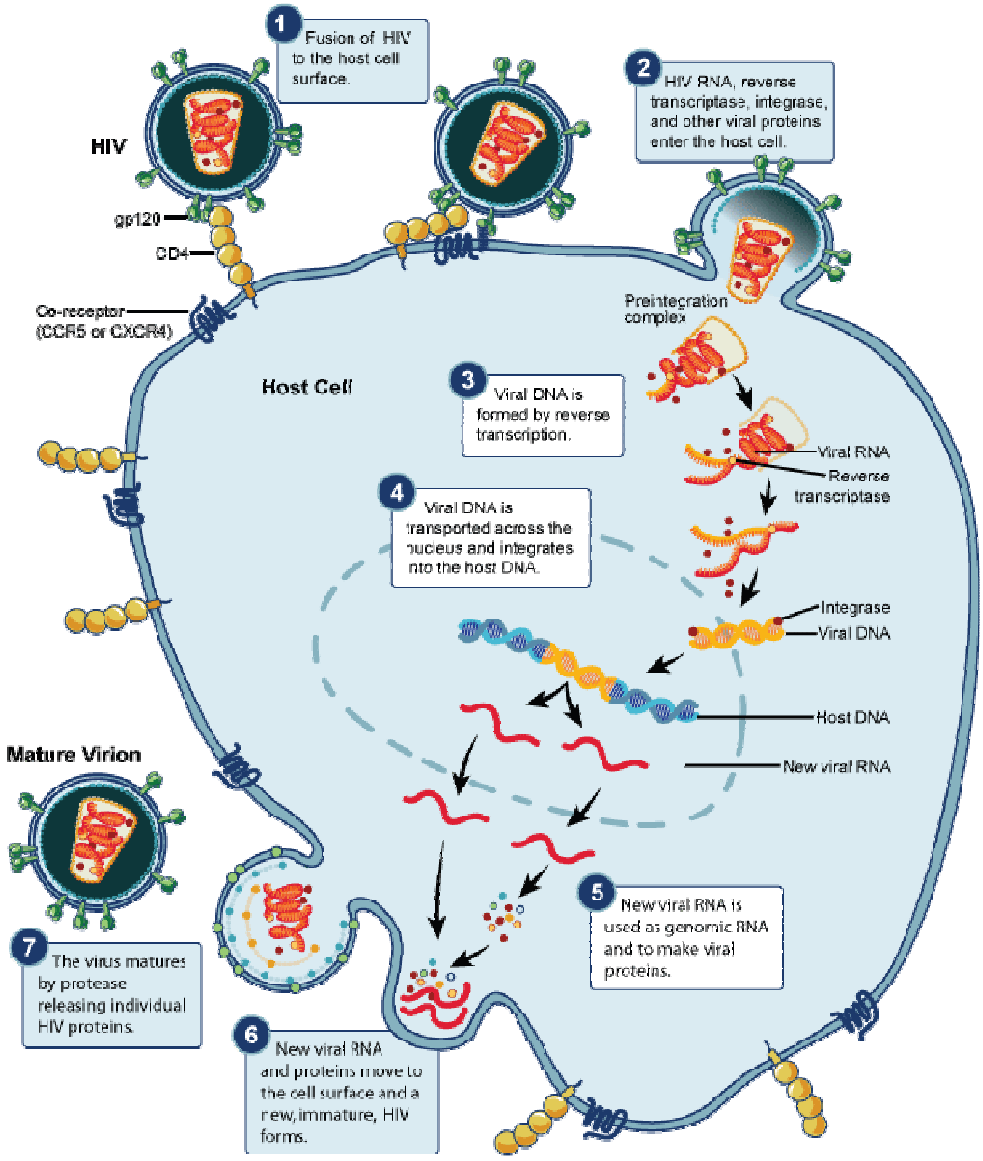

Figure 5 – Schematic overview of the HIV-1 replication cycle (Credit: NIAID).
1) Fusion of the HIV virus to the host's cell surface.
2) Release of the viral content into the host cell and uncoating of the viral capsid.
3) The viral RT enzyme reverse transcribes the two viral ssRNA copies into one viral dsDNA.
4) Integration of viral DNA into the host cell genome; the dsDNA is now referred to as provirus.
5) Synthesis of viral RNA and proteins.
6) Transportation of viral RNA and proteins to the cell surface; assembly of immature virions.
7) Maturation of the virion.

The Env polyprotein (gp160) is processed into the trimeric glycoproteins gp120 and gp41 [20]. Next, the viral RNA genome, proteins like gp120 and gp41 and both Gag and Gag-Pol precursor proteins are assembled in the immature HIV-1 virions [17,41]. Virion maturation occurs after budding when the PR enzyme cleaves the Gag and Gag-Pol polyproteins [41,42]. Cleavage of Gag results in the production of the structural proteins MA, CA, NC, and p6; whereas cleavage of the Pol portion of Gag-Pol results in the independent enzymes PR, RT, and IN [41,43].

1.4. Clinical progression to AIDS

Transmission of the HIV-1 virus may occur upon exposure to contaminated body fluids like vaginal fluids, semen, pre-ejaculate, blood, and breast milk. Ways of transmission include unprotected sexual intercourse, blood exchange, and mother-to-child transmission. Untreated HIV-1 infection undergoes three phases (Figure 6).

Once the virus has entered the body, the acute infection phase follows. During this highly infectious [44] phase in which rapid viral replication occurs, non-specific symptoms may manifest such as fever, headache, and lymphadenopathy. This acute HIV-1 infection phase is characterized by a strong increase in viral load and decrease in CD4 count. The predominant site for HIV replication is the gastrointestinal tract resulting in a massive depletion of gut-associated CD4+ T cells mainly in the first 3-6 weeks [45,46]. In response to the high number of HIV-1 virus present, the immune system activates cytotoxic CD8+ T cells [47] and produces anti-HIV-1 antibodies (seroconversion) [48]. Intervention of the immune system lowers the number of viral copies in the plasma and partly recovers the host's CD4 count. From then on, the clinical or chronic phase commences in which the virus slowly replicates and the CD4 count steadily decreases. When the CD4 count falls below 200 cells/mm^3, the patient is said to have AIDS. The host's immune system is compromised and the patient becomes vulnerable to common opportunistic infections such as Kaposi's sarcoma and tuberculosis. Eventually, death is caused by these infections.

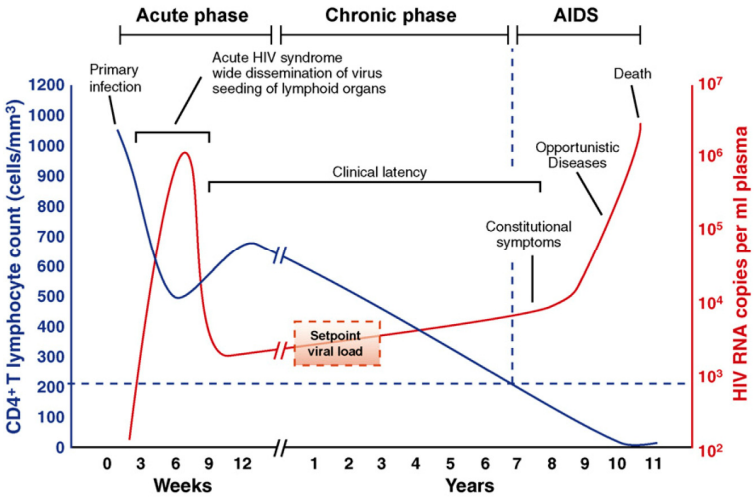

Figure 6 – HIV-1 replication phases. This graph demonstrates the phases of untreated HIV-1 infection as well as the relationship between viral load (HIV RNA copies/ml plasma, red graph) and CD4 count (cells/mm³, blue graph). (adapted from [49]).

1.5. Correlates of protection

The period between HIV-1 infection and the onset of AIDS may vary strongly. On average, the natural course of untreated HIV-1 infection covers a period of 8-10 years in typical progressors. Rapid progressors evolve to AIDS within 4 years. Long-term-non-progressors (LTNP) usually remain HIV-infected for $\geq$ 7 years with consistent low to intermediate levels of plasma viral loads and little or no loss of CD4+ cells [50,51]. They display no disease progression and account for approximately 5-15% of the HIV-1-infected subjects. Elite controllers/suppressors are a small subgroup of HIV-1-infected subjects in which viral load is undetectable with standard assays [52,53].

In general, human immune cells and/or intrinsic proteins aim to remove the HIV-1 virus during the viral replication cycle. While the HIV-1 infection progresses, the host's immune system mostly gets weakened which may lead to opportunistic infections and finally to death. Nevertheless, subjects like LTNP (see above) and HIV-1-exposed seronegatives (see below) seem capable to respectively suppress viral replication and prevent viral transmission. It is of major interest to decipher which correlates of protection are involved in these subjects, as this information may highly contribute to future vaccine development.

2. HIV-1-Exposed Seronegative individuals (HESN)

In this PhD-thesis, we will focus on those individuals who have been shown to remain seronegative despite frequent unprotected exposure to HIV-1 [54,55]. Following terms and abbreviations have been used to describe these subjects: exposed uninfected (EU), highly exposed persistently seronegative (HEPS), HIV resistant (HR), exposed seronegative (ESN) and finally, HIV-1-exposed seronegative (HESN) which has internationally been chosen to uniformly describe these individuals [56]. From hereon, we refer to them as HESN. These HESN can be categorized in 3 major groups based on their sexual exposure [57]. The first group is composed of discordant couples [58,59], which are in general monogamous couples in which the seronegative partner is regularly exposed to HIV-1 by the HIV-infected partner. Beneficial about these HESN cohorts is that larger numbers of these populations can be enrolled. In addition, as these couples move less to other areas, follow-up is more precise. On the other hand, HIV exposure may reduce over time due to e.g. counseling about condom use and antiretroviral treatment of the HIV-infected partner. While interpreting the data, such parameters should be kept in mind. A second valuable group to identify correlates of protection against sexual transmission consists of high-risk sex practitioners including commercial sex workers [60,61] and men having sex with men [62,63]. Unfortunately, a good control group that covers the effects of sex work is often hard to find. The third group encloses individuals who are non-sexually exposed as described for infants born to HIV-1-infected mothers [64,65], health care workers [66,67], intravenous drug users [68,69], and hemophiliacs [70]. These latter study populations allow for the study of systemic rather than mucosal correlates of protection in HESN.

Most likely, multiple factors [71] including innate, intrinsic and adaptive immunity contribute to the apparent protection against HIV-1 infection. Of note, interpretation of results on possible correlates of protection should, if available, take into account HIV-1 infection risk factors like therapy treatment, viral load, and the presence of other sexually transmitted diseases or genital ulcerations.

3. HIV-1 immune restriction mechanisms in HIV-1-infected and HESN subjects

In HESN, a natural protection mechanism against HIV-1 infection is assumed. It is of great interest to understand which specific immune responses are mounted, at what stage they are elicited, and what role they play in the protection against HIV-1 infection. Aside from immune responses elicited, it is hypothesized that intrinsic cell resistance to HIV-1 infection could also contribute to protection against infection in HESN [69,72,73]. The most relevant aspects of innate and adaptive immunity as well as of intrinsic cell resistance possibly involved in HIV-1 immune restriction are summarized below. Of note, HIV-1 restriction mechanisms may vary between distinct HESN cohorts as different mechanisms of resistance can be expected e.g. for commercial sex workers and infants born to HIV-1-infected mothers. Therefore, it is important to discuss data in the context of specific HESN cohorts [57].

3.1. Physical barrier

Based on the high number of HIV-infected subjects worldwide, one would think that the risk of HIV-1 acquisition upon unprotected HIV-1 exposure is high. However, upon genital HIV exposure, host factors prevent over 99% of infections. The range of transmission probability upon unsafe exposure that leads to productive HIV-1 infection is “only” 0.0001-0.0020 per act [74–79]. Both epithelial and mucosal surfaces act as barrier against HIV-1. Yet, the virus can cross these barriers and infect underlying cells especially when physical trauma or pre-existing sexually transmitted infections are present [75,80], or upon exposure to higher levels of viral load [75].

When studying the phenomenon of “HIV-1 resistance” in HESN cohorts, one should try to eliminate as many confounding factors as possible. For the HESN subjects, the HIV infection pressure should be assessed. Details should be gathered on recent sexual behavior and preferentially also on prevalence and incidence of other sexually transmitted infections (STI). If possible, information should be included about viral load and genital co-infections, as these are important sources of transmission heterogeneity [81].

In several HESN cohorts, mucosal immune responses have been associated with anti-inflammatory/regulatory immune responses [82–86], and contradictorily with increased levels of broadly pro-inflammatory immune factors [87–89]. However, one should bear in mind that some of these immune factors are not necessarily correlates of protection. Instead, they may be confounders induced by sexually transmitted infections (STI) other than HIV-1, chronic genital infections, regionally prevalent infections like malaria, and sexual activity which e.g. leads to local inflammation [81].

3.2. Innate immunity

The innate immune system provides a first line of defense against viral infection and tumor development [90]. Upon effective transmission of the HIV-1 virus across the mucosal barrier, the immune system becomes activated and cytokine/chemokine production enhanced. Innate immune cells like plasmacytoid dendritic cells (pDCs) recognize the HIV-1 ssRNA genome by the Toll-like receptors 7 and 8 (TLR7/8) [54,91], after which a cascade of cytokines (Figure 7) is produced [92–94].

The cytokine **interferon-alpha (IFNα)** is of particular interest in the innate immune system. IFNα induces expression of several IFN-stimulated genes [95,96] and proteins [97–102] of the host involved in the HIV-1 replication cycle. Type I interferon also regulates/activates both innate (e.g. NK cells, pDCs) [103,104] and adaptive (e.g. CD4+ T, CD8+ T, and B cells) immune cells [105–108]. Of note, although immune activation may play a role in the control of viral spread during acute HIV-1 infection, persistent stimulation with IFNα may also increase the number of (activated) cells at sites of infection [54,108–110]. In general, these latter cells are susceptible to HIV, thereby facilitating viral replication and disease progression during exposure, acute infection, and chronic infection [94]. Future studies should attempt to understand the critical transient immune response which is elicited e.g. in HESN subjects who are repeatedly exposed to HIV-1 infection. This is a hard task to fulfill as this immune response may have disappeared long before the HESN subject has been identified, unless repeated exposure may “reset” this critical transient innate immune response [111].

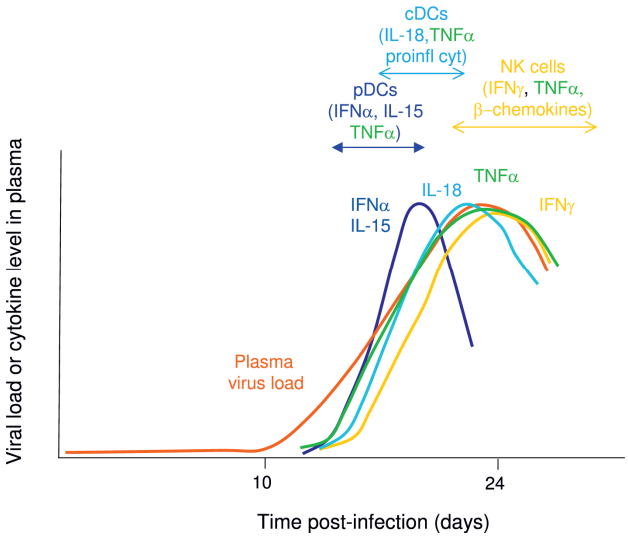

Figure 7 – Innate immunity. This diagram shows the progress in plasma viral load increase (red line) and how this is associated with cytokine and chemokine levels (colored lines) induced during acute HIV-1 infection. Also, systemic activation of pDCs, cDCs and NK cells is depicted relative to the cytokine and chemokine levels induced [92]. pDC: plasmacytoid dendritic cells, cDCs: conventional dendritic cells; NK cells: natural killer cells.

Natural killer (NK) cells also play an important role in the innate immune system. Reciprocal NK-DC interaction allows both cell types to activate one another through direct cell contact or through production of soluble factors [112]. Expansion of highly activated NK cells occurs mainly early in infection, whereas antiviral activity is progressively lost later in infection [113]. NK cells identify and destroy infected cells through cell-mediated cytotoxicity based on KIR/HLA interactions [54,114], or by the secretion of chemokines, cytokines (like IFNγ), perforins, and granzymes [115].

KIR are killer immunoglobulin-like receptors on NK cells which can be either inhibitory or activating. KIR recognize specific major histocompatibility (MHC) class I molecules [116,117]. The MHC class I molecules encode for three human leukocyte antigen proteins (HLA-A, HLA-B, and HLA-C) which are expressed on practically all normal nucleated cells [116]. In HIV-1-infected subjects, the cytolytic activity of NK cells depends on the expression of KIR on NK cells [118,119] or of HLA proteins on cells like lymphocytes [120]. In addition, both KIR and HLA molecules are highly polymorphic [116]. As a consequence, different allotypes of KIR and HLA result in different subsets of the respective cell-types. Particular NK subsets have been described to expand preferentially during acute HIV-1 infection [121,122] and/or to mediate better control of HIV-1 replication [123–126]. Also, specific KIR/HLA interactions have been

reported in the context of resistance against HIV infection [127–132]. Interestingly, the viral envelope of the transmitted virus also contains human HLA antigens from the HIV-1-infected partner, which may mount immune responses in the newly infected person [133]. This phenomenon of HLA discordance has been shown to associate with a reduced risk of HIV-1 transmission in HIV-discordant couples [134,135]. Apart from KIR/HLA interactions, enhanced frequency of NK cells and/or high IFNγ cytokine levels have been reported for HESN of discordant couples and intravascular drug users relative to HIV-1-infected subjects [136,137]. This observation supports the hypothesis that NK cells contribute to the protection against HIV-1 infection.

During infection, HIV-1 exploits at least 250 host-derived HIV dependency factors [138–140]. Several **host genetic factors** are thought to be involved in resistance against HIV-1 infection. Apart from above described KIR and HLA polymorphisms, these factors include chemokine receptors like CCR5 and CXCR4 which are required for viral entry. Mutations in these co-receptors may influence susceptibility to HIV-1 infection and/or disease progression. The internal 32 base pair deletion in the *CCR5* gene was the first correlate of protection reported [141–143], and is still the only correlate identified with certainty. In addition, overproduction of the natural ligands for CCR5 (RANTES and MIP-1α or β) and CXCR4 (SDF-1) is suggested to suppress HIV-1 entry [144]. Binding of these β-chemokines is thought to inhibit HIV-1 entry by competition or steric hindrance, by inducing down-regulation of the receptor, or by receptor dimerization disturbing the fusion process [145]. In several HESN cohorts [144,146–148], β-chemokine up-regulation has been reported although not supported by others [60,149]. Also, Missé et al [150] reported that several innate immune genes like *PRDX2* and *IL-22* would be up-regulated in activated T cells from HESN of discordant couples, and that acute-phase proteins like acute-phase serum amyloid A may contribute to HIV-1 resistance and – in the case of infection [151] – to the control of viral replication.

Recently, certain host factors were intensively investigated for their antiviral activity. They are called **intrinsic immune proteins**. The intrinsic immune system encloses host proteins which are constitutively expressed in the cell. These host proteins may exert antiviral activity

without prior activation. Often described intrinsic immune proteins involved in the HIV-1 replication cycle include the IFN-inducible host restriction factors APOBEC3G, TRIM5α, and tetherin [152,153]. In addition to host restriction factors, host cells also express co-factors. Co-factors like e.g. LEDGF/p75 are intrinsic host proteins that are used by HIV-1 during the replication cycle. The relevance of the host restriction factors APOBEC3G, TRIM5α, and tetherin as well as of the co-factor LEDGF/p75 in the light of HIV-1 infection and/or resistance is discussed more in detail in paragraph 4.

Aside from protein restriction factors, recent findings suggest similar roles played by non-coding RNAs (ncRNA) [153]. These RNAs exert RNA interference activity to "silence" viruses like HIV-1 [154–157]. However, HIV-1 sustains viral replication via its viral protein Tat which is a suppressor of RNA silencing [158–160].

3.3. Acquired immunity

During the acute phase of HIV-1 infection, virus-specific CD4+ T cell responses emerge simultaneously or prior to CD8+ T cell responses. **HIV-specific CD4+ T helper cells** are involved in the CD8+ T cell-mediated control of HIV-1 infection and induction of HIV-1-specific antibody [47]. Distinct studies report HIV-specific CD4+ T cell immune responses in different HESN cohorts [62,66,161–165], although contradicted by others [63].

Furthermore, the CD4+ T cell immune activation status may be a factor involved in cell susceptibility to HIV-1 infection [166]. Activated CD4+ lymphocytes like effector and memory T cells express higher levels of CCR5 than naïve T cells [28]. These activated cells are preferentially infected by the R5-HIV strain which is predominantly transmitted via sexual intercourse [30]. Lower levels of CD4+ T cell activation were observed in three different HESN cohorts [59,167,168], whereas no differences [169] or enhanced levels [60,170–172] were also found. The lower levels of CD4+ T cell activation were associated with reduced susceptibility to HIV-1 infection in HESN [167,168,173]. However, findings by Camara et al [59]

suggest that the lower CD4+ T cell activation levels may be associated with a higher degree of condom use by HESN subjects relative to low-risk control populations.

Early upon infection, **HIV-1-specific CD8+ T cell** immune responses are elicited. Only a limited number of these responses is described to actually suppress HIV-1 replication. Later in infection, these HIV-1-specific responses get progressively impaired [47,174]. CD8+ T cells inhibit HIV-1 replication through lysis of the infected cell upon HLA class I recognition (cytolytic mechanism) or by secretion of soluble factors including cytokines such as IFNα and β-chemokines (non-cytolytic mechanism, see above) [175].

HIV-specific cytotoxic T lymphocyte responses appear in several HESN cohorts [67,68,88,164,176–181] as well as in HIV-1-infected subjects [182,183], targeting different HIV-1 epitopes in both study groups [178]. In a HESN cohort of Kenyan sex workers, constant exposure to HIV-1 seemed to be required to maintain immunity [184]. Also, duration of the exposure was associated with the frequency and strength of the immune response [177]. Intriguingly, the CD8+ T cell response seemed lower in magnitude and breadth in certain HESN cohorts than in HIV-1-infected individuals [185,186]. Also, resistance to HIV-1 infection may require local mucosal cytotoxic T lymphocyte responses [88,187].

In addition, HIV-specific CD8+ T cells may exhibit non-cytolytic activity as observed in several HESN cohorts [188–190]. This non-cytolytic mechanism is mediated by soluble factors produced by CD8+ T cells such as CD8+ T cell antiviral factor (CAF) and certain chemokines. These chemokines may block viral entry e.g. through competition with the virus for the chemokine receptors CCR5, CCR3, and CXCR4.

In contrast to above described immune responses, several studies could not demonstrate the presence of consistent HIV-1 specific CD8+ T cell responses in different HESN cohorts [169,183,191]. Thus, it remains questionable whether CD8+ T responses should be considered a correlate of protective immunity or whether these responses induced by exposure to HIV-1 are functionally protective.

Next, **antibodies** may assist to remove free virus and to prevent virus infection. Specific antibody production happens in subsequent stages during the course of HIV-1 infection [48,192]. Primarily, non-neutralizing antibodies which are generally unable to control virus replication are formed. About 12 weeks post-infection, neutralizing antibodies emerge when the virus is now latently present. Thereafter, broadly neutralizing antibodies can be observed in a limited population of HIV-1-infected persons [193]. In different cohorts of HESN, specific anti-cell IgG [194–197] and mucosal IgA [61,85,198–202] antibodies have been associated with reduced HIV-1 acquisition, although contradicted by others [203,204]. These humoral responses may be elicited upon contact with viral antigen, free virus or HIV-infected cells from another person. These antibodies may serve a role as correlate of protection. In addition to the anti-HIV antibodies, antibodies directed towards various host proteins have been observed too [194–197,205,206]. Both anti-CD4 [194,207] and anti-CCR5 [196,197] antibodies have been detected in varying HESN cohorts.

4. Study of the cellular host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin with regard to HIV-1 infection

During the viral replication cycle, the HIV-1 virus interacts with many cellular host factors of which we selected one co-factor and three restriction factors for further investigation. The restriction factors APOBEC3G and TRIM5α were selected based on previous expression studies [73,97,100,208–210]. Whereas the latter studies focused mainly on mRNA expression profiles, we intended to study APOBEC3G and TRIM5α protein expression specifically in CD4+ lymphocytes and CD14+ monocytes of HESN, healthy control and HIV-1-infected subjects. The co-factor LEDGF/p75 was included based on the emerging interest in this protein, the therapeutic significance of the LEDGF/p75-integrase interaction and the scientific interest to include a cellular co-factor [211,212]. While optimizing the intracellular staining procedure, similar expression profile experiments were published for the restriction factor tetherin [213–217]. As little was known on tetherin expression in specific study populations, we decided to include total and membrane tetherin expression analysis in this PhD-thesis.

As is reviewed below and depicted in Figure 8, each of these cellular HIV-1-related host factors acts on different steps of the HIV-1 replication cycle. It is hypothesized that these intrinsic host factors may be involved in HIV-1 resistance. Functional capacity of HIV-1 restriction factors might be increased while that of HIV-1 co-factors might be decreased in HESN subjects as compared to control subjects. Whereas post-translational modifications or mutations like single nucleotide polymorphisms may also be involved in host factor-related restriction effectiveness, we focus in this PhD-thesis on the hypothesis that protection against HIV-1 infection by cellular host factors may be induced upon higher restriction factor or lower co-factor expression.

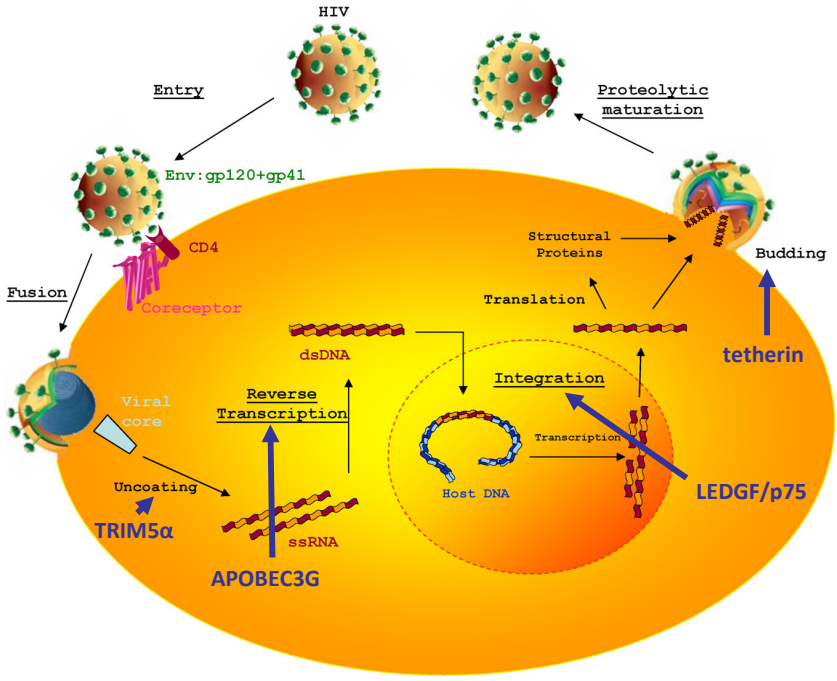

Figure 8 – HIV-1 replication in host cells. The HIV-1 replication cycle in host cells depends on a delicate balance between cellular co-factors required for virus propagation like lens epithelium-derived growth factor (LEDGF/p75) [218] and intrinsic immune factors limiting viral dissemination like apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G), tripartite motif 5alpha (TRIM5α) and tetherin (BST-2). The arrow points to the step of the viral replication cycle that is affected by the host protein of interest (adapted from [219]).

4.1. LEDGF/p75

4.1.1. Background

Over a period of 5 years, from 1998 to 2003, the lens epithelium derived growth factor (LEDGF) was described in four apparently unrelated fields being transcriptional regulation, cell survival, autoimmunity, and virology [212,220–222]. Two LEDGF splice variants with distinct coactivator properties were reported by Ge et al [220] as transcriptional coactivator p75 and p52. Both splice variants belong to the hepatoma-derived growth factor (HDGF) related protein (HRP) family [223]; and are encoded by the *PSIP1* (PC4- and SFRS-interacting protein 1) gene located on chromosome 9p22.3 [224,225]. The two proteins share the same N-terminal region of 325 residues, whereas the C-termini differ from 205 residues for p75 to only 8 residues for p52 [220,224] (Figure 9).

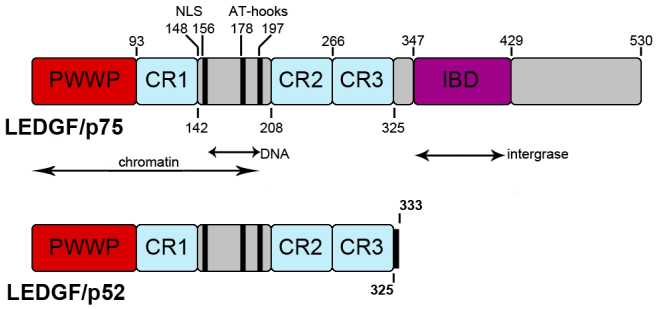

Figure 9 – Host protein LEDGF. Schematic representation of the splice variants LEDGF/p75 (above) and LEDGF/p52 (below): In between both splice variants, binding properties of specific protein domains to chromatin, DNA, and integrase are depicted (adapted from [226]).

The LEDGF/p75 protein is composed of specific domains and/or sequences (Figure 9). The nuclear localization signal (NLS) determines the nuclear distribution of the protein [227–229]. LEDGF/p75 binds to DNA via NLS and both AT hook DNA binding motifs [228,230]. In dividing cells, NLS appears dispensable due to the strong chromatin binding capacity of LEDGF/p75 [229]. The cooperative interaction of the PWWP domain with the two downstream AT hook motifs mediates the chromatin binding [230–232]. The flanking domains (CR1, CR2, and CR3) contribute to a lesser extent to the chromatin binding capacity [231]. The LEDGF/p75 integrase binding domain (IBD) interacts with several proteins including c-Myc interactor JPO2 [233,234], menin/MLL histone methyl transferase complex [235], pogZ (pogo transposable

element with ZNF domain) [236], Cdc7/ASK (Cdc7-activator of S-phase kinase) [237], and lentiviral integrase (IN) [238]. The IBD domain is important for HIV-1 integration [239,240] and for stimulation of HIV-1 IN function *in vitro* [230,238,241–244]. Recently, Tsutsui et al [245] identified a LEDGF/p75 super-coiled DNA-recognition domain involved in HIV-1 integration specifically in active transcription units.

Although the cellular functions of LEDGF/p75 remain largely uncharacterized, the protein is reported to be involved in transcriptional regulation, cell survival, viral replication and autoimmunity [212,220–222,225,246–248]. LEDGF/p75 was observed constitutively expressed in the nucleus [212,249,250]. A sub-nuclear localization study showed that the protein's distribution was cell cycle dependent exhibiting a diffuse nuclear localization during the G1-phase and cytokinesis, and a striated pattern associated with the chromosomes during the metaphase [251]. One study evidenced that LEDGF/p75 would be part of the cytoplasmic HIV-1 pre-integration complex (PIC) [249], although not confirmed by others [252]. LEDGF/p75 is a cellular co-factor involved in viral cDNA integration of lentiviruses like HIV-1 into the host's genetic material [226,239,240,253–256]. Below, we focus on the working mechanism of the HIV-1 IN protein and the contribution of LEDGF/p75 to the HIV-1 integration process.

4.1.2. Host co-factor LEDGF/p75 versus HIV-1 integrase protein

In vitro experiments show that the **HIV-1 IN protein** exerts two consecutive enzymatic functions during the HIV-1 replication cycle [257]. First, IN removes a dinucleotide from each 3' end of the viral cDNA in the cell cytoplasm, after which the viral genome is imported into the cell nucleus [38,258–260]. Next, the IN protein assists in the DNA strand transfer by cutting the host DNA in a staggered fashion, and connecting viral 3' ends of DNA to host 5' ends [261]. HIV-1 IN promotes integration of a single viral cDNA end (i.e. semi-ligation) [244,262,263]. Although the *in vivo* mechanism needs to be further elucidated, IN proteins are also shown to be involved in concerted (i.e. full-site) integration of both viral cDNA ends [242,264–266].

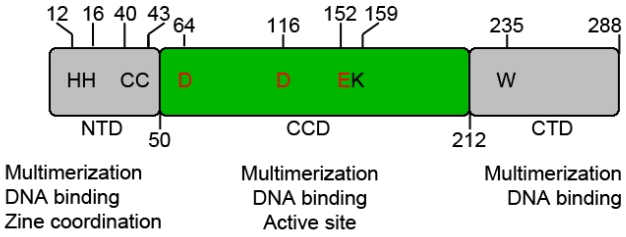

Figure 10 – HIV-1 integrase. HIV-1 integrase protein (IN) is composed of three domains: NTD (N-terminal domain), CCD (catalytic core domain) and CTD (C-terminal domain), each exerting certain functions. Amino acids conserved across the *Retroviridae* are indicated. The Aspartate (D) and Glutamate (E) residues (i.e. DDE motif), as highlighted in red in the CCD region, contribute to the 3' processing and DNA strand transfer activity of the integrase (adapted from [226]).

The IN protein is structurally composed of three domains [260,267–269] (Figure 10).

1) The **N-terminal domain (NTD)** contains a conserved zinc-binding HHCC motif [270,271] involved in folding of the NTD, multimerization of the full-length protein, and protein activity [272,273].
2) The **catalytic core domain (CCD)** is essential for the protein's enzymatic activity and contains several functional domains and residues.
    a. The catalytic DDE-motif coordinates two Mg^{2+} ions within the active site of the IN protein to allow for both enzymatic activities being 3' processing and strand transfer [270,274,275].
    b. The nuclear localization signal (NLS) mediates the nuclear import of the HIV-1 PIC [276].
    c. The KRK multimerization motif is localized at the dimer-dimer interface allowing for tetramerization [277,278].
    d. Many specific residues of the IN protein [269] are involved in interactions with the LEDGF/p75 host protein [241,250,254,279,280].
3) The **C-terminal domain (CTD)** contains a MDBD (minimum DNA-binding domain) region involved in the nonspecific binding of the IN protein with viral and cellular DNA [281–284]. Furthermore, this domain is required for HIV-1 integration and contributes to protein multimerization [283].

Structural investigation showed that separate domains of the IN protein preferentially form dimers, whereas the intact IN protein exists in an equilibrium of monomers, dimers, tetramers and higher order oligomers (Figure 11) [285,286].

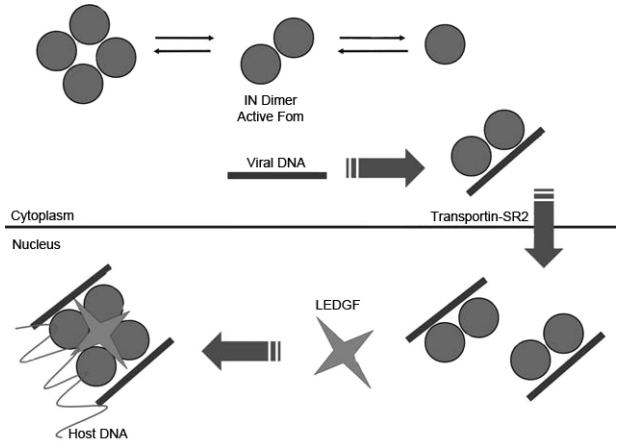

Figure 11 – Structural configuration of the HIV-1 IN protein. In the cytoplasm, equilibrium exists of IN monomers, dimers, tetramers and higher order oligomers. Upon viral DNA binding by the IN dimer, the structure is transported to the nucleus where two DNA-bound dimers interact to form a tetramer with LEDGF/p75 prior to strand transfer of viral DNA into host DNA [286].

The dimer configuration can interact with viral DNA enabling 3' processing in the cytoplasm prior to nuclear import [287,288]. In the nucleus, two DNA-bound dimers form a tetramer in the presence of the nuclear host protein LEDGF/p75 [286–289]. Upon binding of LEDGF/p75 to IN, dynamic IN subunit-subunit interactions are stabilized and tetramerization promoted [263]; and strand transfer activity of the IN protein is enhanced [238,262]. Although further elucidation is required, the IN-LEDGF/p75 complex is suggested to be a symmetrical complex of a pair of integrase tetramers and two subunits of the LEDGF/p75 protein [212]. Interaction of LEDGF/p75 with the viral IN protein [212] is suggested to shield the viral protein from proteasomal degradation [290,291].

Several reports show that specific cellular proteins affect the integration process e.g. to coordinate HIV-1 integration [212] or to repair single-strand discontinuities in the DNA intermediate to complete provirus formation [292,293]. An efficient strand transfer step requires LEDGF/p75 that tethers viral integrase to the chromosomal DNA of the host (Figure 12) [227,229–231,238,241,249,250,253,290,294,294–297].

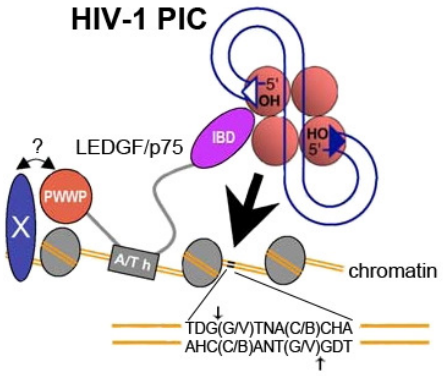

Figure 12 - Model of LEDGF/p75 tethering HIV-1 PIC (pre-integration complex) to the host's chromosomal DNA. LEDGF/p75 binds to the DNA (yellow lines) with its NLS and A/T hook motifs (grey box) and/or to chromatin proteins (nucleosome: gray oval of histone proteins; unknown chromatin factors: blue X-labelled oval) via the PWWP (orange circle) and AT-hook motifs. Binding of the LEDGF/p75 IBD-domain (purple oval) to IN (red circle, depicted as tetramer) activates IN to integrate the viral cDNA preferentially in active transcription units (black arrow) of the host's DNA (adapted from [226]).

The PWWP domain, NLS sequence and A/T hook motifs of LEDGF/p75 are involved in the chromatin tethering activity of LEDGF/p75 [227–231]. The IBD of LEDGF/p75 interacts with the NTD [250] and mainly the CCD [250,279,280] of the viral IN protein present in the HIV-1 PIC. Structural studies [241,289,296] highlight that IBD interactions with the NTD occur via charged residues [289], while a lock-and-key principle and specific residues are essential for the IBD-CCD interaction [241,296]. Mutations of the specific IBD residues I365, D366, or F406 result in disruption of the LEDGF/p75-IN binding [241] and in LEDGF/p75-mutants that lack co-factor activity [239,240,256].

Functionally, chromosomal tethering activity of LEDGF/p75 is required for efficient HIV-1 integration favoring active transcription units [212,240,245,249,250,298–300]. This statement is supported by experiments on LEDGF/p75-deficient cells which continue HIV-1 viral integration at a 10-fold reduced level [239,240] and display diminished integration in active transcription units [240,299,301,302] with palindrome-approximating symmetry [303–305]. Furthermore, these findings clarify that the LEDGF/p75 protein itself is not strictly required for HIV-1 integration [240,306]. It is assumed that HIV-1 integration can also occur independent of LEDGF/p75 e.g. into transcriptionally repressed or gene-poor regions which mainly results in latent viral reservoir formation and thus persistent clinical infection [240,307,308].

4.1.3. Therapeutic importance of LEDGF/p75-IN interactions, LEDGF/p75 expression and LEDGF/p75 polymorphisms

Current HIV therapies combine drugs targeting different steps of the HIV-1 viral life cycle. Recently, one integrase inhibitor called raltegravir was approved by the US FDA (United States Food and Drug Administration) [309]. The working mechanism of this integrase strand transfer inhibitor (InSTI) involves binding to the integrase-viral DNA complex, and interaction with the two magnesium ions in the active site of the integrase enzyme [310,311].

Several studies highlight the interest to **disrupt integrase oligomerization** for drug development [263]. Molteni et al [312] described two small molecules that bind at the IN dimer interface as possible integrase inhibitors. Al-Mawsawi et al [313] mentioned a coumarin derivative that binds the 128-136 region of HIV-1 IN and displays integrase inhibitory activity through disruption of IN oligomerization and of LEDGF/p75-IN interaction. Hayouka et al [314] used synthesized peptides derived from LEDGF/p75. These peptides were reported to shift the IN oligomerization pattern from the active dimer toward the inactive tetramer [314], which is unable to catalyze the 3' processing of the viral cDNA [287,288]. Of note, resistant virus strains may develop rapidly when a therapeutic strategy is used that focuses solely on viral proteins. Therefore, as the cellular environment appears of crucial importance for (retro)viral replication, one should attempt to include cellular proteins like the LEDGF/p75 co-factor as potential therapeutic targets [246,315].

Today, full-length crystal structures of viral integrase and human LEDGF/p75 have not been elucidated yet. However, structural information exists on the interaction site of LEDGF/p75 and IN (Figure 13). Although protein-protein interactions are generally more challenging to target for drug development than enzyme active sites or receptor binding pockets [316], the IBD-CCD interaction is of interest for small molecule therapy [296,312]. Several attempts have been undertaken to **interfere with the LEDGF/p75-IN interaction** [317]. Brin et al [211] mentions a particular peptide that binds to certain IBD contact residues thereby preventing LEDGF/p75 from binding to IN. Du et al [318] performed a small-scale screening for small

molecules to reduce LEDGF/p75-IN binding and found D77 as possible therapeutic molecule. Hou et al [319] observed about 90 compounds with the capacity to inhibit LEDGF/p75-IN binding; further testing is required. Recently, Christ et al [320] designed a series of 2-(quinolin-3-yl) acetic acid derivatives (LEDGINs) that are allosteric inhibitors of the LEDGF/p75-IN binding site. Schrijvers et al [306] added that LEDGINs also interfere with HRP-2 (Hepatoma-derived growth factor related protein 2) which is the only other human protein that contains an IBD and mediates residual replication upon LEDGF/p75 depletion.

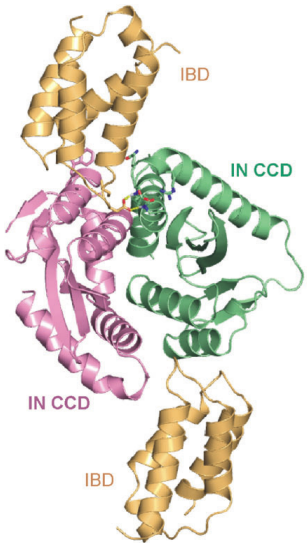

Figure 13 – Crystal structure of the LEDGF/p75-IN interaction site. The IBD of LEDGF/p75 is shown to interact with the CCD of two viral IN proteins via insertion of an IBD inter-helical loop (including hotspot residues like I356, D366 and F406) into a defined pocket at the interface of two CCDs of the IN dimer [296,315,321].

Another approach to abrogate viral replication involves **LEDGF/p75 expression levels** which may influence HIV-1 integration. Several parameters are described to alter LEDGF/p75 expression levels including oxidative and thermal stress [322–324], hormones like LH (gonadotropic luteinizing hormone) and FSH (follicle-stimulating hormone) [325], tumor necrosis factor-alpha (TNF-α) [326], transcription factors like Sp1 [327] and post-translational modification like SUMOylation [328] and acetylation [329]. Recently, Madlala et al [330] demonstrated that LEDGF/p75 mRNA levels were higher in seroconverters prior to HIV-1 infection suggesting that higher LEDGF/p75 levels may enhance HIV-1 susceptibility. In un-manipulated cells, cell-type specific levels of LEDGF/p75 were shown to correlate linearly with the frequency of HIV-1 integration in active transcription units [299]. Several LEDGF/p75 knock-down studies resulted in unaffected or modestly reduced levels of HIV-1 integration [249,302,331,332]. Deeper analysis demonstrated that cells remained fully HIV-1 susceptible as long as more than 3% of baseline LEDGF/p75 levels remained expressed [239]. These findings further suggested the existence of a cellular pool as well as a chromatin-bound

fraction of functionally active LEDGF/p75. Thus, as was further evidenced by LEDGF/p75 knock-out experiments, LEDGF/p75 needs to be fully removed to counteract efficient HIV-1 integration in active transcription units [240,299]. Therefore, as complete removal of total LEDGF/p75 levels is required to prevent HIV-1 integration, gene therapy to knock-down LEDGF/p75 is unlikely to be a sufficiently effective antiviral strategy [247,333].

Alternatively, gene therapy to induce **over-expression of IBD proteins** is of interest as reduced viral replication could be demonstrated both *in vitro* [239,254,334] and *in vivo* [333]. Passage of HIV-1 in cells expressing abundant levels of IBD proteins resulted in virus strains with reduced replication efficiency expressing adaptive escape mutations in the IN dimer interface [254]. Furthermore, the combination of LEDGF/p75 knock-down and over-expression of IBD expression synergistically inhibited viral replication [239]. Two working mechanisms for IBD protein over-expression may exist. On the one hand, IBD proteins may compete with endogenous LEDGF/p75 protein levels for IN binding and abrogate HIV-1 integration [334]. On the other hand, it has been hypothesized that LEDGF/p75 over-expression may induce a conformational change in the cytoplasm from the active IN dimer to the inactive tetramer [314,335,336].

Recently, two research groups demonstrated the interest to investigate LEDGF/p75 **gene polymorphisms**. Madlala et al [330] observed that particular polymorphisms are associated with susceptibility to HIV-1 infection and disease progression. SNP rs12339417 was associated with lower LEDGF/p75 mRNA levels and with a slower decline in CD4 count. Furthermore, Ballana et al [337] described rare LEDGF/p75 genetic variants in white long-term non-progressors (LTNP). Both studies provide *in vivo* evidence that further research on LEDGF/p75 genetic variation and expression levels in particular study populations is of interest.

In conclusion, further investigation is required to address the full potential of the LEDGF/p75 host factor and/or the LEDGF/p75-IN interaction in future drug development strategies.

4.2. APOBEC3G

4.2.1. Background

APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G) was first identified by Sheehy et al [338] as CEM15. This protein is part of the APOBEC family of cytidine deaminases (Figure 14) [339]. Each family member contains a short α-helical domain, a cytidine deaminase (CDA) domain, a linker region, and a pseudo-active site. In APOBEC3B, APOBEC3DE, APOBEC3F, and APOBEC3G, these 4 domains are present in duplicate.

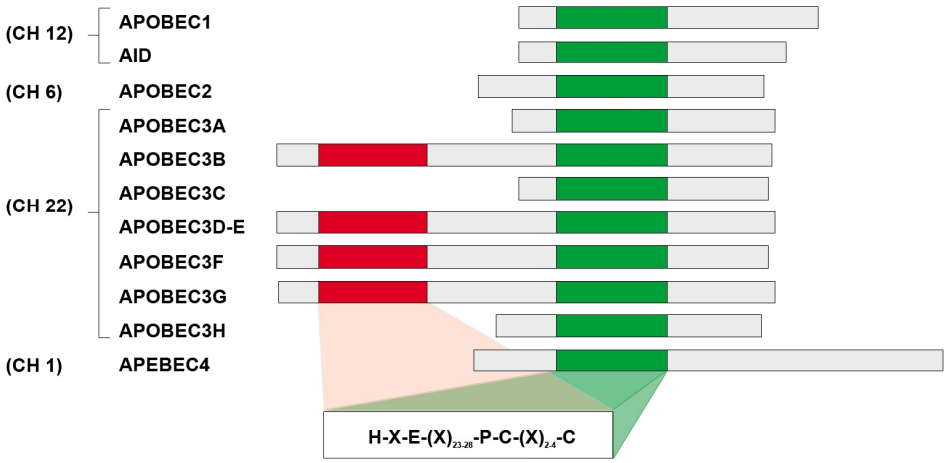

Figure 14 – Members of the human APOBEC protein family. Chromosomal localization is mentioned on the left (between brackets). Catalytically active cytidine deaminase domains (CDA) are depicted in green and inactive CDA domains which are involved in RNA binding and encapsidation in red. The consensus sequence for the CDA domains is highlighted at the bottom of the figure (adapted from [340]).

The APOBEC3 gene family, present on chromosome 22, exists of seven genes evolved by tandem duplication [341]. The expansion of this gene family is most likely driven by evolutionary pressures to defend against genomic assaults by a broad spectrum of retrovirus-like parasites [342–345]. Both human APOBEC3G and HIV-1 viral infectivity factor (Vif) have been described to evolve rapidly by positive selection [345–347]. As mentioned above, APOBEC3 proteins contain either one or two cytidine deaminase domains (CDAs). For each CDA domain, a consensus sequence of His-Xaa-Glu-Xaa_{23-28}-Pro-Cys-Xaa_{2-4}-Cys was observed

[341]. At this conserved motif, cytidine deaminase activity is exerted by 2 cysteines and 1 histidine which coordinate a zinc ion and water molecule, and one glutamate. The glutamate contributes to the proton shuttle during the deamination reaction (Figure 15), after which a uridine ring is formed [341,348–350].

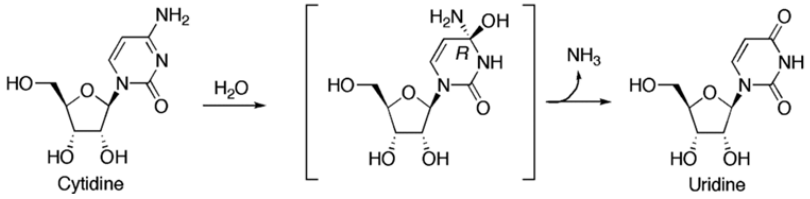

Figure 15 - Cytidine deaminase reaction resulting in uridine.

The APOBEC3G protein has two CDA domains, one catalytically active C-terminal and one inactive N-terminal domain [351,352]. The C-terminal domain of APOBEC3G preferentially deaminates multiple C-residues in a CCC motif on one single viral ssDNA-strain (single-stranded DNA) during reverse transcription [353–355]. This phenomenon occurs with a 3' to 5' polarity [356,357]. These multiple deamination events finally result in G-to-A hypermutation of the viral genome. Of note, APOBEC3G selectively targets HIV-1 minus-ssDNA, although it can also bind dsDNA (double-stranded DNA), ssRNA or dsRNA [340]. The catalytically inactive domain is involved in nucleic acid binding, as well as in interaction with HIV-1 Gag, virus encapsidation and dimerization [351,352,358,359]. Formation of homo- and heterodimers has been reported for certain family members including APOBEC3G and APOBEC3F [360,361].

APOBEC3G is observed in the cell cytoplasm of different cell-types [362], probably due to a cytoplasmic retention signal located at the N-terminus of the protein [363,364]. APOBEC3G was found in P-bodies [365–368], and is shown to redistribute into stress granules upon exposure to stress signals [340,365]. Both P-bodies and stress granules are thought to be part of the high molecular mass (HMM) APOBEC3G complex [340], possibly explaining the rather constant expression of APOBEC3G in the cytoplasm of non-stimulated peripheral blood mononuclear cells (PBMC).

4.2.2. Host restriction factor APOBEC3G versus HIV-1 Vif protein

The human APOBEC3G protein was first identified as the host factor inhibiting replication of Vif-deficient HIV-1 viruses in non-permissive cells like primary T cells, macrophages, and CEM cell lines [338]. Several antiviral working mechanisms are suggested for the **APOBEC3G protein**. The most often described mechanism involves hypermutation of the viral HIV genome during viral reverse transcription via the APOBEC3G active CDA domain [354,355,369]. This action occurs upon infection with a virion carrying APOBEC3G of the previously infected cell. No more than 7 copies of such transported APOBEC3G proteins appear sufficient to restrict HIV-1 infection [370]. This deaminase-dependent antiviral mechanism (Figure 16) may lead to the formation of mutated viral ssDNA which is prone to endonuclease activity, impaired plus-strand DNA synthesis, and formation of non-functional viral genes [219]. In addition, as also shown in the figure, APOBEC3G may exert antiviral activity in a deaminase-independent manner by inhibiting reverse transcription, by reducing the ability of $tRNA^{Lys3}$ primers to initiate reverse transcription [371], and/or by causing defects in $tRNA^{Lys3}$ cleavage during plus-strand DNA transfer resulting in aberrant viral DNA ends and difficulties in chromosomal integration [372]. Thus far, normal cellular functions for APOBEC3G other than suppression of retrotransposition of endogenous retroelements [373] and above discussed antiviral capacities which may result in viral sequence variation and indirectly in antibody diversification [374] are unknown [375].

The inactive CDA domain assists APOBEC3G in binding to RNA and in the process of encapsidation. The RNA binding property appears also to be involved in the formation of enzymatically inactive high molecular mass (HMM) APOBEC3G ribonucleoprotein complexes which contain at least 95 different proteins [365,366,373]. These complexes were predominantly found in activated CD4+ T lymphocytes, lymphoid residing resting CD4+ T cells, macrophages and CD16+ monocytes, whereas the enzymatically active low molecular mass (LMM) APOBEC3G was observed in circulating resting CD4+ T lymphocytes and monocytes [376–378]. Upon stimulation of particular cell-types with endogenous factors like interferon

and IL-2, APOBEC3G can shift from the enzymatically active LMM to the inactive HMM APOBEC3G form [98,210,378].

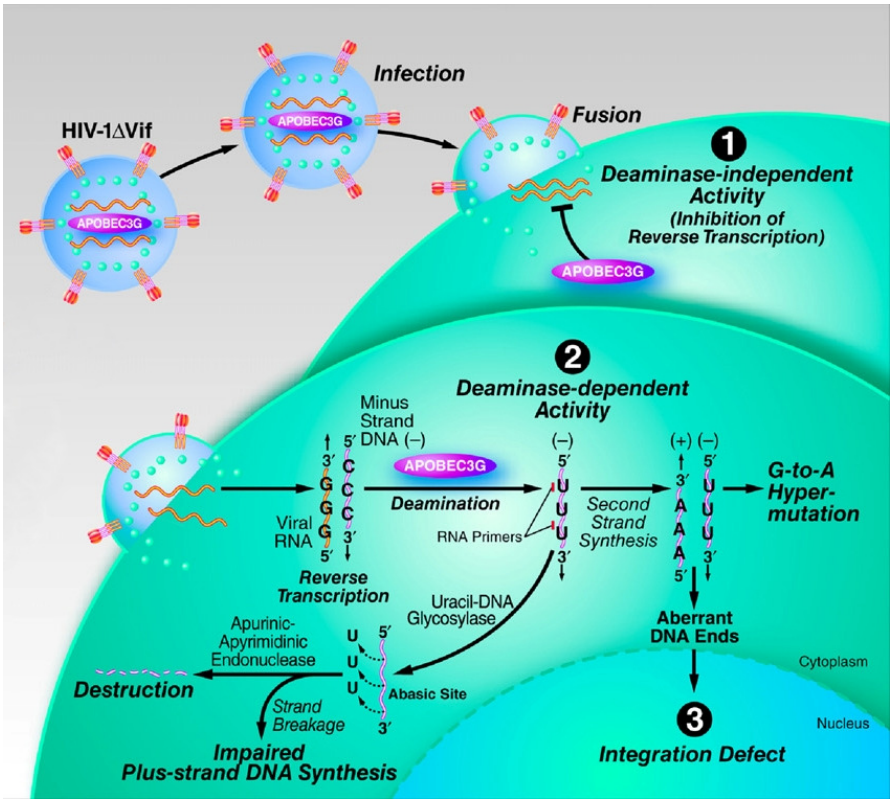

Figure 16 – Antiviral activity mechanisms of APOBEC3G in the absence of Vif.
Once APOBEC3G is incorporated in the transmitted HIV-1 virion:
1) APOBEC3G affects the efficiency of reverse transcription, possibly due to steric hindrance by binding to the RNA and ssDNA templates.
2) APOBEC3G induces G-to-A hypermutations in the proviral DNA.
3) The mutated DNA leads to nonfunctional viral genes, and APOBEC3G might induce defects in tRNALys3 cleavage leading to aberrant DNA ends and integration defects.
(adapted from [219,379])

The **viral infectivity factor (Vif)** is shown to counteract the antiviral activity of APOBEC3G by preventing incorporation of APOBEC3G into newly formed virions through different mechanisms (Figure 17). Primarily, Vif reduces APOBEC3G protein levels through proteasomal degradation [340,380–384]. Next, Vif partially impairs translation of APOBEC3G mRNA into

proteins [383]. Finally, Vif prevents APOBEC3G encapsidation via competition for APOBEC3G binding sites to viral components like the nucleocapsid region of Gag polyprotein or viral genomic RNA during viral assembly/budding [379,385–389]; and by promoting transition of enzymatically active LMM APOBEC3G into the enzymatically inactive HMM form [390].

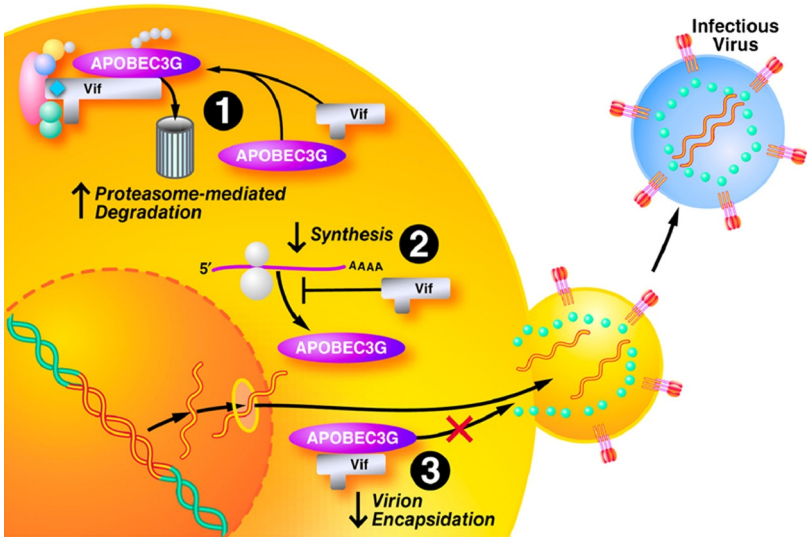

Figure 17 - Mechanisms by which HIV-1 Vif interferes with cellular APOBEC3G.
1) Vif interacts with APOBEC3G resulting in proteasomal degradation.
2) Vif interferes with mRNA translation of APOBEC3G into protein.
3) Vif prevents APOBEC3G encapsidation into newly formed virions. (adapted from [219,379])

Vif is thought to circumvent encapsidation especially of the enzymatically active LMM APOBEC3G [389]. In case LMM APOBEC3G does become encapsidated, the protein seems to assemble in large intra-virion complexes and to lose its antiviral activity through interaction with HIV genomic RNA. The antiviral activity of the APOBEC3G protein is regained during HIV-1 reverse transcription in the newly infected host cell through the action of RNase H which generates viral minus-strand DNA and removes inhibitory RNA bound to APOBEC3G [379,389].

4.2.3. Therapeutic importance of APOBEC3G-Vif interactions, APOBEC3G expression and APOBEC3G polymorphisms

Today, HIV therapy does not integrate the potential of host APOBEC3G protein. Below, we summarize the interest of the APOBEC3G protein in future HIV therapy development.

One possible approach to preserve APOBEC3G antiviral activity is to **interfere with the APOBEC3G-Vif complex** (Figure 18). Therapeutic strategies that may attack described interactions include small-molecule inhibitors and gene therapy [219,340,375,391]. Currently, structural information on full-length APOBEC3G and Vif is not available [375]. Nevertheless, several interaction sites of APOBEC3G and Vif observed through e.g. mutational analyses are considered of interest in the search for small-molecule inhibitors. For APOBEC3G, the DPD motif (amino acids 128-130) is involved in binding to Vif, whereas R122 and W127 are required for efficient encapsidation [392–395]. For Vif, the residues 33 to 88 which contain the YRHHY motif (residues 40 to 44) are critical for interaction with APOBEC3G [396,397]. Recently, Nathans et al [398] identified a small molecule, RN-18, that interferes with Vif function and targets the Vif-APOBEC3G axis. However, further investigation is required to better understand the exact working mechanism of the compound. Protein kinase A (PKA) was shown to reduce Vif-APOBEC3G binding affinity upon phosphorylation of APOBEC3G thereby preventing APOBEC3G from ubiquitinylation and degradation [399] but also suppressing the intrinsic DNA deaminase activity of APOBEC3G [400]. The latter observation suggests that phosphorylation of APOBEC3G may not be the preferred approach to prevent APOBEC3G from binding to Vif.

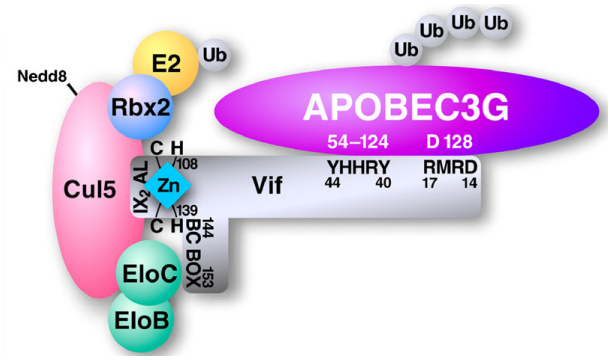

Figure 18 – APOBEC3G-Vif-ubiquitin ligase complex. Interaction of Vif with APOBEC3G results in recruitment of the ubiquitin ligase complex to Vif and efficient ubiquitination of APOBEC3G. A couple of functional motifs of Vif important for interactions with APOBEC3G and the ubiquitin ligase complex are highlighted [219].

Alternative to disruption of the APOBEC3G-Vif interaction, one can also focus on **the interface of the Vif-ubiquitin ligase complex** (Figure 18) [401] or on **the CBF-β-Vif interface** [384]. However, both latter approaches may lead to uncertain effects on further HIV-1 replication and/or APOBEC3G antiviral events as Vif may remain bound to APOBEC3G, resulting in co-packaging in newly formed virions [219]. Moreover, to prevent Vif activity, one may consider **disrupting the Vif dimerization site** (residues 156 to 164) [402–404]. This disruption has been shown to also result in enhanced encapsidation of APOBEC3G [403].

Apart from targeting APOBEC3G-Vif, Vif-Ubiquitin ligase complex, the CBF-β-Vif interface, and/or Vif-Vif interactions, one may consider **to target host APOBEC3G or viral Vif expression levels.**

Gene therapy [391,405] may be a valuable approach **to prevent degradation of APOBEC3G**. Studies on APOBEC3G proteins derived from other species like e.g. African green monkey demonstrated that the D128K substitution in human APOBEC3G may protect this host protein from Vif binding [392–395]. Thus, one strategy may be to alter Vif-binding sites in APOBEC3G. An alternative focus may be to fuse APOBEC3G to a stabilization signal like UBA-2 (ubiquitin-associated domain 2 [406]) that protects APOBEC3G from degradation. Also, gene therapy may be of interest to selectively direct APOBEC3G to HIV-1 virions. Different study groups showed that fusion of APOBEC3 proteins to viral peptides enhanced incorporation of these APOBEC3 proteins into virions [407–409]. Elimination of the Vif-sensitive N-terminal domain of APOBEC3G and subsequent fusion to Vpr was sufficient for APOBEC3G encapsidation [408]. Fusion of APOBEC3G to the mutant derivative Nef7 of HIV-1 also resulted in elevated APOBEC3G-mediated restriction [409]. Of note, further improvements on safety and efficacy of gene delivery and cell therapy are required prior to application of above described gene therapeutic strategies.

Next, although further investigation on effects of increased levels of APOBEC3G on the normal physiological state of the organism is warranted [375,391,410], **boosting cellular APOBEC3G levels** appears a valuable therapeutic approach [340].

APOBEC3G mRNA levels were demonstrated to correlate positively with CD4 count and negatively with viremia in therapy-naïve HIV-1-infected subjects [209,411,412]. These data suggest that APOBEC3G would aid in the control of HIV-1 infection, although other studies do not support these observations [208,413,414]. In addition, APOBEC3G expression was demonstrated to be enhanced in HIV-1-exposed seronegative (HESN) subjects compared to healthy controls, which further hints to the antiviral function of APOBEC3G [73,411]. However, we and others did not observe a difference in APOBEC3G expression between HESN and HC [414] (see also Chapter 6 and [415]). Of interest, Xu et al [370] described that the amount of APOBEC3G incorporated in the newly formed virion is proportional to its expression level in the virus producing cell. In addition, they concluded that a limited number of APOBEC3G proteins would be sufficient to restrict HIV-1 infection. Thus, these observations support the idea that APOBEC3G expression levels may contribute to HIV-1 restriction.

Apart from expression studies in different study populations, studies in distinct cell-types were also conducted. One study found higher levels of APOBEC3G in the Th1 subset of activated CD4+ T cells [416]. The authors reported that augmented expression would result in higher levels of APOBEC3G encapsidation and finally in lower viral infectivity. Another study links higher expression of APOBEC3G to the relative resistance of monocytes against HIV-1 infection when compared to differentiated macrophages [417]. In addition, upregulation of APOBEC3 proteins by IFNα, and to a lesser extent, IFNγ has been associated with increased resistance to HIV-1 infection [100,417,418].

Based on above observations, a possible pharmaceutical approach to increase APOBEC3G protein levels could involve cell stimulation via interferon-alpha treatment as described amongst others by Peng et al [100]. However, as IFNα influences many cellular mechanisms independent of APOBEC3G, further research is mandatory to obtain a method that can selectively increase APOBEC3G expression.

In addition, apart from expression studies, different human **APOBEC3G polymorphism** studies have been performed. However, until now, knowledge on the relevance of such

polymorphisms remains limited [391]. One group described that the C40693T allele in intron 4 was associated with an increased risk of infection [419]. In another cohort, a H186R mutation correlated with CD4+ cell depletion and rapid progression to AIDS-defining conditions [420], a finding that could be confirmed by our laboratory (unpublished data). As the frequency of this allele is higher in African than in European or American subjects [420], this observation suggests that African subjects may be more susceptible to faster disease progression. Moreover, based on simian APOBEC3G, substitution of D128K in human APOBEC3G is shown to render APOBEC3G resistant to Vif-mediated restriction [421]. Above results in combination with the positive selection under which APOBEC3 proteins have evolved give reason to believe that polymorphisms may affect HIV-1 replication *in vivo* [391]. Therefore, further research is warranted.

4.3. TRIM5alpha

4.3.1. Background

The TRIM5α (tripartite motif 5 alpha) protein is considered to be a potential intrinsic immunity protein as anti-HIV-1 activity was shown in many primate species [422–427], cows [428,429] and rabbits [430] due to TRIM5α orthologues. In the human genome, more than 70 tripartite motif genes of the TRIM family are described. TRIM family members are involved in various cellular processes like cell cycle regulation, apoptosis, viral response, and innate immunity [431–435]. The majority of TRIM genes is scattered throughout the genome, except for two clusters located in the HLA region on chromosome 6p21-23 (TRIM10, 15, 26, 27 and 31) and 11p15 (TRIM5, 6, 21, 22, 34 and TRIMψ) [436]. Six different isoforms of TRIM5 (TRIM5α, β, γ, δ, ε and ζ) have been described in mammals [410,436,437].

Each TRIM family member expresses a TRIM or RBCC motif in front of a variable C-terminal domain (Figure 19) [432]. The RBCC motif is composed of a RING (really interesting new gene) domain, one or two B-boxes, and a CC (coiled-coil) domain. These domains are arranged in this typical order from N- to C-terminus. The RING finger domain exerts E3 ubiquitin ligase

activity both *in vitro* and *in vivo* [438–440], including auto-ubiquitination. Previous reports describe that the RING domain would be important – but not essential – in TRIM5-mediated restriction. This apparent contradiction may be explained by its involvement in different processes of reverse transcription and nuclear import [441–443]. The BCC region of TRIM5α, composed of a B-box2 and coiled-coil domain, promotes homo-multimerization and is required for anti-HIV-1 restriction activity [436,444,445].

In addition to the RBCC motif, TRIM5α specifically expresses the C-terminal (PRY-)SPRY or B30.2 domain [446] which exerts weak viral capsid (CA-)binding activity [447–449]. Human TRIM5α exerts antiviral activity against retroviruses [422,450] like N-tropic murine leukemia virus (N-MLV), but has insufficient activity against HIV-1 as opposed to e.g. rhesus macaque TRIM5α [426]. Observed species-specificity of TRIM5α for retroviruses appears dependent on specific amino acid changes in the coiled-coil and more prominent in the PRY-SPRY domains [451–454]. Of particular interest are the residues 328, 330, and 332 as exemplified by the restriction activity of the single amino acid substitution R332P in human TRIM5α analogous to rhesus macaque and African green monkey TRIM5α [452,453].

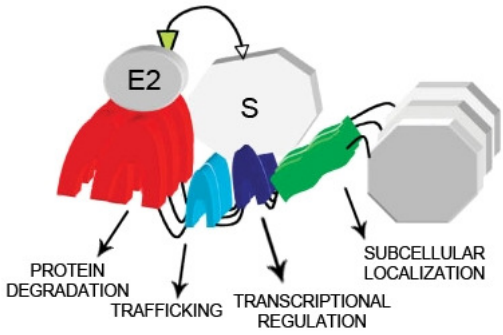

Figure 19 – Schematic ubiquitination model of TRIM proteins. A homo-trimer of TRIM/RBCC protein is depicted defined by the typical N-terminal RING (red), B-box1 (light blue) and/or B-box2 (dark blue) and coiled-coil domain (green) followed by a variable C-terminal domain (grey) with each domain having its own functionality. The ubiquitin-conjugating enzyme (E2) binds to the RING domain and the substrate (S) to the pocket formed by the BCC domain, after which the ubiquitin (or ubiquitin-like) molecule (light green triangle) is transferred from the E2 protein to the substrate (adapted from [432]).

Based on *in vitro* experiments, TRIM5α protein localization is described in mobile, highly dynamic cytoplasmic bodies [426,455]. Moreover, the protein appears to move rapidly between different bodies and cell cytoplasm. In transfected cell lines, low levels of TRIM5α have been observed diffusely expressed across the cell cytoplasm and in fewer and smaller

cytoplasmic bodies [455]. Based on this latter observation as well as on our confocal images visualizing endogenous TRIM5α (see Chapter 3 and [456]), we conclude that TRIM5α proteins may have a predominant diffuse cytoplasmic distribution *in vivo*.

4.3.2. Host restriction factor TRIM5α versus HIV-1 capsid proteins

Thus far, it is unknown how TRIM5α exerts its retroviral restriction activity. Different mechanisms have been proposed, which are summarized below.

Upon infection, the C-terminal domain of TRIM5α is shown to interact specifically with certain retroviral capsids [447,449,452,453,457,458]. In 2005, Mische et al [459] described how trimeric TRIM5α protein may bind to HIV-1 capsid hexamers (Figure 20A). Now, 6 years later, the group of Ganser-Pornillos et al [460] suggests a hexagonal assembly of TRIM5α dimer proteins (Figure 20B-C) in HIV-1 restriction. Upon interaction of host TRIM5α with viral capsid proteins, premature disassembly of the incoming viral capsid is induced and subsequent transportation of the viral DNA towards the nucleus is disturbed [447,449,458,461,462]. In humans, the interaction of TRIM5α with viral CA-proteins is insufficient to restrict infection [152,463].

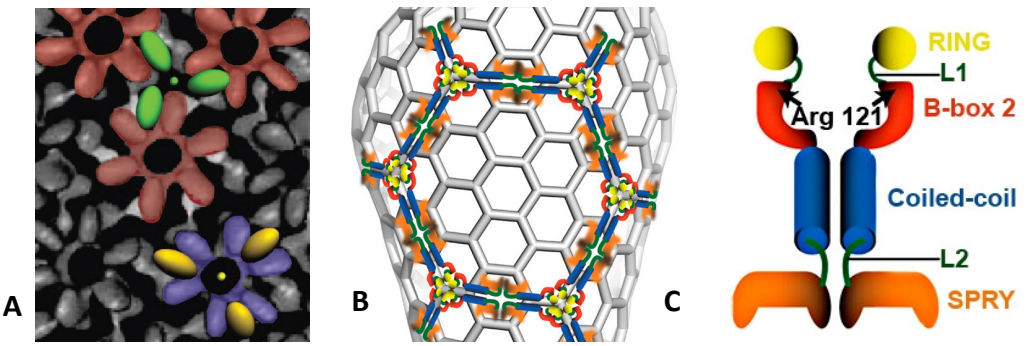

Figure 20 – Models of TRIM5α protein interaction with HIV-1 capsid proteins. (A) Hexameric HIV-1 capsid proteins (grey, purple, and red) are depicted as reported by Li et al [464]. Two possible binding modes of trimer TRIM5α protein are displayed (yellow, and green) (adapted from [459]). (B) Speculation on how TRIM5α (dimers, hexagonally assembled) interacts with the HIV-1 capsid (grey). (C) Schematic representation of a TRIM5α dimer, the suggested building block of the hexagonal assembly in (B) (Both B and C were adapted from [460]).

The coiled-coil domain [438,458,459,465–468] as well as a hydrophobic patch and the arginine residue 121 in the B-box2 domain [445,469] are thought to mediate TRIM5α multimerization, which appears essential for inhibition. Also, the N-terminal RING finger domain is described to enhance the potency with which TRIM5α inhibits infection, although the exact mechanism remains to be elucidated [457,470]. One hypothesis considers the E3 ligase activity of the TRIM5α RING domain which is involved in (auto)ubiquitination and subsequent proteasomal degradation of the capsid protein [440,471,472]. Although proteasome activity was described to assist in the destruction of HIV-1 capsid proteins [473–475], the only loss of protein observed during viral restriction was of TRIM5α itself [475]. Several groups observed that TRIM5α-mediated restriction does not affect the total amount of any of the viral proteins [476,477] including cytoplasmic capsid proteins [424,449,477–480], and occurs independently of the proteasome system [442,449,461,473,475,481,482].

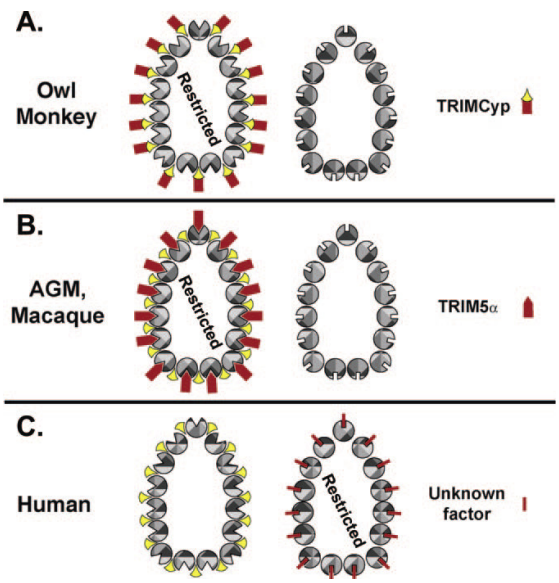

Figure 21 – Hypothesis on the species-specific effects of CypA on HIV-1 restriction. (A) The fusion protein TRIMCyp is expressed in owl monkeys, and acts by binding HIV-1 CA in *cis* to restrict HIV-1. (B) Both CypA and TRIM5α are expressed non-fused in African green monkeys (AGM) or macaques. In these species, CypA acts in *trans* to alter the conformation of HIV-1 CA so that the HIV-1 virion core is more susceptible to TRIM5α-mediated recognition and restriction. (C) In human cells, CypA seems to protect HIV-1 from an unknown restriction factor. The CypA action is considered similar to the action observed in rhesus macaques and AGM, resulting in conformational changes of the HIV-1 CA proteins. Yellow wedges: CypA – Gray circles: HIV-1 CA proteins – Red symbols: TRIM or unknown restriction factor [483].

Another hypothesis regarding species-specific TRIM5α-mediated restriction considers the involvement of other proteins like Cyclophilin A protein (CypA) (Figure 21). In owl monkeys, a fusion protein called TRIMCyp was found to restrict HIV-1 infection by binding to the HIV-1 capsid [425]. Therefore, the non-fused proteins TRIM5α and CypA, both present in other primate species, were further investigated. In African green monkeys (AGM) and rhesus

macaques, CypA alters the HIV-1 CA conformation so that TRIM5α can recognize and restrict the virus [483]. In humans however, CypA interaction with HIV-1 CA proteins seems to protect HIV-1 from TRIM5α [483] which is shown to restrict HIV-1 only modestly [484–487]. Although disruption of the CypA-CA interaction or elimination of CypA protein resulted in diminished HIV-1 infectivity, this was not the result of a more efficient TRIM5α activity [484–486,488]. These results suggest that, in human cells, CypA protects HIV-1 from antiviral activity exerted by an unknown antiviral factor other than TRIM5α [483]. In addition, a number of studies anticipated that TRIM5α and/or the above mentioned CypA-inhibited factor may depend on an unknown co-factor(s) in their retroviral restriction pathway (Figure 22) [476,483,485,488].

Although the exact working mechanism of the TRIM5α protein is unknown, TRIM5α is generally believed to induce premature disassembly of incoming retrovirus capsids. Strangely, however, application of proteasome inhibitors does not reverse rhesus TRIM5α-inhibited HIV-1-infection, while it does prevent TRIM5α-induced capsid disassembly and rescues TRIM5α-inhibited synthesis of viral DNA [442,479,482]. These observations suggest that proteasome activity, inhibition of reverse transcription, as well as involvement in the “uncoating” process are not essential for the TRIM5α protein to block retroviral infection [152]. Others observed that the formation of cytoplasmic bodies would also not be required for retroviral restriction [457,489].

In addition to the above described mechanisms of post-entry inhibition, TRIM5α has also been postulated to interfere with late events in the viral life cycle. Sakuma et al [490] described that rhesus TRIM5α would promote the degradation of Gag polyproteins during or before Gag assembly, thereby affecting virion release. This observation was true with rhesus but not with human TRIM5α [490], although not supported by others [491]. Recently, however, similar to findings by Sakuma et al [490], human TRIM5α was reported to exert an inhibitory effect later in the HIV-1 replication cycle too [487].

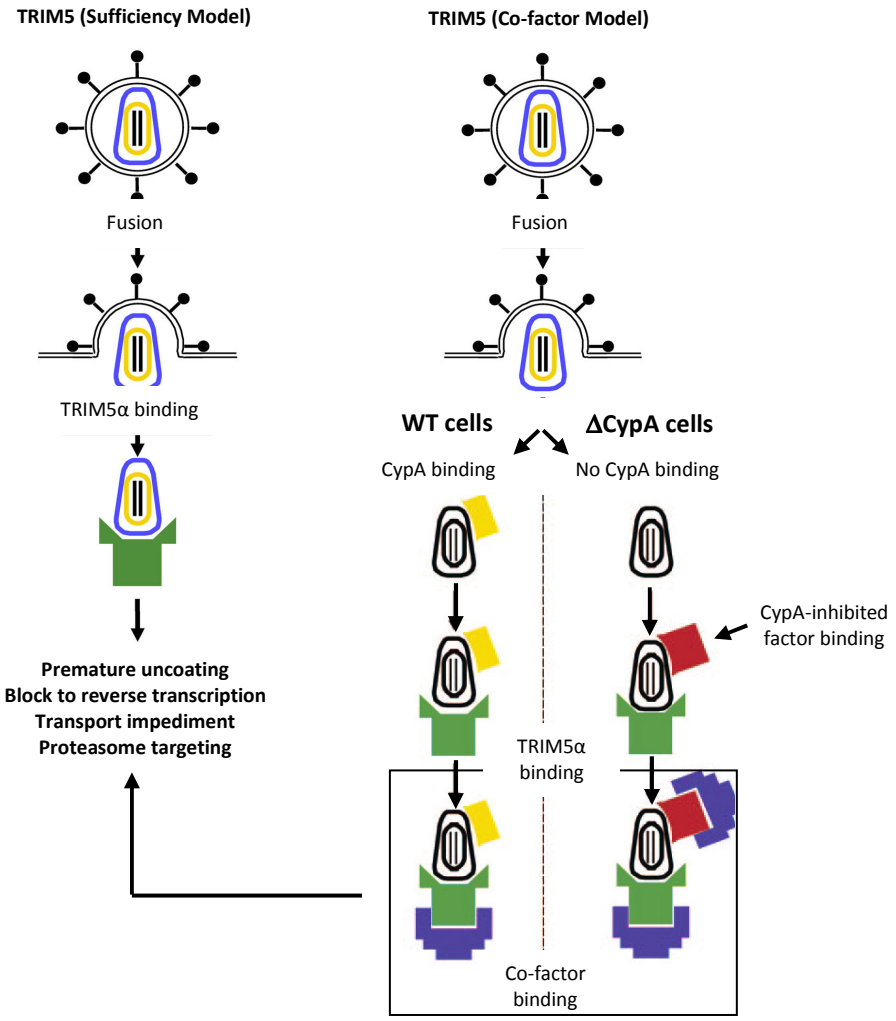

Figure 22 – Models to explain TRIM5α-mediated HIV-1 restriction pathways in human cells. Some investigators consider binding of TRIM5α (green) to the incoming virion sufficient to disrupt infection (sufficiency model). Others suggest that the antiviral effects of TRIM5α require cellular co-factors (blue) (co-factor model). This latter hypothesis gets even more complicated when CypA is considered. In the presence of CypA (yellow), TRIM5α modestly restricts HIV-1 requiring an as yet to be identified but necessary co-factor. In the absence of CypA, a second unknown factor (red) also recognizes the viral capsid. Both TRIM5α and this CypA-inhibited factor then interact with the same saturable co-factor to restrict HIV-1 (adapted from [153,483]).

In 2011, a functional contribution of human TRIM5α in innate immunity was added. Tareen et al [434] described human TRIM5α to exert two opposing effects on the NF-κB pathway. On the one hand, TRIM5α can reduce TAB2 (TAK1 binding protein 2) levels in a non-proteasomal manner, leading to abrogation of TAB2-dependent NF-κB activation. On the other hand, TRIM5α is shown to activate NF-κB-driven transcription in a dose-dependent manner. Distinct domains of human TRIM5α are responsible for TAB2 level modulation, NF-κB regulation, and recognition of retroviral capsids. Pertel et al [433] added that TRIM5α can constitutively promote innate immune signaling and that the protein acts as pattern recognition receptor specific for the retrovirus capsid structure.

4.3.3. Therapeutic importance of TRIM5α-capsid interactions, TRIM5α expression and TRIM5α polymorphisms

The host restriction factor TRIM5α may assist in the development of novel antiretroviral approaches that target the incoming viral core and disrupt early events in HIV-1 replication [16]. Most strategies focus on the virus-host interaction site as the PRY-SPRY domain of TRIM5α interacts with the retroviral capsid in a species-specific manner [447,449,452,453,457,458]. Non-human primate TRIM5α proteins like rhesus TRIM5α have been shown to exert a more potent restriction activity against HIV-1 than human TRIM5α (Figure 23).

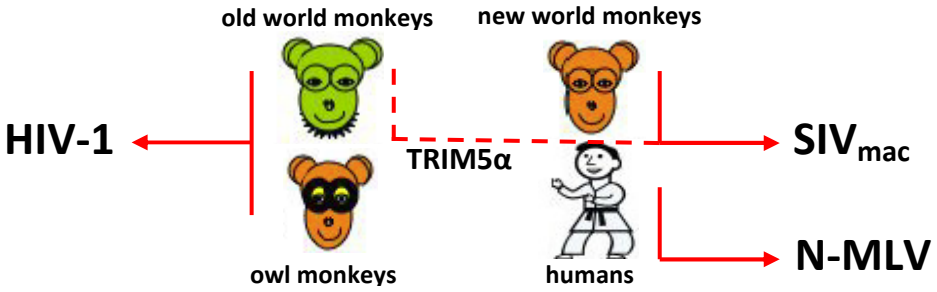

Figure 23 – Species-specific TRIM5α proteins versus restriction of viral infection. TRIM5α proteins from old world monkeys (like rhesus macaques, African green monkeys, cynomolgus, and sooty mangabeys) and owl monkeys restrict HIV-1 infection. TRIM5α proteins from African green monkeys and new world monkeys (like squirrel monkeys and common marmosets) excluding owl monkeys restrict SIV_{mac} infection. Human TRIM5α exerts antiviral activity against the N-tropic murine leukemia retrovirus (N-MLV) (adapted from [410,454,492]).

Due to species-specific restriction capacity, one may consider a **gene delivery strategy with a more potent TRIM5α homolog** to inhibit HIV-1 infection. Initial *in vitro* results applying this strategy are promising [493]. However, since these "foreign" TRIM5α proteins may provoke an immune response, genetically engineered human TRIM5α proteins may be used instead. As such, human TRIM5α proteins may be genetically modified to express a HIV-1 CA-recognizing PRY-SPRY domain. Thus, one may replace sections of the human PRY-SPRY domain with restriction-specific regions from non-human primates [452], or simply introduce the R332P single amino acid change in human TRIM5α [452,453,494,495]. Another challenging approach may be to modify human TRIM5α to mimic rhesus TRIM5α by pharmacologically neutralizing the positive charge of the arginine residue at position 332 [453,495].

Alternatively, **small molecules** may be designed to affect viral core stability and/or to prevent HIV assembly. The minimal design requirement for TRIM5α-like small molecules to restrict HIV-1 in a CA-dependent manner seems to be the presence of a multimer with CA-binding capacity [468]. The successful design of such a TRIM5α-like molecule that maintains restriction activity once delivered into the cell, appears unrealistic [219]. Potential therapeutic HIV capsid (CA) targets include the CA N-terminal domain, the CA C-terminal domain, or the CA-SP1 cleavage site [496]. For the N-terminal domain, Tang et al [497] described the first small-molecule inhibitors of HIV assembly being CAP-1 and CAP-2. Recently, the compound PF-74 was described which interferes with protein-protein interactions in the mature capsid resulting in premature post-entry core dissociation in the early phase or failed maturation in the late phase of the replication cycle [498,499]. Potential molecules that target the C-terminal domain of HIV capsids include NYAD-1 and CAI (capsid assembly inhibitor) which dismantles *in vitro* preassembled CA tubes to inhibit virus assembly and maturation [500,501]. Another molecule concerned bevirimat (also called DSB or PA-457) which prevents CA-SP1 cleavage in Gag-processing but may also stabilize the immature CA lattice [496,502].

Next, several studies mention the interest of **TRIM5α expression levels**. Sastri et al [477] described e.g. a modest but reproducible degree of HIV-1 restriction upon human TRIM5α over-expression in human cells or human TRIM5α expression in cell lines lacking a TRIM5α gene. Sewram et al [503] observed significantly higher levels of TRIM5α in non-seroconverters (HESN sex workers) relative to seroconverters (HIV-1-infected individuals). Of note, pre- and post-infection levels of TRIM5α were similar in the study population of seroconverters. Thus, higher levels of TRIM5α appear associated with reduced susceptibility to HIV-1 infection in HESN. On the other hand, TRIM5α expression does not seem to control HIV-1 infection, as no correlations were observed between levels of TRIM5α and viral load or CD4 count. One may consider increasing human TRIM5α levels with specifically designed small molecules [219] or stimulation agents like IFNα [97,101,504,505]. Of note, as was also mentioned for APOBEC3G, selective induction of TRIM5α should be aimed for, instead of a general induction by IFN. Although this approach is unlikely to be therapeutically effective for human TRIM5α, a combination therapy targeting human TRIM5α levels and modifying human TRIM5α to behave like rhesus TRIM5α may be a valuable therapeutic solution [219].

In addition, multiple studies have been conducted to identify **TRIM5α polymorphisms**. In humans, different TRIM5α polymorphisms with neutral or reduced antiviral activity have been observed. The single nucleotide polymorphisms (SNPs) V112F, R136Q, E238W, G249D, and H419Y are examples of SNPs with neutral antiviral activity, as these SNPs resulted in irreproducible small alterations in HIV-1 infection [506–510]. The SNP H43Y is an example of a TRIM5α protein with impaired ability to restrict HIV-1 [507,508]. Individuals homozygous for this allele were shown to exhibit accelerated disease progression [511], and tended to express higher viral set-point levels [509]. Opposed to these results, Nakayama et al [512] observed no influence on disease progression for this mutation, whereas Liu et al [513] suggested a protective effect among homozygous individuals. Recently, TRIM5α polymorphisms with increased antiviral activity were observed in rhesus macaques [514–517] as well as in humans [518,519]. Of note, the majority of human TRIM5α polymorphisms are localized in the RBCC motif and do not modulate TRIM5α specificity to individual retroviruses [506–509], whereas rhesus macaque SNPs are predominantly observed in the CC and PRY-SPRY domain which

dictate the TRIM5α restriction specificity [514–517] (Figure 24). The observation that human and rhesus TRIM5α express different SNPs should be taken into account when designing and subsequently interpreting studies in primates [477].

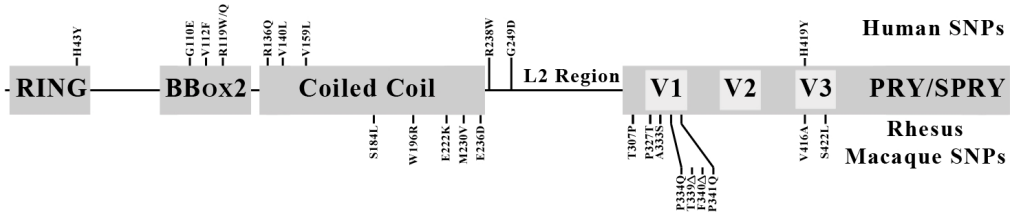

Figure 24 – Single Nucleotide Polymorphisms in TRIM5α. SNP's of TRIM5α in humans (above the domain structure) and rhesus macaques (below the domain structure) (adapted from [477]).

In conclusion, the TRIM5α-mediated cross-species restriction mechanism needs to be further elucidated to develop a successful therapeutic strategy. Obtaining full insight in the structure of TRIM5α protein bound to the HIV-1 viral core will be of great importance. Also, the observation that TRIM5α levels associate with susceptibility to HIV-1 infection but not with disease progression support the interest to evaluate TRIM5α and other endogenous TRIMs specifically at the primary site of infection.

4.4. Tetherin

4.4.1. Background

In 2008, tetherin (also referred to as BST-2, CD317 or HM1.24) has been identified as a novel host restriction factor. This glycosylated protein is shown to inhibit the release of newly formed virions in a Vpu sensitive manner [520,521]. A single copy of the tetherin gene is located on chromosome 19 [522]. Several studies suggest that tetherin, similar to APOBEC3G and TRIM5α, has experienced positive selection during evolution through exposure to ancient viral antagonists [346,523,524]. A recent study specifies that tetherin as a whole has been under neutral rather than positive evolution, whereas specific regions of tetherin that interact with viral proteins have undergone positive selection [525].

The 30 to 36kDa type II transmembrane protein [522] is composed of an N-terminal transmembrane region with cytoplasmic tail, an extracellular coiled-coil domain, three extracellular conserved cysteines, and a C-terminal glycosylphosphatidylinositol (GPI) group as second membrane anchor (Figure 25) [344,526]. The transmembrane domain determines the sensitivity of tetherin for Vpu [524,527,528]. Based on X-ray crystallography, a model of tetherin as disulfide-bonded coiled-coil homodimer was confirmed [529–531]. Dimerization appears very important in the restriction of HIV-1 infection [532]. The GPI-moiety serves to recruit tetherin to lipid rafts [526].

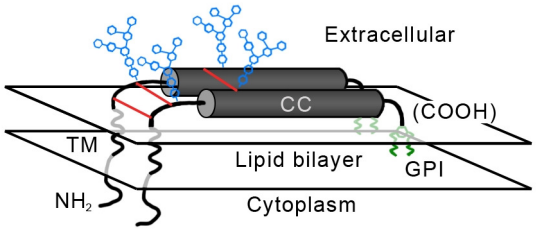

Figure 25 – Model of the tetherin dimer. A monomer is composed of a transmembrane (TM) domain, coiled-coil (CC) domain, and GPI-anchor (green) (from N- to C-terminus). Glycosylation modifications are depicted in blue. The red stripes highlight the 3 possible disulfide bonds (adapted from [533]).

Tetherin exerts antiviral activity against distinct classes of virus including retroviruses, filoviruses, arenaviruses, and herpesviruses [525,534–541]. Over time, several viral antagonists like Vpu (HIV-1), Nef (SIV), Env (HIV-2, SIV, Ebola virus) and K5 protein (KSHV, Kaposi's sarcoma-associated herpesvirus) evolved to counteract tetherin's activity to inhibit virion release [344].

Localization of the transmembrane protein tetherin is highly dynamic. The protein is shown to reside in a plasma membrane pool associated with lipid rafts, in early and recycling endosomes, and in the trans-Golgi network [526,536,542–545]. In general, the protein is expressed on certain cell types only [546–548], whereas a broad expression is observed following stimulation with IFNα via the JAK/STAT signaling pathway [538,549]. Of note, various response elements are observed in the tetherin gene promoter suggesting that other inflammatory cytokines like IL-6 and TNFα may also enhance its expression [549].

4.4.2. Host restriction factor tetherin versus HIV-1 Vpu protein

Early studies on the HIV-1 Vpu protein raised the hypothesis that Vpu would assist in virion release and prevent virion internalization [536,543,550]. Recently, in the absence of Vpu, intracellular virions were observed primarily in CD63+ endosomes [538,542] as a result of internalization from the cell surface [538,542,551,552]. When endocytosis was blocked, newly formed virions were retained at the cell surface and released upon protease treatment [538,542]. The attachment of fully formed and mature virions was further enhanced upon stimulation with IFNα [214,538]. Above observations suggested the existence of a human IFNα-inducible membrane protein "tethering" newly formed virions, which is counteracted by the viral Vpu protein [520,550]. Recently, this protein was uncovered as bone marrow stromal cell antigen 2 (BST-2), or tetherin [521,542].

Most likely, **tetherin** is part of a broad antiviral defense system as it is induced by IFNα [214,538,553]. The exact working mechanism of tetherin in restricting the release of newly formed HIV-1 virions remains thus far unclear. It appears relatively non-specific as tetherin affects the release of many different viruses. Moreover, Perez-Caballero et al [533] designed an artificial protein composed of similar domains as tetherin but with limited sequence homology that could also restrict HIV-1 release. Thus, the structural configuration of tetherin appeared of greater importance than its amino acid sequence.

Accumulating evidence postulates that tetherin may physically prevent the release of fully assembled virions by connecting virions to the plasma membrane and to each other [344,526]. Different tethering models have been suggested (Figure 26). The parallel homodimer model is preferred since tetherin antiviral activity could be abrogated upon removal of either cytoplasmic tail or GPI anchor [520,533,554,555]. However, Fitzpatrick et al [556] observed that enzymatic cleavage of the GPI-anchor did not result in the release of retained virions. Furthermore, the distance between two linked membranes is often larger than calculated based on currently available structural data [520,529–531,556,557]. Likely, other tethering models are involved.

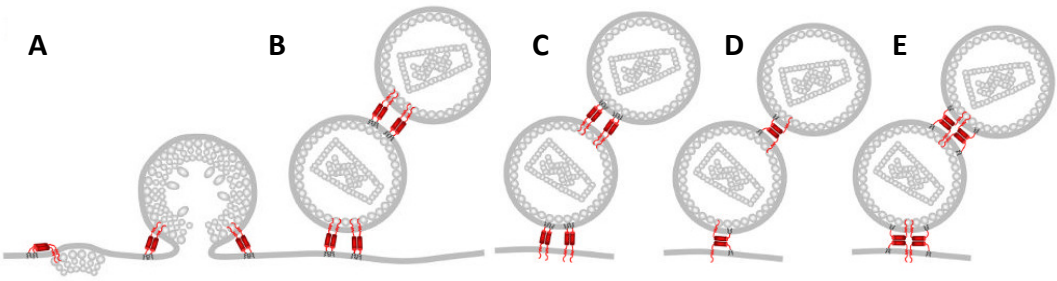

Figure 26 – Models of tetherin restricting virion release. (A) The process of HIV-1 assembly including tetherin involvement. (B-C) Models of membrane spanning homodimers (parallel). (D) One monomer of the tetherin dimer is anchored in the viral membrane, and the other in the plasma or another viral membrane. Both molecules interact via their coiled-coil domains. (E) One tetherin dimer interacts with another dimer via coiled-coil based interactions. The red line depicts the N-terminal transmembrane domain with cytoplasmic tail, the grey V-shape depicts the C-terminal GPI-anchor, and the red rectangles highlight the extracellular domains of tetherin (adapted from [533]). Although not shown, an anti-parallel spanning dimer may be another working model [554,556].

In infected cells, tetherin is shown to inhibit virion release, to diminish viral infectivity [520,521,558–560] and to impair direct cell-to-cell spread [344,540,561,562]. Opposed to this latter observation, at least two studies suggest that the accumulation of virions at the cell membrane may promote cell-to-cell spread e.g. by enhancing fusogenicity or regulating viral synapse integrity [563,564]. Furthermore, when expressed on uninfected target cells, tetherin is suggested to promote viral transfer and transmission by cell-to-cell contact [562]. In conclusion, tetherin reduces virion release in the absence of Vpu, but does not necessarily prevent viral spread [344].

In addition to above described observations on tetherin versus viral spread, the protein is postulated to influence the host's immune response in two distinct manners. On the one hand, tetherin is thought to allow a better presentation of viral antigens to the adaptive immune system through the retention of budding viruses on the cell surface [344,540]. On the other hand, tetherin is described to regulate innate immune cells independent of its "tethering" capacity. Cao et al [565] showed that tetherin can inhibit the production of IFN and pro-inflammatory cytokines in a negative feedback loop (Figure 27). Matsuda et al [566] identified tetherin to activate NF-κB and MAPK signaling pathways. Thus far it remains unclear whether Vpu interferes with these immunomodulatory functions of tetherin.

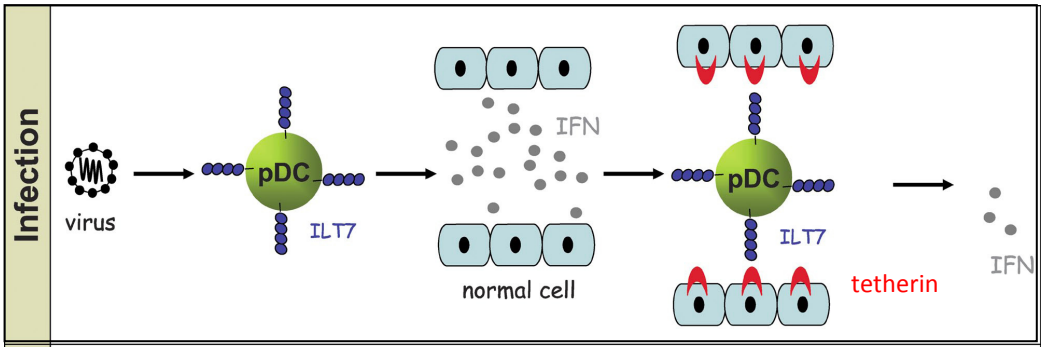

Figure 27 - Model of IFN negative feedback regulation by tetherin. Upon virus infection, plasmacytoid dendritic cells (pDCs) start to produce large amounts of type I IFN via TLR7 or TLR9 activation. Type I IFN induces tetherin levels which can interact with immunoglobulin-like transcript 7 (ILT7) on pDCs. Next, tetherin-ILT7 interaction down-regulates the IFN response (adapted from [565]).

The 16kDa **HIV-1 Vpu protein** exerts two major biological activities in the HIV-1 life cycle [543,554,563,564,567–569].

(1) Vpu targets newly synthesized CD4 molecules bound to Env in the endoplasmic reticulum (ER) for degradation [570,571]. This ubiquitin-proteasome dependent degradation process needs Vpu to associate with CD4 and to recruit β-TrCP, a component of the E3 ubiquitin ligase [567,568]. Of note, HIV-1 is suggested to down-regulate CD4 receptor expression to prevent super-infection and to promote the production of fully infectious viruses, despite the primary receptor role of CD4 during HIV-1 entry [572–574].

(2) Vpu counteracts human tetherin in restrictive cells, thereby promoting HIV-1 virion release [520,521,538]. Tetherin is sensitive to Vpu based on a number of positively selected determinants in its transmembrane domain [523,524,528,558,575]. Thus far, no consensus has been reached on how exactly Vpu interferes with tetherin antiviral activity. One hypothesis mentions that Vpu may act by the removal of tetherin from its site of tethering action. This recently challenged model [547] is supported by (a) Vpu decreasing cell-surface levels of tetherin [521] and (b) Vpu preventing tetherin and Gag co-localization at the site of virion assembly [215,520,537]. Although a number of studies explain the Vpu-induced tetherin degradation through a β-TrCP sensitive process of proteasomal and/or lysosomal degradation [213,215,523,558,576,577], tetherin removal alone can not fully clarify the Vpu anti-tetherin activity. A second hypothesis suggests that Vpu may affect the resupply or surface delivery of tetherin. Recently, several groups [578–580] observed that Vpu may

promote virion release by slowing-down transportation of recycled or newly formed tetherin towards the plasma membrane, without affecting overall tetherin expression or turnover (Figure 28). Dubé et al [578] confirmed that Vpu and tetherin most likely associate via their transmembrane domains, which appears necessary to counteract virion tethering and to re-locate tetherin from the plasma membrane to the trans-Golgi network away from membrane virus assembly sites. Degradation of tetherin can also occur in the trans-Golgi network.

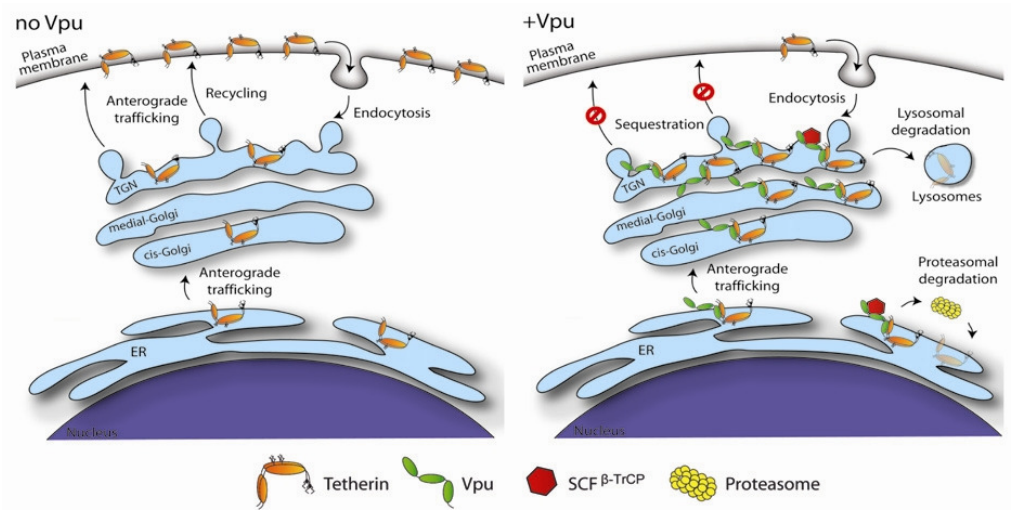

Figure 28 - Scheme of Vpu-mediated cell-surface tetherin downregulation. In the absence of Vpu (left), tetherin follows the anterograde trafficking pathway. Once expressed on the cell surface, tetherin is endocytosed, transported to the trans-Golgi network (TGN) and most likely recycled to the plasma membrane. In the presence of Vpu (right), tetherin is trapped in the TGN by the viral protein. Vpu-mediated proteasomal and/or lysosomal degradation of tetherin may represent a complementary mechanism (from [554]).

In addition, Varthakavi et al [581] found Vpu to localize in the recycling endosome compartment, which is important in virion assembly or release and may act as potential Vpu-tetherin interaction site. Vpu-induced redistribution of tetherin from early and recycling endosomes to the trans-Golgi network may be a third working hypothesis for Vpu [521,544,578,582].

Above described roles of Vpu versus CD4 and tetherin occur in different subcellular compartments [583] and rely on distinct structural regions within Vpu [554,584].

Of note, at least three cross-species transmissions from chimpanzee to human led to the HIV-1 groups M, N and O [1,585]; each expressing a Vpu protein with different functional properties. The pandemic spread of HIV-1 group M may have been facilitated due to the expression of a fully competent Vpu protein able to mediate CD4 degradation as well as to antagonize tetherin [559], whereas the Vpu protein from HIV-1 group O mediates CD4 degradation and is unable to antagonize tetherin [586].

4.4.3. Therapeutic importance of tetherin-Vpu interactions, tetherin expression and tetherin polymorphisms

Although the exact working mechanism of HIV-1 Vpu to counteract human tetherin remains unknown, recent studies revealed a role for Vpu's β-TrCP-binding capacity in tetherin degradation [213,215,558,576] and for Vpu's transmembrane domain in tetherin binding [576,587]. Further investigation is required to identify specific residues e.g. in the transmembrane domain of Vpu and/or tetherin that may be of importance in the putative Vpu-tetherin binding. Different strategies have been suggested in establishing Vpu or tetherin as potential therapeutic targets.

Vpu's ion channel function is thought to participate in Vpu-induced virion release [588,589]. Several findings demonstrate that blocking Vpu ion channel formation may be a potential anti-HIV strategy [590–593]. Waheed et al [594] speculated that the compound AME (amphotericin B methyl ester) disrupts HIV-1 particle release in a Vpu-dependent manner by blocking Vpu's ion channel function. Recently, Bolduan et al [595] reported that the S23A mutation which abrogates Vpu's ion channel function did not affect virion release, suggesting that the ion channel activity of Vpu is not required to augment HIV-1 release.

A second approach may be to target the **Vpu-tetherin interface** [555,575,577,596] by means of e.g. small molecule inhibitors that interfere with the putative interaction between Vpu and tetherin [152,597]. Next, one could consider targeting the **interface between Vpu and e.g. β-TrCP,** a host factor that helps mediate proteasomal or lysosomal degradation of tetherin [16].

Unfortunately, the latter interface-targeting strategies will not satisfy as HIV-1 is shown to replicate even when Vpu is completely lost [217,538]. Therefore, another approach to target tetherin as potential therapeutic mean may be to enhance **tetherin expression levels** at the cell surface [549]. Early *in vivo* experiments showed that administration of IFNα led to the reduction of the HIV-1 viral load in some patients [598,599]. Several cell culture experiments added evidence that IFNα may inhibit HIV-1 replication in one or another way [600–604]. One common observation showed that IFNα could block virion release and lead to the accumulation of newly formed virions in primary cell types like macrophages and primary CD4+ T cells [602,605–608]. The IFNα-inducible protein tetherin was recently described as possible mediator of this IFNα-effect [214,520,538]. Although wild-type HIV-1 strains that express Vpu appear considerably resistant to this type I IFN-induced virion retention at the cell surface [538], several *in vitro* studies showed Vpu-independent residual inhibition of virion release upon over-expression of tetherin [521,524,609].

Also, several sequence analysis studies have been undertaken to study tetherin **polymorphisms**. Although tetherin appears relatively non-polymorphic [579], several interesting observations have been done. (a) Species-specific residues of tetherin are involved in virus-mediated antagonism [610]. Within the transmembrane domain of human tetherin, certain residues have been identified that influence HIV-1 Vpu-mediated downregulation [523,524,528,596]. (b) Species-specific residues of tetherin are not important for the antiviral function of tetherin [610]. Moreover, maintenance of the structural configuration and the intracellular transport appear of greater importance than the amino acid sequence [533,610]. Inhibition of virion release does not require specific interactions between tetherin and proteins of the retained virus [537,538,582]. (c) Plausible binding sites for IFN-regulated transcription factors as well as a variety of SNPs have been observed in the tetherin promoter site [152].

In conclusion, to gain further knowledge on tetherin and Vpu in HIV-1 infection, additional research is required to elucidate the working mechanism and potential therapeutic interest of both proteins [16,344,549,554,610].

Chapter 2 – Aim and design of the study

At the Laboratory of Immunology at the Institute of Tropical Medicine (ITM, Antwerp, Belgium), a study on correlates of protective immunity (COPRIM) was established in collaboration with the "Centre du Traitement Ambulatoire" at Fann hospital, and with the "Laboratoire de Virologie-Bacteriologie" at Le Dantec hospital, both in Dakar, Senegal. The aim of the COPRIM study is to identify and characterize biological factors that mediate protection against HIV infection among HIV-1-exposed seronegative (HESN) subjects. For this purpose, a cohort consisting of HIV-discordant, HIV-concordant and HIV-negative couples was established.

In the context of my PhD thesis, collaboration was started between the Laboratory of Immunology at the ITM and the Proteinscience, Proteomics and Epigenetic Signalling (PPES) laboratory at the University of Antwerp. Our objective was to study the impact of intrinsic, cellular HIV-1-related host factor expression in HIV-1 restriction and/or replication. Initially, the co-factor LEDGF/p75 and the restriction factors APOBEC3G and TRIM5α were selected for detailed analysis. During the course of the PhD-thesis, the newly identified restriction factor tetherin was added to the panel of host factors under investigation. The expression profiles are studied in HESN, healthy control and HIV-1-infected subjects.

As our goal was to investigate intrinsic *in vivo* protein expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in CD4+ immune cells being CD4+ lymphocytes and CD14+ monocytes, our interest went out for a flow cytometry-based method. One major advantage of the flow cytometer is its capacity to obtain information on expression levels in different cell subtypes simultaneously, based on fluorescently labelled antibodies.

The specific aims of this PhD thesis can therefore be summarized as follows:

1) To optimize a flow cytometry-based intracellular staining method to study intrinsic protein expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin at the level of the single cell.
2) To optimize a flow cytometry-based intracellular staining method to study mRNA levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin at the level of a single cell.
3) To compare protein and mRNA expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin between HESN individuals and healthy controls, and between HIV-infected individuals and healthy controls. These comparisons are done to unravel the mechanisms that lead to *in vivo* resistance of HESN persons and that may contribute to the development of new antiviral therapeutic strategies.

Chapter 3 – Intracellular detection of differential APOBEC3G, TRIM5alpha, and LEDGF/p75 protein expression in peripheral blood by flow cytometry

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Reprinted from:

Mous, K., Jennes, W., De Roo, A., Pintelon, I., Kestens, L. and Van Ostade, X. *J. Immunol. Methods* 2011; S0022-1759(11)00152-9 [pii];10.1016/j.jim.2011.06.028 [doi

1. Abstract

Objective: Expression studies on specific host proteins predominantly use quantitative PCR and western blotting assays. In this study, we optimized a flow cytometry-based assay to study intracellular expression levels of three important host proteins involved in HIV-1 replication: apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G, A3G), tripartite motif 5alpha (TRIM5α), and lens epithelium-derived growth factor (LEDGF/p75).

Methods: An indirect intracellular staining (ICS) method was optimized using antibodies designed for other applications like enzyme-linked immunosorbent assay (ELISA), confocal imaging, and western blotting.

Results: The median fluorescence intensity (MFI) value – a measure for the protein expression level – increased upon higher antibody concentration and longer incubation time, and was reduced following preincubation with recombinant proteins. Staining of stably transfected or knock-down cell lines supported the method's specificity. Moreover, confocal microscopy analysis of peripheral blood mononuclear cells (PBMC), when stained according to the ICS method, confirmed the localization of APOBEC3G and TRIM5α in the cytoplasm, and of LEDGF/p75 in the nucleus. Also, stimulation with mitogen, interferon-alpha, or interferon-beta resulted in detectable, albeit weak, increases in intracellular expression of APOBEC3G and TRIM5α. After optimization, the method was applied to healthy control and HIV-1-infected subjects. For all subjects studied, the memory subset of CD4+ T cells showed significantly higher expression levels of APOBEC3G, TRIM5α, and LEDGF/p75, while the CD16+ subset of monocytes was characterized by higher expression levels of LEDGF/p75. In addition, we observed that therapy-naïve HIV-1 patients tended to have lower expression levels of APOBEC3G and TRIM5α than HIV-1 negative controls.

Conclusions: In summary, our data provide proof-of-principle for the detection of specific host factors at the level of a single cell, which may prove useful for our further understanding of their role in virus-host interactions.

2. Introduction

Human immunodeficiency virus type I (HIV-1) interacts with several cellular host proteins during its replication cycle. Some of these factors have antiviral activity whereas others are HIV-1 co-factors essential for HIV-1 replication. Recently, apolipoprotein B mRNA-editing catalytic polypeptide-like 3G [611] (APOBEC3G, A3G) and tripartite motif 5alpha [612] (TRIM5α) were shown to display antiviral activities, whereas lens epithelium-derived growth factor [613] (LEDGF/p75) was demonstrated to aid HIV-1 replication. Although alternative mechanisms of action are described [219], the main working mechanisms can be summarized as follows. APOBEC3G is packaged into newly formed viral particles following infection with a HIV-1 virus deficient for viral infectivity factor (Vif), leading to viral genome hypermutation during viral reverse transcription in the freshly infected cell [355,614–617]. TRIM5α is thought to disorder the retroviral capsid, thereby interrupting the natural "uncoating" process and subsequent transportation of the viral DNA towards the cell nucleus in a species-specific manner [618,619]. For example, rhesus macaque TRIM5α restricts HIV-1 infection, whereas human TRIM5α inhibits e.g. N-tropic murine leukemia virus and feline immunodeficiency virus [452,619]. LEDGF/p75, finally, assists in the integration of the viral cDNA into specific regions of the host's genetic material [620].

Previous reports have proposed that expression levels of APOBEC3G and TRIM5α could influence the differential tropism of HIV-1 for peripheral blood mononuclear cell (PBMC) subsets [417,621,622]. For instance, some studies suggested a pivotal role for APOBEC3G in the restriction of HIV-1 replication in resting CD4+ T cells, but these data could not be confirmed [623–626]. Different studies predicted APOBEC3G, TRIM5α, and LEDGF/p75 expression levels by the extrapolation of oligonucleotide microarrays [417,627] and quantitative real-time polymerase chain reaction (PCR) data [100,208,621,622,628–634]. However, since mRNA expression levels do not necessarily correspond with protein expression levels, data based on mRNA expression must be interpreted with care. Protein-based methods are better suited to address the *in vivo* protein expression levels of HIV-1-related host proteins. This is supported by the notion that viral Vif degrades host APOBEC3G

protein and impairs its *de novo* synthesis, while leaving APOBEC3G mRNA undisturbed [635]. Thus far, ELISA [621] and western blotting [100,622,628–630,632,634] have been used to analyze protein expression. However, these techniques are not fully appropriate to simultaneously study protein expression levels in distinct cell types and at the single-cell level. Here, we optimized an intracellular staining (ICS) assay based on commercially available monoclonal antibodies to study APOBEC3G, TRIM5α, and LEDGF/p75 expression at the single-cell level by flow cytometry. Next, we applied this method to study APOBEC3G, TRIM5α, and LEDGF/p75 protein expression levels in peripheral blood from HIV-1-infected subjects and controls.

3. Methods

3.1. Study population

Blood samples were obtained from twenty HIV-1 positive patients as well as ten HIV-1 negative controls (HC), all recruited at the Institute of Tropical Medicine (ITM, Antwerp) (Table 1). The HIV-1 positive study group consisted of 10 therapy-naïve patients (HIV-untreated, HIV-UT) and 10 patients treated with antiretroviral therapy (HIV-ART, viral load < 50 copies/ml). All individuals included in the study were of Caucasian ethnicity and signed the informed consent form as approved by the ITM institutional review board and the ethical committee of the University Hospital of Antwerp (UZA). Blood samples obtained from one HIV-negative control subject were used to optimize the intracellular staining method.

In addition, ten healthy control samples were recruited at the Department of Infectious Diseases at the Fann University Teaching Hospital, Dakar, Senegal as described elsewhere [59]. The subjects gave written informed consent for participation in the study approved by the Internal Review Board of the Institute of Tropical Medicine (Antwerp, Belgium) and by the Ethical committees of the Senegalese Ministry of Health (Dakar, Senegal) and the University Hospital of Antwerp (Belgium). The samples were used to investigate whether mRNA and protein levels of APOBEC3G, TRIM5α, and LEDGF/p75 correlate.

Table 1a - Clinical and demographic characteristics of the study population.

	HC (n=10)		HIV-UT (n=10)		HIV-ART (n=10)	
	Median	IQR	Median	IQR	Median	IQR
Age (years)	26	(25-34)	39	(33-42)	43	(41-46)
Sex (% Male)	30	-	80	-	100	-
% CD4+ T Cells	43.8	(39.5-48.5)	30.2	(27.2-34.0)	28.7	(18.4-33.1)
CD4 count (cells/µl)	-	-	581	(410-693)	612	(336-686)
VL (log10 copies/ml)	-	-	4.5	(4.2-5.3)	<1.69	-

HC: HIV-1 seronegative individuals; HIV-UT: Therapy-naïve HIV-1 seropositive patients; HIV-ART: antiretroviral therapy-treated HIV-1 seropositive patients; IQR: Interquartile Range; VL: viral load

Table 1b - Clinical and demographic characteristics: Statistics.

	p-value		
	HC vs HIV-UT	HC vs HIV-ART	HIV-UT vs HIV-ART
Age (years)	0.008	0.001	0.151
Sex (% Male)	0.028	0.001	0.146
% CD4+ T Cells	0.002	0.001	0.45
CD4 count (cells/µl)	-	-	0.683
VL (log10 copies/ml)	-	-	< 0.001

HC: HIV-1 seronegative individuals; HIV-UT: Therapy-naïve HIV-1 seropositive patients; HIV-ART: antiretroviral therapy-treated HIV-1 seropositive patients; P-values were calculated using Mann-Whitney *U* tests; VL: viral load

3.2. Sample collection and processing

Blood samples were obtained in EDTA-coated blood tubes (BD Biosciences). Plasma was tested for HIV-1 infection by ELISA and western blotting, while HIV-1 viral loads were quantified by the Amplicor HIV-1 Monitor assay, version 1.5 (Roche Diagnostics GmbH). CD4+ T cell counts were determined in whole blood using a FACSCalibur flow cytometer (BD Biosciences). Peripheral blood mononuclear cells (PBMC) were separated by density gradient

separation using Ficoll Paque™ PLUS (GE Healthcare Biosciences AB), washed in Hank's balanced salt solution (HBSS, Invitrogen) and re-suspended in complete RPMI medium at the desired final concentration. Complete RPMI medium consisted of RPMI medium (Lonza) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (P/S, Roche Diagnostics GmbH), and 10% fetal bovine serum (FBS, Lonza).

From each Senegalese healthy control subject, PBMC were separated as described above. Next, PBMC were frozen in liquid nitrogen and transported to Belgium. Then, PBMC were thawed (37°C water bath), washed twice and counted prior to protein staining and mRNA analysis. Prior to mRNA analysis, CD4+ T cells were isolated from the PBMC with the Dynal CD4 Positive Isolation Kit (Invitrogen) according to the manufacturer's protocol.

3.3. Reagents

The washing buffer was composed of phosphate buffered saline (PBS, Lonza), 0.1% bovine serum albumin (Acros Organics), and 0.05% sodium azide (Merck). Reagents A and B of the Leucoperm kit (AbD Serotec) were used to fix and permeabilize the PBMC, respectively. The fixative contained paraformaldehyde and the permeabilization reagent saponin. Primary unlabeled high-affinity mouse anti-human IgG1 monoclonal antibodies for APOBEC3G and TRIM5α (clone 9.3.1) were obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Primary rat anti-HA antibody was purchased from Roche. Primary unlabeled mouse IgG1 antibodies for human LEDGF/p75 (clone 26) and non-human keyhole limpet hemocyanin protein (clone X40) were purchased from BD Biosciences. This latter antibody is referred to as the isotype mouse IgG1 antibody. Indirect staining required the addition of the secondary rat anti-mouse IgG1 fluorescein isothiocyanate (FITC)-labeled antibody (clone A85-1, BD Pharmingen). The membrane antibodies anti-CD4-phycoërythrin (PE), anti-CD3-allophycocyanin (APC), anti-CD4-peridinin-chlorophyl protein (PerCP), anti-CD45RO-APC, anti-CD14-APC, and anti-CD16-PE were all purchased from BD Biosciences. The recombinant proteins human APOBEC3G (expressed in *E. coli*) and TRIM5α (expressed in Baculovirus) were obtained through the AIDS Research and Reference Reagent Program

(Division of AIDS, NIAID, NIH). Recombinant human LEDGF/p75 (expressed in *E. coli*) was kindly provided by Zeger Debyser [212] (Catholic University of Leuven, KUL, Belgium). The stimulation agents used were phorbol myristate acetate in combination with ionomycin (P/I, both from Sigma-Aldrich), interferon-alpha and interferon-beta (IFNα, IFNβ, both from Peprotech). Normal mouse serum was purchased from eBioscience. The final fixation solution contained 1% paraformaldehyde (Sigma-Aldrich) in PBS.

3.4. Intracellular protein staining

The intracellular protein staining (ICS) method used to study the expression of the HIV-1-related host proteins APOBEC3G, TRIM5α, and LEDGF/p75 was adapted from established intracellular cytokine staining protocols [636,637], with omission of both cell stimulation and inhibition of cytokine secretion. PBMC were added to 96-microtiterplate wells, washed (with washing buffer, 630 g, 10 min, RT), fixed (10 min, RT), and washed twice. If required, the second washing step was paused by placing the fixed cells overnight in washing buffer at 4°C and performing the centrifugation step the next day. Subsequently, cells were permeabilized (30 min, 4°C), washed, and incubated with unlabeled primary antibody (24 h, 4°C). The next day, cells were washed twice prior to incubation with an excess (1 µg) of secondary FITC-labeled antibody supplemented with the permeabilization agent (30 min, 4°C). Two washing steps were performed, followed by incubation with 1:5 diluted normal mouse serum (10 min, 4°C). Subsequently, cells were incubated with the desired fluorescently labeled membrane antibodies (20 min, 4°C). Finally, two washing steps were performed, followed by the addition of 1% paraformaldehyde prior to data acquisition and analysis with the FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences). Protein expression levels were studied based on median fluorescence intensity (MFI) values. This value reflects the amount of antibody bound to the intracellular protein and thus the protein's abundance. For all results described, host protein MFI-values were corrected for non-specific background staining by subtracting the isotype MFI.

3.5. Specificity testing of the ICS method

Primary antibody (2.5 µg, 2.5 µg, and 5 µg per million cells for APOBEC3G, TRIM5α, and LEDGF/p75, respectively) was pre-incubated with a 0-, 10- or 50-fold excess of its respective recombinant protein for 3 h in two independent blocking experiments. The molar excess of recombinant protein was calculated considering the molecular mass of both antibody and recombinant protein, and based on the consensus that IgG antibodies can simultaneously interact with two antigens. Recombinant protein concentrations were 15 µg, 25 µg, and 50 µg per million cells for APOBEC3G, TRIM5α, and LEDGF/p75 respectively (10-fold excess); and 75 µg, 125 µg, and 250 µg per million cells for APOBEC3G, TRIM5α, and LEDGF/p75 respectively (50-fold excess). The fixed and permeabilized PBMC were then incubated with the mixture of antibody and recombinant protein for 24 h, after which the protocol proceeded as described above (Section 3.4.).

Specificity of the method was also studied in specific cell lines. APOBEC3G was studied in HeLa cells from Dr. Richard Axel [638] and HeLa-A3G cells from Drs. Klaus Strebel and Eri Miyagi (both obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). Cells were cultured in DMEM with L-glutamine and 4.5g/l glucose (Lonza), supplemented with 10% FBS, and 1% P/S; for HeLa-A3G, 400µg/ml of G418 (Invitrogen) was added. TRIM5α expression was addressed in Cf2Th-pLPCX and Cf2Th-TRIM5α cells as kindly provided by Dr. Joseph Sodroski (Boston, USA). The latter cell-type expresses HA-tagged human TRIM5α. Cells were cultured in DMEM with L-glutamine (Lonza) supplemented with 10% FBS, 1% P/S, and 5µg/ml puromycin (invitrogen). LEDGF/p75 was studied in LEDGF/p75 knock-down (KD) and wild-type (WT) HeLa cells as kindly provided by Dr. Zeger Debyser (KUL, Belgium). Cells were cultured in DMEM with 4.5g/l glucose and L-glutamine (Lonza) supplemented with 7% FBS, 1% P/S, 500µg/ml of geneticin (Invitrogen), and 50µg/ml of gentamicin (Invitrogen); for the KD cells, 200µg/ml of zeocin (Invitrogen) was also added. Protein expression was studied in above described cell lines with the intracellular staining protocol but without membrane staining (Section 3.4.), and with western blotting (Section 3.6.).

3.6. Western blotting analysis

Both cell line as well as CD4+ cells were lysed using RIPA buffer pH 7.4 composed of 150 mM NaCl (Merck), 5 mM TRIS (Boehringer Mannheim), 1 mM EDTA (across organics), 1% Triton X 100 (Merck), 1% Na-deoxycholate (Merck), and 1 tablet of protease inhibitor (Roche). Proteins were mixed with sample buffer pH 6.8 composed of 250 mM Tris-HCl, 8% SDS, 40% glycerol, 2% β-mercaptoethanol, and 0.2% bromophenol blue supplemented with 10 mM NaF, 26.7 mM DTT, 200 mM $NaVO_3$ and 0.5% β-mercaptoethanol. Then, proteins were resolved by 12.5% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene fluoride (PVDF) transfer membranes (Immobilon-P, Millipore). Subsequently, membranes were incubated with APOBEC3G (1:5000), TRIM5α (1:10000), HA (1:5000) or LEDGF/p75 (1:5000) antibody overnight at 4°C, prior to incubation with horseradish peroxidase-conjugated anti-mouse or anti-rat Ig antibody (1:2000 – DakoCytomation) for 1 h at room temperature (RT). The antibody-protein interactions were detected using Immobilon™ Western Chemiluminescent Horse Radish Peroxidase Substrate (Millipore). In order to quantify APOBEC3G, TRIM5α or LEDGF/p75 expression, membranes were stripped with 0.2 M NaOH, and subjected to the same protocol as above, substituting primary antibody with mouse monoclonal antibody to GAPDH (1:30000 – 1 h incubation at RT) (Novus Biologicals, Inc.).

3.7. Microscopic analysis of intracellular stained PBMC

PBMC stained according to the ICS method were cytospinned onto a slide glass with the Cytospin3 centrifuge (Shandon) at 800 rpm for 5 minutes. Then, cells were washed with PBS, incubated with 5 μM propidium iodide (Sigma-Aldrich) for one minute, washed three times with PBS, and covered with a drop of citifluor (Ted Pella) and a cover glass. All detailed images included in this work were obtained using a microlens-enhanced dual spinning disk confocal microscope (Ultra*VIEW* ERS, PerkinElmer LAS) equipped with a three-line (488, 568, and 647nm) argon-krypton laser. Images were processed using the reconstruction facilities of the Volocity 5.2.2 software (Improvision).

3.8. Cell stimulation with P/I, IFNα or IFNβ

One healthy person's PBMC were cultured in complete RPMI medium with or without the addition of stimulation agents. APOBEC3G, TRIM5α, and LEDGF/p75 expression was studied in two independent experiments in CD4+ T cells and monocytes after 5 h of stimulation with P/I or 48 h of stimulation with IFNα or IFNβ. Mitogen concentrations added to the cells were 0.2 ng/ml PMA combined with 10 ng/ml ionomycin or 2 ng/ml PMA combined with 100 ng/ml ionomycin. Type I IFN concentrations of 0, 250, 500, 1000, or 2000 U/ml were added to the PBMC as well as to the CD4+ cells obtained by isolation with the Dynal CD4 Positive Isolation Kit (Invitrogen) according to the manufacturer's protocol. Corresponding to experiments by Chen et al [639], we also collected CD4+ cells through negative selection from three healthy donors' PBMC by means of the Dynabeads Untouched^{TM} Human CD4 T cells kit (Invitrogen). The next day, cells were stimulated with IFNα at concentrations of 0, 50, 300, 600, and 1000 U/ml for 7 h.

3.9. Reproducibility testing of the ICS method

Aliquots of $5x10^4$ PBMC from one control blood sample were stained 10 times in parallel for APOBEC3G, TRIM5α, or LEDGF/p75 to test the method's reproducibility. Coefficients of variance (CV) were calculated based on the collective MFI-values from the 10 protein stainings. This value had to be less than 10% to consider the method reproducible.

3.10. mRNA analysis of APOBEC3G, TRIM5α, and LEDGF/p75 in CD4+ T cells

The reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is described in Chapter 6 and [415]. In brief, the following steps were undertaken. CD4+ T cells were stored in TRIzol® Reagent (Invitrogen), RNA was isolated according to the TRIzol-protocol, and genomic DNA removed with the DNA-free Kit (Ambion). Total RNA levels were quantified with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific) prior to reverse transcription of the RNA with the iScript cDNA synthesis Kit (Bio-Rad Laboratories NV-SA). Messenger RNA

(mRNA) expression levels were quantified by real-time PCR using the Bio-Rad CFX96 system and the iQ SYBR Green Supermix (Bio-Rad). All the kits were used according to the manufacturer's protocol. Obtained results (Cq-values) were exported from the Bio-Rad CFX Manager version 1.5 and analyzed with the qbasePLUS software version 1.5 from BioGazelle (Ghent, Belgium). Finally, CNRQ (Calibrated Normalized Relative Quantity) values were exported and statistically correlated to MFI-values using SPSS as described in section 3.2.12.

3.11. Measurement of APOBEC3G, TRIM5α, and LEDGF/p75 protein expression

APOBEC3G, TRIM5α, and LEDGF/p75 protein expression levels were investigated in CD4+ T cell and CD14+ monocyte subsets based on simultaneous intracellular and cell surface staining of PBMC. Membrane staining for CD4/CD45RO and CD14/CD16 was performed to study APOBEC3G, TRIM5α, and LEDGF/p75 expression in CD45RO- (naïve) and CD45RO+ (memory) CD4+ T cells, and in CD16- and CD16+ CD14+ monocytes, respectively. Expression levels were determined in 10 HIV-1 negative (healthy control, HC), 10 therapy-naïve HIV-1 positive (HIV-UT), and 10 ART-treated HIV-1 positive (HIV-ART) subjects.

3.12. Statistical analyses

The influence of cell stimulation was studied with the non-parametric Friedman test. Differences in host protein expression levels between different cell-types as well as between different stimulation conditions were studied with non-parametric paired-sample Wilcoxon signed rank tests. Differences in expression levels between the study populations were investigated with non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Correlations were studied with non-parametric Spearman's rank correlation tests. Because of the relatively small sample sizes (for each group, n = 10), non-parametric tests were applied. Observations were considered statistically significant when $p < 0.05$. Statistical analyses were performed with SPSS, version 14.0, and graphs were drawn with GraphPad Prism, version 5.

4. Results

4.1. Optimization of the intracellular protein staining protocol

4.1.1. Determination of primary antibody incubation time and concentration

Cells were stained with anti-APOBEC3G, anti-TRIM5α, and anti-LEDGF/p75 mouse IgG1 primary antibodies. Optimization of the method demonstrated that prolonged incubation times with primary protein antibody resulted in increasing MFI-values (Figure 1A). Higher primary antibody concentrations resulted in further increases in protein MFI-values, albeit without reaching a saturation point (Figure 1B). In contrast, MFI-values remained low for isotype staining regardless of antibody incubation time and concentration. The isotype control (mouse IgG1 antibody directed against non-human keyhole limpet hemocyanin protein) was used at the same concentration as the specific antibodies to correct for non-specific background staining. Results for CD4+ T cells are shown and similar staining patterns were observed for the monocyte subset (data not shown). Since no saturation in MFI-value could be obtained with primary antibody concentrations titrated up to 10 μg/10^6 cells, we selected optimal primary antibody concentrations resulting in more than 90% of cells being positively stained relative to the isotype control. Thus, antibody concentrations of 2.5 μg/10^6 cells for APOBEC3G, 2.5 μg/10^6 cells for TRIM5α, and 5 μg/10^6 cells for LEDGF/p75 were obtained (Figure 1C). Under optimized conditions, intracellular protein staining resulted in a clear positive homogeneous cell staining as compared to isotype control staining (Figure 1D). Notably, all cells within lymphocyte and monocyte subsets stained positive for the three proteins of interest.

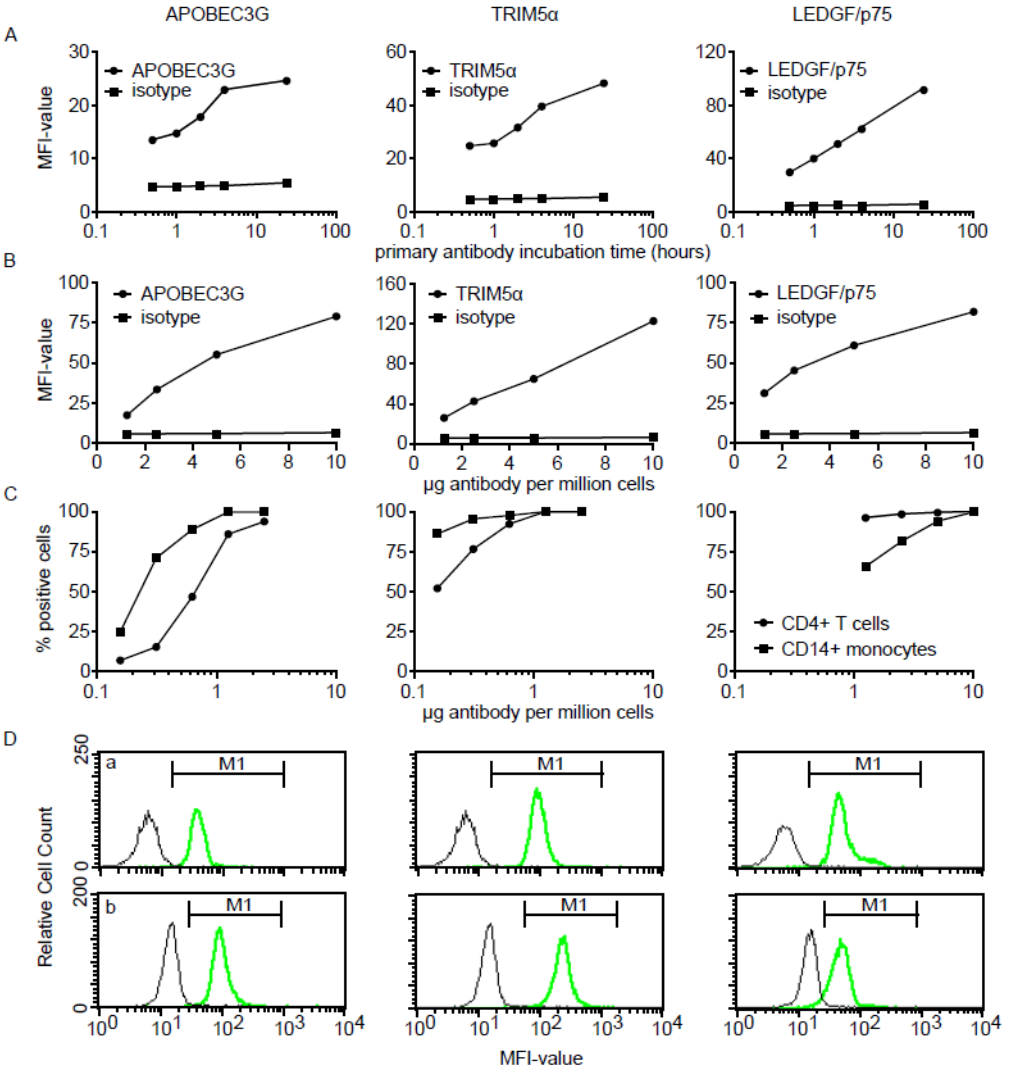

Figure 1 – Optimization of intracellular staining for APOBEC3G, TRIM5α, and LEDGF/p75. (A) Variation in primary antibody incubation time. Graphs show MFI-values of APOBEC3G, TRIM5α, and LEDGF/p75 versus isotype background staining in CD4+ T cells following primary antibody incubation for 0.5 h, 1 h, 2 h, 4 h and 24 h. (B) Titration of primary antibody concentration versus MFI-value. Primary antibody concentrations ranging from 1.25 µg/10^6 cells up to 10 µg/10^6 cells were used for APOBEC3G, TRIM5α, and LEDGF/p75 versus isotype staining. Graphs demonstrate protein levels expressed in MFI-value (y-axis) versus antibody concentration (x-axis) in CD4+ T cells. (C) Titration of primary antibody concentration versus percentage positive cells. The percentage of cells positively stained (y-axis) for APOBEC3G, TRIM5α, and LEDGF/p75 are depicted versus the concentration of primary antibody (x-axis) in both CD4+ T cells and monocytes. This percentage is calculated based on the number of positively stained cells enclosed by the M1 marker (as depicted in Figure 1D) versus all stained cells. (D) Histograms depict fluorescence intensity (x-axis) of APOBEC3G, TRIM5α, and LEDGF/p75 (green lines) next to isotype (black lines) staining versus Relative Cell Count (y-axis). Histograms are shown for CD4+ T cells (a) and monocytes (b).

4.1.2. Determination of the specificity of the ICS method

To assess the specificity of the staining, excess amounts of recombinant APOBEC3G (purified from E. coli), TRIM5α (purified from baculovirus-infected insect cells), and LEDGF/p75 (purified from E. coli) were added to the respective primary antibodies, three hours prior to incubation with PBMC (Figure 2A). Intracellular protein staining of APOBEC3G, TRIM5α, and LEDGF/p75 in CD4+ T cells was partially blocked by 39%, 38%, and 63%, respectively. These decreases in MFI-value suggest specific intracellular protein staining by the antibodies.

In addition, the method's specificity was addressed by expression analysis in cell lines expressing distinct levels of the respective protein. APOBEC3G was studied in HeLa cells with (HeLa-A3G) and without (HeLa) APOBEC3G expression. TRIM5α was addressed in Cf2Th cells with (Cf2Th-TRIM5α) and without (Cf2Th-pLPCX) HA-tagged human TRIM5α. HeLa cells with regular (WT) and reduced (KD) LEDGF/p75 levels were used to study LEDGF/p75. Intracellular protein expression (Figure 2B) of APOBEC3G was on average 63% higher in HeLa-A3G when compared to HeLa cells. TRIM5α staining was on average 29% enhanced in Cf2Th-TRIM5α versus control Cf2Th cells, and expression of LEDGF/p75 was found to be on average 38% lower in LEDGF/p75-KD HeLa cells as compared to WT HeLa cells. Moreover, the corresponding western blots (Figure 2C) confirmed specific interactions of all three antibodies with their antigen, although non-specific bands were also observed.

Finally, PBMC were stained according to the ICS method and visualized by confocal fluorescence microscopy. Results showed expression of APOBEC3G and TRIM5α located mainly in the cytoplasm, whereas LEDGF/p75 was predominantly present in the nucleus (Figure 2D).

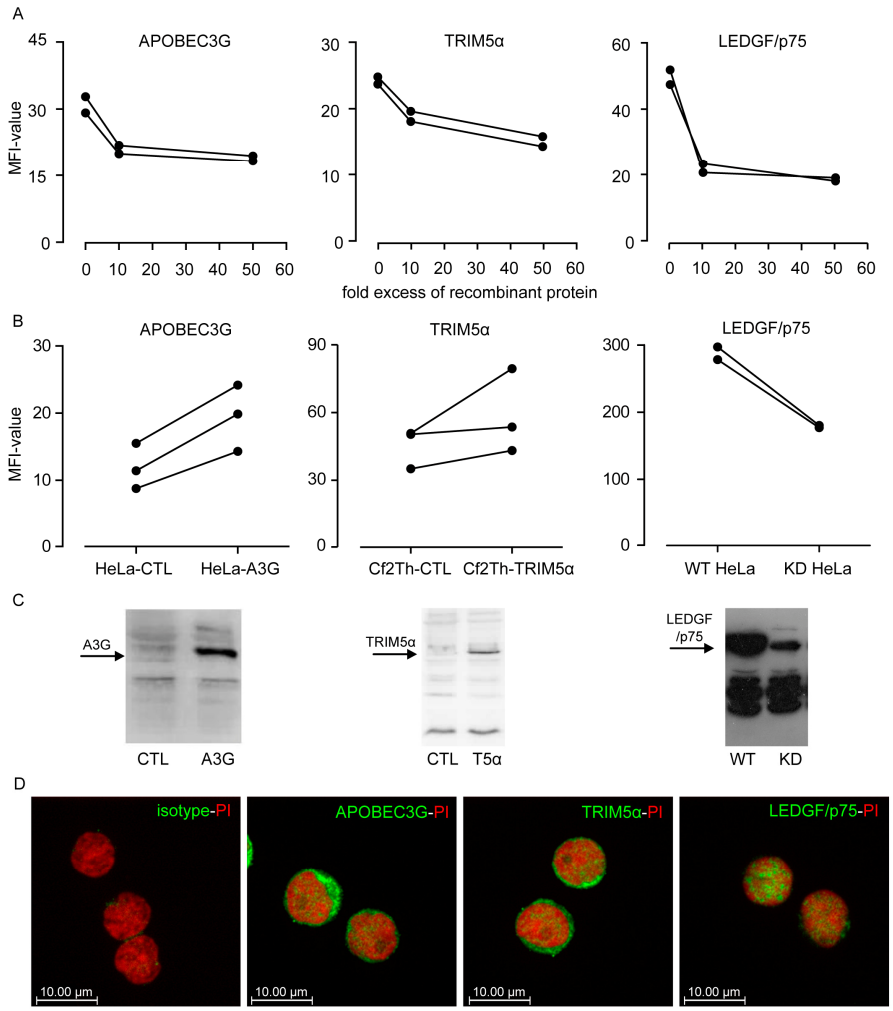

Figure 2 - Specificity of the intracellular staining for APOBEC3G, TRIM5α, and LEDGF/p75. (A) CD4+ T cells were stained for the expression of APOBEC3G, TRIM5α, and LEDGF/p75 in the presence of 0-, 10- or 50-fold molar excess of corresponding recombinant protein. MFI-values of duplicate results (y-axis) are represented versus the concentration (in fold excess) of recombinant protein added (x-axis). Next, APOBEC3G, TRIM5α, and LEDGF/p75 expression levels were studied in specific cell lines through ICS (B) or western blotting (C). APOBEC3G was studied in triplicate in control HeLa cells (CTL) and HeLa-A3G cells (A3G). TRIM5α was studied in triplicate in control Cf2Th cells (CTL) and Cf2Th cells expressing human TRIM5α (T5α). LEDGF/p75 was investigated in duplicate in HeLa (WT) and LEDGF/p75-KD (KD) HeLa cells. The graphs depict the ICS results with MFI-value in the y-axis and the cell line studied in the x-axis. One representative blot is shown for each protein studied. The arrow points to the protein band of interest. (D) Single confocal images of PBMC at the level of the cell nucleus show the fluorescent staining of isotype (negative control), APOBEC3G, TRIM5α, and LEDGF/p75 (FITC, green) versus nuclear heterochromatin (PI, red). Scale bars represent 10.00 µm.

4.1.3. Determination of APOBEC3G, TRIM5α, and LEDGF/p75 expression levels following stimulation

Stimulation experiments were set up to investigate whether the expression of APOBEC3G, TRIM5α, and LEDGF/p75 expression levels altered following cell stimulation. Primarily, one healthy person's PBMC were stimulated in duplicate with increasing concentrations of phorbol myristate acetate (PMA) in combination with ionomycin (P/I) for 5 hours (Figure 3A). The mitogens (P/I) were chosen based on their general ability to stimulate T cells. The stimulation showed a trend (although non-significant) towards increased expression levels of APOBEC3G and TRIM5α in CD4+ T cells, while no difference in expression was observed for LEDGF/p75.

Also, stimulation experiments with type I IFN were implemented. Initially, PBMC were stimulated in duplicate with increasing concentrations of interferon-alpha (IFNα) or interferon-beta (IFNβ) for 48 hours. In parallel, based on literature, naïve CD4+ T cells of three healthy donors were stimulated with increasing concentrations of IFNα for 7 hours prior to intracellular staining of APOBEC3G [98]. Following stimulation of PBMC with type I IFN, APOBEC3G expression increased weakly in CD4+ lymphocytes (Figure 3B), whereas APOBEC3G expression levels remained relatively constant following stimulation of purified CD4+ T cells with IFNα (Figure 3C) or IFNβ (data not shown). Based on both ICS and western blotting, a limited induction in TRIM5α expression was observed in CD4+ T cells following stimulation with IFNα (Figure 3D) and IFNβ (data not shown).

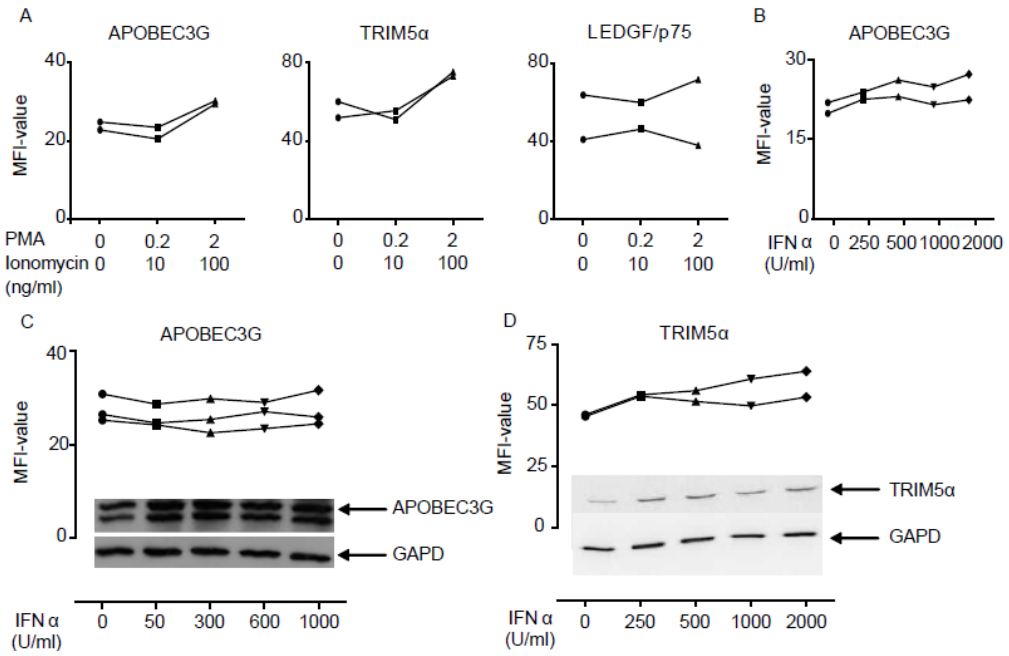

Figure 3 – Expression of APOBEC3G, TRIM5α, and LEDGF/p75 following stimulation. The graphs depict protein expression levels described by MFI-value (y-axis) versus increasing concentrations of stimulation agent (x-axis) in CD4+ T cells. Western blotting images show protein bands of CD4+ cells. (A) One healthy person's PBMC were stimulated for 5 hours, in duplicate, with PMA and ionomycin and stained for APOBEC3G, TRIM5α, and LEDGF/p75. (B) One healthy person's PBMC were stimulated, in duplicate, for 48 hours with IFNα and intracellularly stained for APOBEC3G. (C) Three healthy donors' CD4+ T cells were stained for APOBEC3G by ICS and western blotting following 7 hours of stimulation with IFNα as similar to the experiment of Chen et al [639]. One representative blot is depicted. (D) TRIM5α protein staining of the CD4+ cells stimulated with IFNα was also studied by ICS and western blotting. For both western blots, GAPDH staining was included as loading control.

4.1.4. Determination of the reproducibility of the ICS method

In order to test the intra-assay variation, the staining procedure for APOBEC3G, TRIM5α, and LEDGF/p75 was repeated 10 times on the same control blood sample. The percentage of deviation in MFI-value versus the mean MFI-value is shown in Figure 4. Overall, the coefficients of variance (CVs) for APOBEC3G, TRIM5α, and LEDGF/p75 were consistently below 10% (values in CD4+ T cells were 2.75%, 3.96%, and 7.91%, respectively), implying that the method was reproducible.

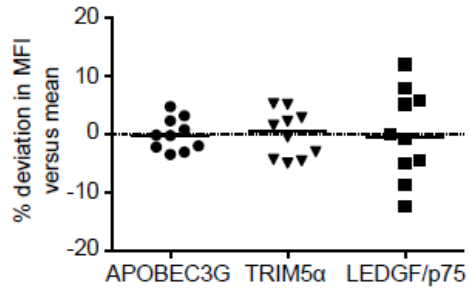

Figure 4 – Reproducibility of the method. Staining of APOBEC3G, TRIM5α, and LEDGF/p75 was repeated ten times using PBMC from one healthy control subject. The graph presents the percentage of deviation in MFI-values in CD4+ T cells versus the mean MFI (y-axis) for each protein studied. This value was calculated by the formula: (MFI / mean MFI * 100) – 100. The median values of the 10 measurements are represented by the horizontal lines.

4.1.5. Determination of the correlation between mRNA and protein expression

An RT-qPCR experiment was conducted to verify whether mRNA and protein expression levels of the three host factors correspond. Both mRNA and protein levels were measured in CD4+ T cells of 10 Senegalese healthy controls. No significant correlations were observed between mRNA and protein levels of APOBEC3G ($p = 0.676$), TRIM5α ($p = 0.260$), and LEDGF/p75 ($p = 0.489$) as depicted in Figure 5.

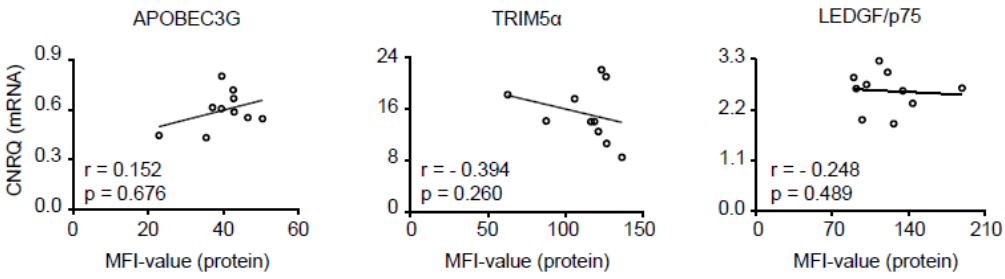

Figure 5 – Correlation between mRNA and protein expression levels. APOBEC3G, TRIM5α, and LEDGF/p75 mRNA and protein expression levels were studied in CD4+ T cells of ten healthy controls, respectively by RT-qPCR and the indirect intracellular staining method. Graphs show the correlation between the CNRQ- and MFI-values of APOBEC3G, TRIM5α, and LEDGF/p75 (for each $n = 10$). Correlations were analyzed with the non-parametric Spearman's rank correlation test. $P < 0.05$: significant difference; r: correlation coefficient.

4.2. Application of the intracellular protein staining protocol

4.2.1. APOBEC3G, TRIM5α, and LEDGF/p75 expression in CD4+ T cell and monocyte subsets

Distinct PBMC subsets exert different levels of susceptibility to HIV-1 infection. CD4+ T cells and the monocyte-macrophage lineage are the main targets for HIV-1 infection [640], of which CD4+ T cells show greater susceptibility to infection than monocytes [641]. Within these cell types, memory CD4+ T cells are preferentially infected by HIV-1 [642]; and CD16+ monocytes, known to modulate inflammatory responses, also display a relatively increased susceptibility [643]. To compare APOBEC3G, TRIM5α, and LEDGF/p75 protein expression levels between different cell subtypes, intracellular staining was combined with specific membrane staining prior to flow cytometry. Staining for CD45RO was used to discriminate naïve from memory CD4+ T cells (Figure 6A), while staining for CD16 was used to subdivide CD14+ monocytes into CD16- and CD16+ subsets (Figure 6B).

Within the CD4+ T cell subset of ten healthy individuals, memory CD4+ T cells showed significantly higher expression of APOBEC3G, TRIM5α, and LEDGF/p75 than naïve CD4+ T cells ($p = 0.005$ for all, Figure 6C). Within the monocyte subset, CD16+ monocytes showed significantly higher expression levels of LEDGF/p75 than CD16- monocytes ($p = 0.005$), whereas no differences were observed in APOBEC3G or TRIM5α expression (Figure 6D). Similar results were obtained for both therapy-naïve and ART-treated HIV-1 seropositive patient groups (data not shown).

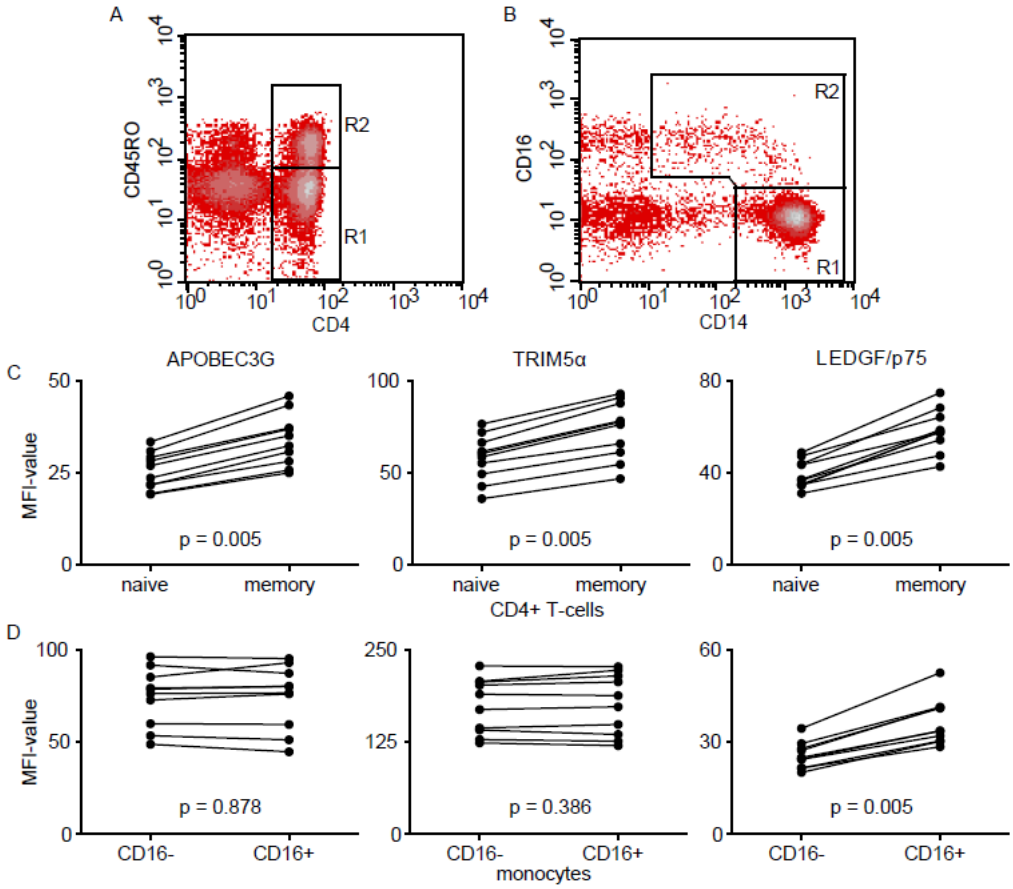

Figure 6 – Differential expression of APOBEC3G, TRIM5α, and LEDGF/p75 in different cell subtypes. (A) PBMC were stained with anti-CD4-PE and anti-CD45RO-APC to discriminate the lymphocytes into CD4+ T cells (combination of R1 and R2), naïve CD4+ T cells (CD45RO-, R1), and memory CD4+ T cells (CD45RO+, R2). (B) PBMC were stained with anti-CD14-APC and anti-CD16-PE to discriminate the monocytes into CD14+ monocytes (combination of R1 and R2), CD16- CD14+ monocytes (R1), and CD16+ CD14+ monocytes (R2). (A, B) Cell density is represented by red (low density) to grey (high density) color coding. (C) Graphs show the distribution in protein expression (MFI, y-axis) of APOBEC3G, TRIM5α, and LEDGF/p75 in ten HIV-1 negative persons between naïve and memory CD4+ T cells. (D) Graphs show the distribution in protein expression (MFI, y-axis) of APOBEC3G, TRIM5α, and LEDGF/p75 in ten HIV-1 negative persons between CD16- and CD16+ monocytes. (C-D) Black lines connect MFI-values measured in the same person. P-values were obtained by paired Wilcoxon signed rank tests.

4.2.2. APOBEC3G, TRIM5α, and LEDGF/p75 expression in HIV-1 patients and controls

Next, we analyzed the expression levels of APOBEC3G, TRIM5α, and LEDGF/p75 in therapy-naïve HIV-1 patients, ART-treated HIV-1 patients, and healthy controls (Figure 7). The characteristics of the different study populations are shown in Table 1. We did not observe a statistically significant difference in APOBEC3G, TRIM5α, and LEDGF/p75 expression level between the different study groups. Nevertheless it remains worthwhile mentioning that we observed a trend towards reduced expression levels of APOBEC3G and TRIM5α, but not of LEDGF/p75, in the CD4+ T cells of therapy-naïve HIV-1 patients when compared with healthy controls and ART-treated HIV-1 patients. Similar trends were found for the monocytes and for the different CD4+ T cell and monocyte subsets (data not shown).

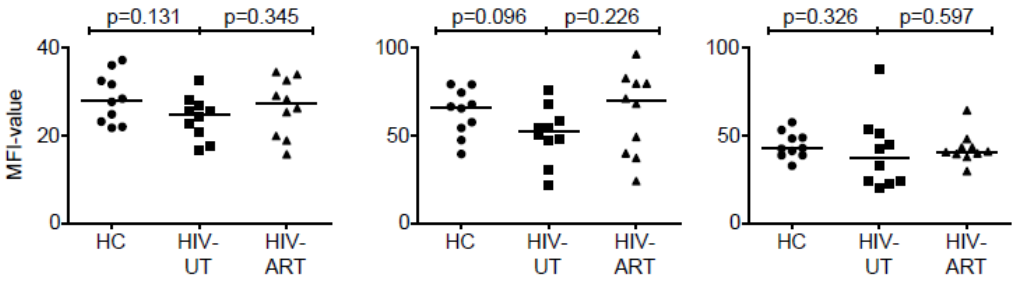

Figure 7 – Differential expression of APOBEC3G, TRIM5α, and LEDGF/p75 in different study populations. Expression of APOBEC3G, TRIM5α, and LEDGF/p75 in CD4+ T cells of 10 HIV-1 seronegative persons (HC), 10 therapy-naïve (HIV-UT) and 10 ART-treated (HIV-ART) HIV-1 seropositive patients. P-values are calculated with Mann Whitney *U* tests. Median MFI-values are represented by the horizontal lines.

4.2.3. Correlation analyses of expression levels of APOBEC3G, TRIM5α, and LEDGF/p75

Finally, we analyzed the degree of correlation between APOBEC3G, TRIM5α, and LEDGF/p75 expression in the peripheral blood CD4+ T cells and the monocytes of HIV-1 patients and controls. In CD4+ T cells, APOBEC3G and TRIM5α expression showed the strongest correlation, while correlations of APOBEC3G and TRIM5α with LEDGF/p75 were weaker or non-existent (Figure 8). Similar results were obtained for the monocytes. However, within the group of therapy-naïve HIV-1 patients, correlations between APOBEC3G and LEDGF/p75, as well as between TRIM5α and LEDGF/p75, were also apparent (data not shown).

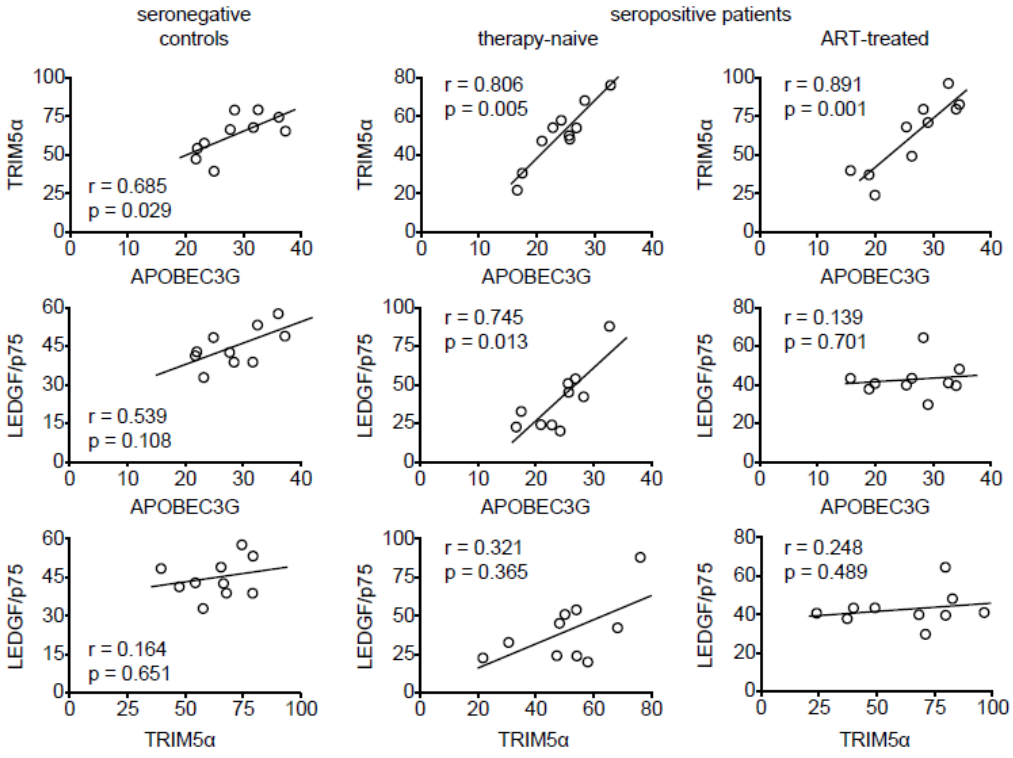

Figure 8 – Correlations between APOBEC3G, TRIM5α, and LEDGF/p75 expression. Graphs show the correlation between the MFI-values of APOBEC3G versus TRIM5α, of APOBEC3G versus LEDGF/p75, and of TRIM5α versus LEDGF/p75 from seronegative persons, therapy-naïve and ART-treated HIV-1 seropositive subjects (for each n = 10) in CD4+ T cells. Correlations were analyzed with the non-parametric Spearman's rank correlation test. $P < 0.05$: significant difference; r: correlation coefficient.

5. Discussion

Thus far, expression levels of host factors like APOBEC3G, TRIM5α, and LEDGF/p75 have been studied predominantly by mRNA-based quantitative PCR [100,208,621,622,628–634] and oligonucleotide microarrays [417,627], as well as by protein-based western blotting [100,622,628–630,632,634] and ELISA [621]. These techniques are less appropriate to study protein expression levels in distinct cell types. The present proof-of-principle study describes an ICS assay to measure expression of the host proteins APOBEC3G, TRIM5α, and LEDGF/p75 at single-cell level, based on intracellular fluorescent staining in combination with flow cytometry.

The intracellular staining protocol was optimized to yield clear positive protein staining patterns relative to background staining. Visualization of these intracellular proteins through confocal imaging supports the overall sub-cellular localization of APOBEC3G [644] and TRIM5α [489] in the cytoplasm, and of LEDGF/p75 [645] in the nucleus. Pre-incubation of the antibodies with recombinant proteins resulted in blocking of intracellular staining. Also, differential staining patterns could be observed between stably transfected or knock-down cell lines relative to control cell lines for APOBEC3G, TRIM5α, and LEDGF/p75, as validated by western blotting. In addition, stimulation with mitogen, interferon-alpha, or interferon-beta resulted in detectable, albeit weak, increases in intracellular expression of APOBEC3G and TRIM5α. All the above experiments support the notion that the optimized intracellular staining method is relatively specific. Furthermore, the staining method was shown to be very reproducible as demonstrated by low intra-run coefficients of variance.

However, although fluorescence signals could be augmented after overnight incubation with primary antibody and with increasing primary antibody concentrations, intracellular staining could not be fully saturated. Similarly, the staining could only be partially blocked, background staining was observed following ICS and western blotting experiments on cell lines, and induction of protein levels was relatively weak following stimulation. These observations might be explained by the selected antibodies which exhibit some degree of cross-reactivity

with abundantly expressed and/or related proteins belonging to the same protein family, e.g. APOBEC3B or APOBEC3F in the case of APOBEC3G [646], TRIM5δ, γ, or ε isoforms or family members like TRIM4 and TRIM21 in the case of TRIM5α [647,648], and LEDGF/p52 in the case of LEDGF/p75 [649]. Indeed, ICS experiments in our lab showed that the recombinant proteins APOBEC3B and APOBEC3F can also partially block APOBEC3G intracellular staining (data not shown). Also, based on some of the western blotting experiments performed, proteins with similar molecular weight like TRIM5α sometimes appeared in the control Cf2Th cell line (data not shown). Hence, to further improve the performance of this intracellular staining assay, antibodies with narrower specificities for the proteins of interest should be selected when they become available.

As for the IFN experiments, conflicting results are described in literature about whether APOBEC3G expression levels increase [639] or remain constant [632,634] following stimulation of CD4+ lymphocytes with IFNα. Interestingly, when we attempted to enhance APOBEC3G expression in CD4+ lymphocytes by stimulation with IFNα according to Chen et al. [639], we could not confirm the induction they observed neither by intracellular staining nor by western blotting (Figure 3C). Although this result was unexpected, our findings are in agreement with previously observed unaltered APOBEC3G protein expression levels in T cells after IFNα stimulation [632,634,650–652]. Nevertheless, we observed a weak but detectable increase of APOBEC3G in CD4+ lymphocytes after type I IFN stimulation of total PBMC. Also, expression of TRIM5α in CD4+ cells appeared weakly induced following stimulation of the cells with IFN as observed by ICS and western blotting. Both these results correspond to previously observed enhancements of APOBEC3G and TRIM5α by IFN [100,628,629,639,653–655].

In addition, we could not find a statistically significant correlation between mRNA and protein expression levels of the three host proteins in CD4+ T cells of ten healthy controls (Figure 5). This observation is supported by the knowledge that mRNA expression does not necessarily guarantee translation into (functional) protein [656]. A partial explanation for this discrepancy could be that certain external factors like e.g. Vif may act differently upon protein and mRNA

levels [635]. Moreover, as proteins are considered to be the "work horses" of the cell, they are of greater relevance in the "direct" fight against HIV- infection. Above arguments underscore the need to include protein data instead of focusing solely on mRNA expression.

Although the precise mechanisms by which HIV-1 replication is restricted in particular cell-types and/or study populations remain unidentified, previous reports have suggested a role for APOBEC3G and TRIM5α [417,621,622,629]. Therefore, intracellular expression of APOBEC3G, TRIM5α, and LEDGF/p75 was studied by intracellular staining of subsets of peripheral blood mononuclear cells from HIV-1 patients and controls. Results demonstrated higher expression levels of LEDGF/p75 in memory CD4+ T cells than in naïve CD4+ T cells, which is in line with the differences in virus susceptibility that have been noted for these subsets [642,657,658]. However, we found that memory CD4+ T cells also showed higher levels of APOBEC3G and TRIM5α expression than naïve CD4+ T cells, which does not fit this model. This suggests that the expression levels of these two factors do not correlate with the preferential tropism of HIV-1 for memory CD4+ T cells, or that their antiviral effects may be masked by LEDGF/p75.

Previously, CD16+ monocytes were demonstrated to be more permissive to HIV-1 infection than their CD16- counterparts [643,659], which was proposed to be the consequence of elevated expression of the co-receptor CCR5 (CC-chemokine receptor 5) [660] and/or expression of APOBEC3G in its enzymatically inactive high molecular mass form [643]. In our study, we observed significantly higher expression levels of cellular co-factor LEDGF/p75 in CD16+ monocytes, which confirms and extends previous findings regarding susceptibility. In contrast, we did not detect any differences in APOBEC3G and TRIM5α staining between CD16+ and CD16- monocytes, suggesting that the expression levels of these proteins do not play a role in these cell types.

Next, differential expression levels were studied between healthy controls, ART-treated and treatment-naïve HIV-1 positive individuals. Each study population was represented only by ten persons, which might explain the relatively low power of the statistical comparisons

made. Although statistically not underscored, we did observe a trend towards reduced APOBEC3G and TRIM5α protein expression in therapy-naïve HIV-1 patients as compared to healthy controls, which is in agreement with the observed reduction in APOBEC3G and TRIM5α mRNA levels in treatment-naïve HIV-1 patients [208,631,661]. Clearly, experiments on higher patient numbers are needed to verify this finding. Furthermore, we found that APOBEC3G and TRIM5α protein levels in therapy-naïve HIV-1 patients tended to inversely correlate with the viral load and directly correlate with the CD4 count (data not shown), which also confirms the findings of a previous mRNA expression study [631]. These data support the notion that APOBEC3G and TRIM5α exhibit antiviral activities resulting in reduced viral load and enhanced CD4 count. Alternatively, one might reason that HIV-1 is capable of suppressing expression and function of the intrinsic restriction factors APOBEC3G and TRIM5α. Indeed, HIV-1 Vif is known to induce ubiquitin-dependent degradation of APOBEC3G, prevent its incorporation into virus particles, and impair its de novo synthesis [635]. In addition, decreases in intrinsic factor levels can also be a sign of ongoing virus replication as was recently shown for TRIM5α, which is rapidly sent for proteasomal degradation after forming complexes with incoming viral capsids [475].

Finally, we demonstrated direct correlations between APOBEC3G and TRIM5α levels in the CD4+ T cell and monocyte subsets of HIV-1 patients and controls, while weak or absent correlations were observed with LEDGF/p75. These correlation data suggest that these intrinsic antiviral factors might share a common signal transduction pathway, distinct from the one that regulates LEDGF/p75.

In conclusion, we show that measuring intracellular expression levels of APOBEC3G, TRIM5α, and LEDGF/p75 via a flow cytometry-based assay is a valuable approach that needs further attention. Although the procedure should be optimized further when more specific antibodies become available, we were able to observe differential expression levels of APOBEC3G, TRIM5α, and LEDGF/p75 in peripheral blood mononuclear cell subsets of HIV-1 patients and controls. Hereby, we underline proof-of-principle of the method allowing simultaneous analysis of specific host factors at single-cell level, which may prove useful for further

understanding of the complex virus-host interactions in a more holistic manner. Possibly, this type of knowledge may contribute to the development of immune intervention strategies for the prevention or treatment of HIV-1 infection.

6. Acknowledgements

We thank the individuals who kindly participated in the study and Katrien Fransen, Annelies Van Den Heuvel and Tine Vermoesen from the ITM AIDS Reference Laboratory for sample collection and viral load testing. We are grateful to the Department of Infectious Diseases (Fann University Teaching hospital, Dakar, Senegal) for the provision of the ten Senegalese healthy control PBMC samples, to Joseph Sodroski (Harvard Medical School, Boston, USA) for the Cf2Th-pLPCX and Cf2Th-TRIM5α cell lines and to Zeger Debyser (Catholic University of Leuven, KUL, Belgium) for the recombinant LEDGF/p75 protein, the LEDGF/p75 knock-down and wild-type HeLa cell lines. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Rockville, MD): monoclonal antibodies against APOBEC3G and TRIM5α, recombinant APOBEC3G and TRIM5α proteins, HeLa and HeLa-A3G cell lines. Also, we would like to thank Géraldine Daneau from the ITM for her intellectual help with the RT-qPCR experiment.
This work is supported by the Fund for Scientific Research Flanders (FWO) (grant G.0660.06) and an FWO doctoral fellowship to KM.

Chapter 4: Optimization of the expression analysis of total and membrane tetherin protein levels by flow cytometry

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(*Unpublished data*)

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1. Abstract

Objective: In the previous chapter, we described the optimization procedure for a flow cytometry-based intracellular staining (ICS) assay to study expression levels of three HIV-1-related intracellular host proteins being APOBEC3G, TRIM5α, and LEDGF/p75. Now, we extend this application to study the expression of both total and membrane tetherin (also known as BST2, CD317 or HM1.24).

Methods: The indirect protein staining method was further optimized to study total (ICS, intracellular staining) as well as membrane (ECS, extracellular staining) expression of tetherin. Mouse IgG2a antibody against human tetherin was used, as kindly provided by Chugai Pharmaceutical Co., Japan.

Results: Tetherin titration experiments showed saturation of median fluorescence intensity (MFI-)values at higher antibody concentrations. Stimulation experiments with IFNα induced tetherin expression levels. Blocking of the tetherin staining with recombinant tetherin resulted in reduced MFI-values. ICS/ECS and western blotting experiments on stably tetherin transfected HEK293T cell-lines supported the method's specificity. Confocal microscopy analysis of stained peripheral blood mononuclear cells (PBMC) showed distinct patches of membrane tetherin relative to diffuse cytoplasmic expression of total tetherin. Visualization of IFNα-induced tetherin expression showed increased numbers of such membrane tetherin patches.

Conclusions: The ICS/ECS method appears useful in the detection of constitutively expressed and stimulation induced tetherin. Knowledge on expression patterns of the HIV-1-related host proteins LEDGF/p75, APOBEC3G, TRIM5α, and tetherin may provide further insight in the role of these proteins in HIV-1 infection.

2. Introduction

Viruses like HIV-1 interact with different cellular host proteins during their replication cycle. Recently, the host restriction protein tetherin was demonstrated to inhibit the release of newly formed virions by tethering newly formed retrovirus particles to the cell membrane [520]. The significance of the expression profile of tetherin as possible correlate of protection against HIV remains to be determined. Here, we optimized a protein staining protocol to study total and membrane tetherin. Therefore, we used procedures described in literature [213–217] and the ICS-protocol as previously optimized for the detection of APOBEC3G, TRIM5α, and LEDGF/p75 (see Chapter 3 and [456]). We succeeded in staining both total and membrane tetherin specifically. In Chapter 6, this ICS method was applied to study LEDGF/p75, APOBEC3G, TRIM5α, and tetherin expression patterns in study populations including HIV-exposed seronegative subjects, healthy controls, and HIV-1-infected subjects.

3. Methods

3.1 Sample collection and processing

To optimize the membrane and total tetherin staining protocol, fresh blood cells from healthy volunteers were used. Fresh whole blood was collected in EDTA-coated blood tubes (BD Biosciences). Peripheral blood mononuclear cells (PBMC) were separated by density gradient separation using Ficoll Paque^{TM} PLUS (GE Healthcare Biosciences AB), washed in Hank's balanced salt solution (HBSS, Invitrogen) and re-suspended in complete RPMI medium at the desired final concentration, as also described in Chapter 3.

3.2 Reagents

The washing buffer, Leukoperm reagents A and B, IFNα, normal mouse serum, and final fixation solution (1% paraformaldehyde in PBS) were equal to the reagents described in Chapter 3. Primary unlabeled high-affinity mouse anti-human tetherin IgG2a monoclonal antibody was kindly provided by Chugai Pharmaceutical Co., Japan.

The isotype mouse IgG2a antibody against non-human keyhole limpet hemocyanin protein, the secondary rat anti-mouse IgG2a FITC-labeled antibody, and the membrane antibodies anti-CD3-PE, anti-CD14-APC, and anti-CD4-PerCP were all purchased from BD Biosciences. The recombinant human tetherin protein (P02) was purchased from Abnova.

3.3 Intra- and extracellular protein staining

Anti-tetherin or isotype mouse IgG2a antibodies were used as primary antibody and an excess of FITC-labeled anti-IgG2a mouse antibody as secondary antibody respectively to stain tetherin or to control for background staining. To study total tetherin levels, the intracellular staining (ICS) protocol was applied in which cells were fixed, permeabilized, indirectly stained with unlabeled primary antibody (5 µg/million cells for 24 h at 4°C) and FITC-labeled secondary antibody (1µg for 30 min at 4°C) respectively, blocked with normal mouse serum (10 min at 4°C), and then stained with fluorescently labeled membrane antibodies (20 min at 4°C). To study surface expression levels of tetherin, PBMC were processed according to the extracellular staining (ECS) protocol in which cells are directly stained with unlabeled primary antibody (1 µg/million cells for 30 min at 4°C) and then with an excess of FITC-labeled secondary antibody (1µg for 30 min at 4°C). Finally, a blocking step with normal mouse serum (10 min at 4°C) was performed followed by incubation with fluorescently labeled membrane antibodies (20 min at 4°C).

For both ICS and ECS protocol, protein expression levels were studied based on median fluorescence intensity (MFI-)values. For all results described, host protein MFI-values were corrected for non-specific background staining by subtracting the isotype MFI.

3.4 Cell stimulation with IFNα

PBMC were cultured in complete RPMI medium for 24 h with or without the addition of 500 U/ml or 2000 U/ml of IFNα. Stimulation experiments were performed in duplicate prior to total or membrane tetherin staining.

3.5 Specificity testing

Blocking experiments required pre-incubation of the primary antibody (5 µg/10^6 cells for total tetherin; 1 µg/10^6 cells for membrane tetherin) with a 0-, 10- or 50-fold molar excess of its respective recombinant protein for 2.5 h. Next, fresh PBMC and PBMC stimulated with IFNα (2000 U/ml, 24 hours) were stained with one of the antibody/recombinant protein mixtures according to the above described ICS and ECS protocols. The experiment was performed in duplicate.

Cell line experiments were performed using a control HEK293T cell line (clone 23), and two HEK293T cell lines that stably overexpress tetherin (clone 17 and 6.2) as kindly provided by Dr. J. Guatelli (University of California San Diego, USA). The two variants of stable tetherin-overexpressing cell lines expressed a different level of tetherin. Cells were cultured in EMEM medium supplemented with 10% FBS, 1% P/S, and 100 µg/ml zeocin (invitrogen).

Western blotting analyses were performed according to the protocol as described in Chapter 3. Western blots were incubated with anti-tetherin antibody (1:5000) overnight at 4°C, prior to incubation with horseradish peroxidase-conjugated anti-mouse Ig antibody (1:2000) 1 h at RT.

Confocal fluorescent images from fresh PBMC and PBMC stimulated with IFNα (2000 U/ml, 24 hours) stained according to the ICS or ECS method were obtained as described in Chapter 3.

4. Results

4.1 Primary antibody titration

Cells were stained with anti-tetherin IgG2a primary antibody using the ICS and ECS protocols. Optimization of the method demonstrated that increasing primary antibody concentrations resulted in saturation of tetherin MFI-values (Figure 1). Isotype MFI-values remained constant upon titration in CD4+ T cells during the ECS protocol, whereas these values increased slightly when the ICS protocol was applied and/or when monocytes were stained. This observation suggests somewhat higher non-specific binding of the mouse IgG2a antibody, especially upon intracellular and/or monocyte staining. Antibody concentrations of 5 µg/10^6 cells for total tetherin and of 1 µg/10^6 cells for membrane tetherin were used in subsequent experiments. Of note, all cells within lymphocyte and monocyte subsets stained positive for tetherin, even though MFI-values were low particularly in CD4+ lymphocytes.

4.2 Determination of the method's specificity

As intrinsic tetherin expression levels in PBMC were low, stimulation experiments were set up. Indeed, previous reports have shown that interferon can augment tetherin expression [217,524,547]. In case our staining techniques would demonstrate this increase, this would be a first indication for the specificity of the method. One healthy person's PBMC were stimulated in duplicate with increasing concentrations of IFNα for 24 hours (Figure 2). The stimulation clearly showed increased expression levels of tetherin.

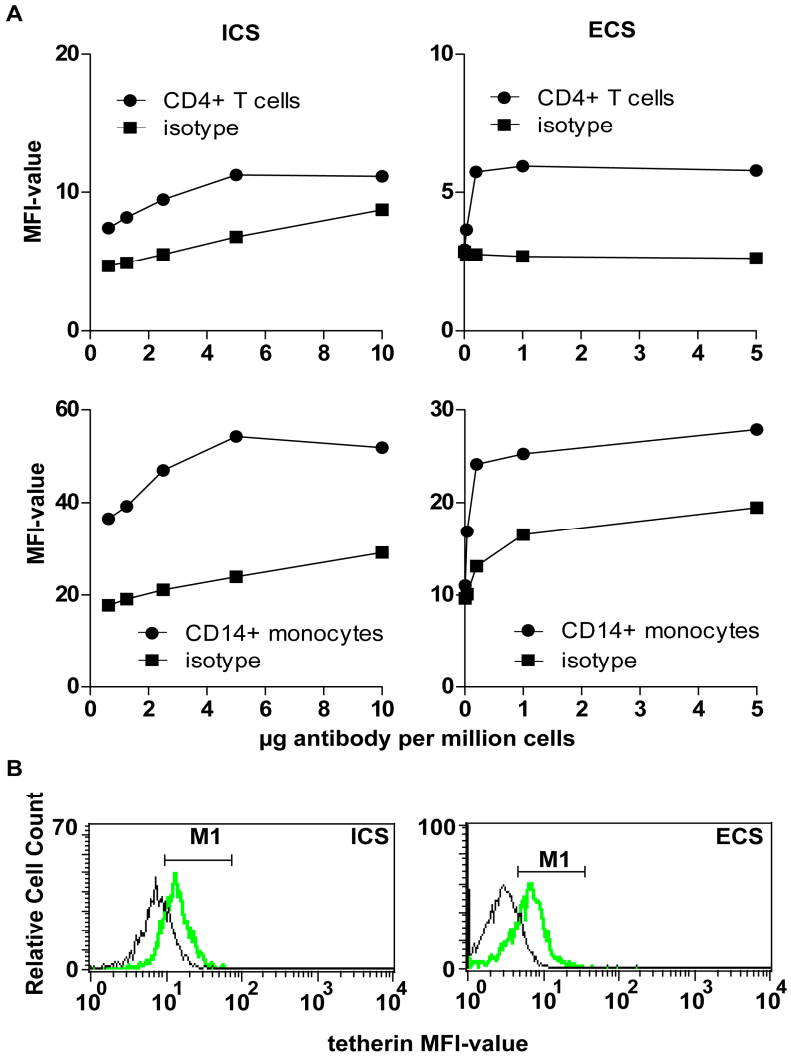

Figure 1 – Titration of primary antibody concentration versus MFI-value. (A) Primary antibody concentrations ranging from 0.625 µg/10^6 cells up to 10 µg/10^6 cells were used for tetherin versus isotype staining of total tetherin (ICS protocol; left). Primary antibody concentrations ranging from 0.008 µg/10^6 cells up to 5 µg/10^6 cells were used for tetherin versus isotype staining of membrane tetherin (ECS protocol; right). Graphs depict protein levels expressed in MFI-value (y-axis) versus antibody concentration (x-axis) in CD4+ T cells (upper) and CD14+ monocytes (below). (B) Histograms depict fluorescence intensity (x-axis) of tetherin (green lines) next to isotype (black lines) staining versus Relative Cell Count (y-axis). Histograms are shown for total tetherin (ICS) and membrane tetherin (ECS) measured in CD4+ T cells.

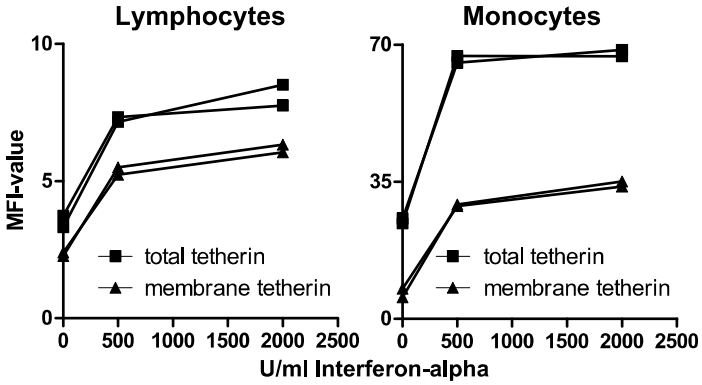

Figure 2 – Stimulation with Interferon-alpha. Tetherin MFI-values (y-axis, in duplicate) of total (square) or membrane (triangle) tetherin in lymphocytes (left) and monocytes (right) following stimulation of PBMC with 0, 500 or 2000 U/ml (x-axis) of IFNα for 24 hours.

Blocking experiments were set-up to further determine the method's specificity. Anti-tetherin antibody was mixed with excess amounts of recombinant tetherin; the mixture was used to stain PBMC. On average, total and membrane tetherin staining in IFNα-stimulated PBMC could be blocked for 71% and 96%, respectively (Figure 3A). Similar results were observed for baseline tetherin levels in non-stimulated PBMC (data not shown). Observed decreases in MFI-value suggest specific protein staining by the anti-tetherin antibody.

We further enquired into the method's specificity via expression analysis of cell lines expressing distinct levels of the tetherin protein (Figure 3B). Three HEK293T cell lines expressing high (clone 6.2), low (clone 17) and undetectable (clone 23) levels of tetherin were kindly provided by Dr. J. Guatelli. Expression of both total and membrane tetherin levels were clearly higher in the HEK293T cells over-expressing high levels of tetherin (clone 6.2) compared to control HEK293T cells (clone 23). The corresponding western blot depicts total tetherin staining (Figure 3C), which supports specific interactions of the tetherin antibody.

Next, unstimulated and IFNα-stimulated PBMC were stained according to the ECS or ICS method and visualized by confocal fluorescence microscopy (Figure 4). In unstimulated PBMC, tetherin appeared aggregated on the cell surface (membrane tetherin) and more diffusely spread in the cytoplasm (total tetherin). Upon stimulation (2000 U/ml IFNα - 24 hours), the number of membrane tetherin patches increased mostly on the membrane and possibly also in the cytoplasm.

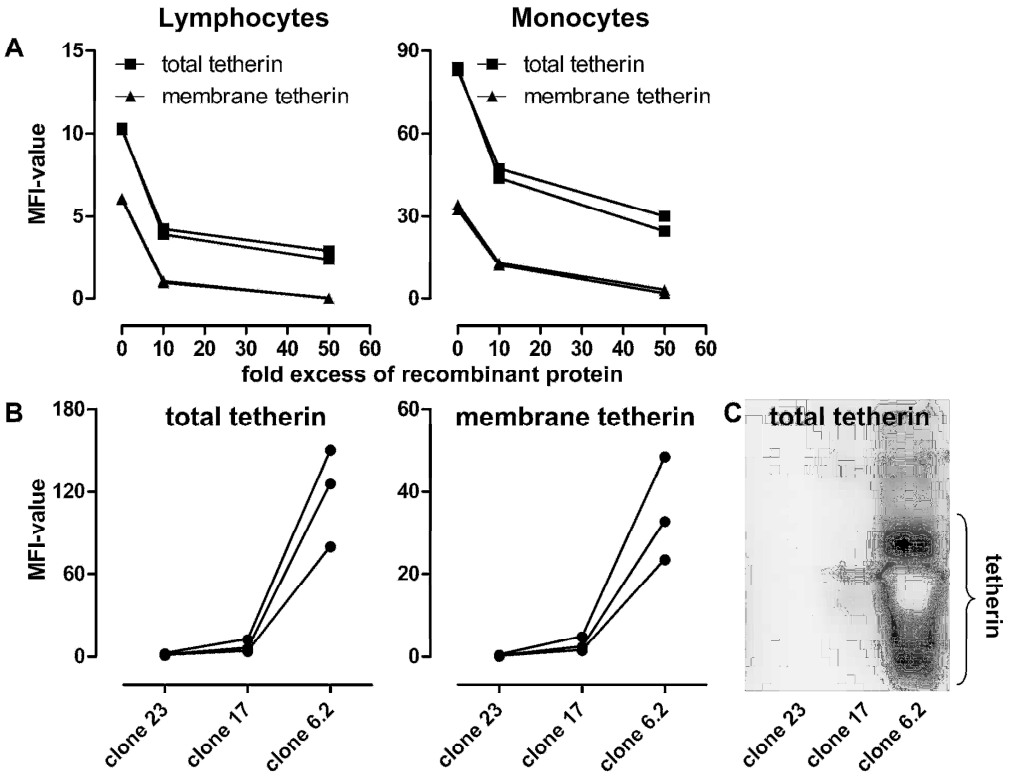

Figure 3 – Blocking and cell line experiment. (A) Duplicate staining of total (square) or membrane (triangle) tetherin in IFNα-stimulated lymphocytes (left) and monocytes (right) following addition of 0-, 10- or 50- fold molar excess of recombinant tetherin protein. (B) Triplicate staining of total (left) or membrane (right) tetherin in control HEK293T cells (clone 23), in HEK293T stably over-expressing low levels of tetherin (clone 17), and in HEK293T stably over-expressing high levels of tetherin (clone 6.2). (C) Western blot stained with anti-tetherin antibody and loaded with the cell-lines mentioned in figure 3B. Tetherin migrates as a heterogeneous smear around 30-36kDa in reducing SDS-PAGE, as also previously described by several others [524,533,538,541,547,558,610,662].

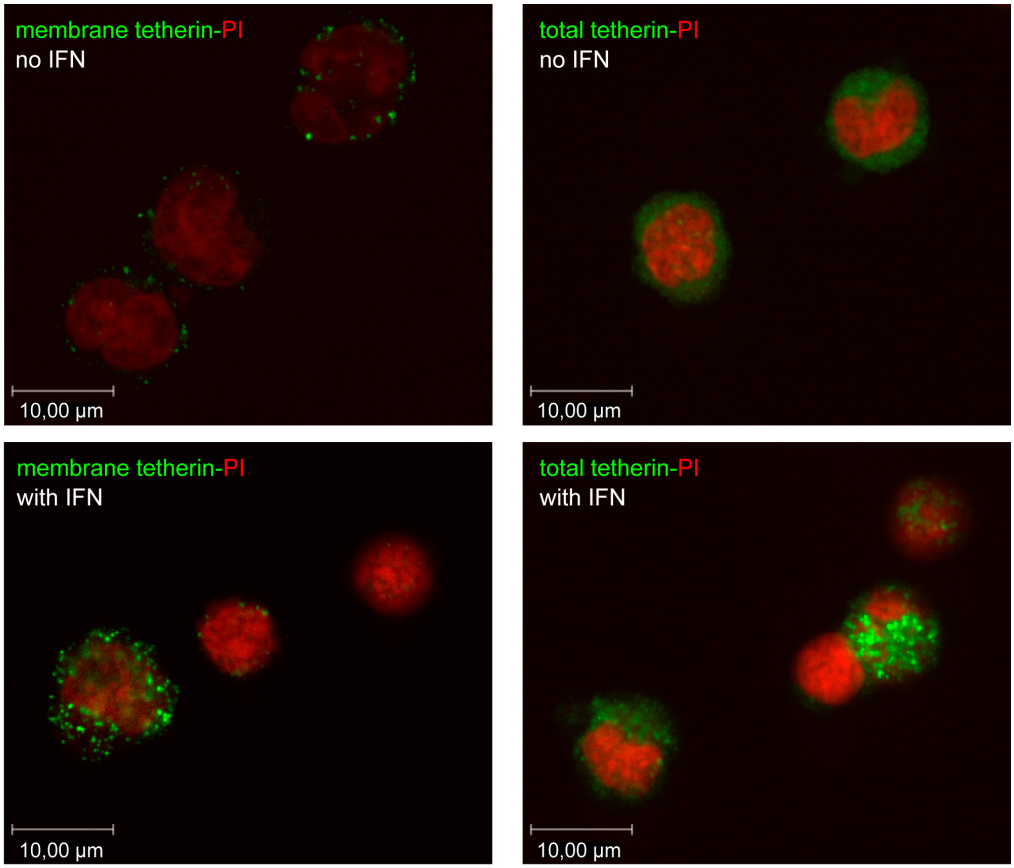

Figure 4 – Confocal imaging. Single confocal images of PBMC at the level of the cell nucleus show the fluorescent staining of membrane tetherin (left) and total tetherin (right) versus nuclear heterochromatin (PI, red) without (upper) or with (below) stimulation with IFNα. Scale bars represent 10.00 µm.

5. Discussion

In Chapter 3, we described a flow cytometry-based method to measure expression levels of the HIV-1-related host proteins APOBEC3G, TRIM5α, and LEDGF/p75. Recently, another host restriction factor called tetherin was described in literature [520]. Total tetherin expression levels were investigated with western blotting [216,217,524,533,538,541,547,558,662] or a flow cytometry-based method similar to the one described in this chapter [213,215]. However, most studies focused on membrane tetherin as the membrane protein inhibits virion release by tethering newly formed virions to the cell membrane [152,663]. To study membrane tetherin levels, many study groups used an extracellular flow cytometry-based staining protocol [213–217,532,547,562,564,578,662–664]. Nevertheless, total tetherin levels are also of interest considering amongst others the anterograde trafficking pathway via which intracellular tetherin proteins may become membrane tetherin. In this chapter, we optimized the ICS as well as ECS method to study respectively total and membrane tetherin expression patterns.

In first instance, we used an anti-tetherin antibody purchased from eBioscience. Experiments performed to verify the applicability of this antibody in the ECS as well as the ICS protocol led to flow cytometric images showing lack of staining (data not shown). Upon correspondence with the company from whom we purchased the antibody, the staining capacity of the antibody was further investigated by eBioscience and found to be insufficient. Consequently, eBioscience removed the antibody from their catalogue. Next, we requested the non-commercial anti-tetherin antibody from Chugai Pharmaceutical Co. (Japan), since many study groups had used this antibody before.

Optimization experiments showed that the “Chugai” anti-tetherin antibody led to satisfactory staining patterns. Indeed, a positive staining was demonstrated on CD4+ T-cells and CD14+ monocytes when using the ICS and the ECS protocols. Furthermore, we measured augmented tetherin levels with both methods upon stimulation with IFNα, supporting previous results [214,538,549]. Next, tetherin staining could be reduced up to 96% after co-incubation of the

antibody with recombinant tetherin protein indicating that the antibody is specific for tetherin in both staining methods. Further evidence on the method's specificity was provided by both cell line and confocal imaging experiments. The cell line experiments clearly showed higher tetherin levels in the tetherin-overexpressing HEK293T cells (clone 6.2) relative to the control HEK293T cells (clone 23) as also confirmed by western blot analysis (Figure 3C). Of note, we observed a smear of tetherin protein bands which was in correspondence with previous observations [524,533,538,541,547,558,610,662]. This smear may be explained by the presence of post-translational modifications like glycosylation [533,534] and/or by (partial) degradation. Finally, confocal imaging of cells fluorescently stained according to the ICS method resulted mainly in a diffuse cytoplasmic staining, whereas the ECS method led to the observation of distinct membrane patches. Interestingly, the formation of patches on the membrane becomes even more prominent upon stimulation with IFNα. Others observed the highly dynamic transmembrane protein tetherin residing in plasma membrane pools associated with lipid rafts, in early and recycling endosomes, and in the trans-Golgi network [526,536,542–545]. As can be deduced from the confocal images in Figure 4, not all cell-types within PBMC express the same level of tetherin. This latter observation is also supported by our flow cytometric data (Figure 1-3) showing higher tetherin levels in human monocytes relative to CD4+ lymphocytes suggesting that monocytes may restrict virion release more efficiently than CD4+ lymphocytes.

Today, the host protein tetherin is mostly described to prevent virion release by tethering the newly formed particle to the cell membrane. Nevertheless, controversy exists on its working mechanism. Several study groups reported that tetherin-induced accumulation of unreleased virions at the cell membrane would not restrict cell-to-cell spread of the virus [553], could actually promote cell-to-cell spread, e.g., by enhancing fusogenicity or regulating the integrity of the viral synapse [563,564], and may trigger further increases in immune activation [344,540]. In 2010, Kuhl et al [562] hypothesized that tetherin may restrict virion release in HIV-1-infected cells, whereas it may play a role in cell-cell contacts and virological synapses in uninfected 'target' cells.

In conclusion, we succeeded in optimizing the intra- and extracellular staining method with the "Chugai" antibody to study protein levels of total and membrane tetherin. In Chapter 6, the flow cytometry-based method to study the expression of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin will be used in parallel to the mRNA-based reverse transcriptase-quantitative polymerase chain reaction method (RT-qPCR) to investigate expression patterns of these four HIV-1-related host proteins in different study populations.

6. Acknowledgements

We are grateful to Chugai Pharmaceutical Co., Japan for the anti-tetherin antibody and to Dr. John Guatelli (University of California San Diego, Boston, USA) for the HEK293T cells expressing different levels of tetherin (clone 6.2/17/23). This work is supported by the Fund for Scientific Research Flanders (FWO) (grant G.0660.06) and an FWO doctoral fellowship to KM.

Chapter 5: Intracellular detection of differential TRIM5alpha mRNA expression in peripheral blood by flow cytometry

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(*Unpublished data*)

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1. Abstract

Objective: In the previous Chapters 3 and 4, we described the successful optimization of a flow cytometry-based intracellular staining (ICS) assay to study protein expression levels of the cellular host proteins LEDGF/p75, APOBEC3G, TRIM5α, and tetherin. Here, we attempted to extend the ICS method to enable mRNA expression analysis simultaneously in different cell-types.

Methods: We included several in situ hybridization settings to set up a flow-cytometry based mRNA ICS method. One keyhole limpet hemocyanin (isotype) and four TRIM5α-specific fluorescein-labelled probes were designed and tested.

Results: We examined three different protocols to develop a cellular mRNA expression analysis assay. Titration experiments resulted in increased Median Fluorescence Intensity (MFI-)levels for one of the four TRIM5α-probes studied. Specificity of the latter probe was demonstrated following blocking and cell stimulation experiments. However, deeper analysis showed that obtained results were hard to confirm/reproduce.

Conclusions: An assay that combines in situ hybridization and flow cytometry to quantify host mRNA levels appeared more difficult to optimize than expected. Consequently, we implement the reverse transcriptase real-time quantitative polymerase chain reaction (RT-qPCR) method to quantify mRNA levels instead (see Chapter 6).

2. Introduction

In literature, mRNA expression levels are mostly studied with techniques like real-time quantitative polymerase chain reaction (qPCR), and micro-array analysis [665–667]. These techniques require cell purification steps to address mRNA expression patterns in specific cell-types and/or subsets. In this study, we attempted to circumvent this cumbersome separation step by combining in situ hybridization settings [668–675] with flow cytometry. Similar mRNA ICS experiments have been described by several others [676–691]. The mRNA ICS method, also referred to as flow-FISH, is very comparable to the protein ICS method [456] since both techniques use fluorescent labels to quantify the host factor of interest (at mRNA or protein level) in fixed and permeabilized cells thereby using flow cytometry. We looked into three different mRNA ICS protocols to investigate differential mRNA expression levels.

3. Methods

3.1. Sample collection and processing

To address mRNA expression levels, fresh blood cells from healthy and/or HIV-1-infected volunteers were used. All blood samples were collected in EDTA-coated blood tubes (BD Biosciences). Whole blood samples were used in a limited number of experiments (protocol 3). Peripheral blood mononuclear cells (PBMC) were obtained by density gradient separation.

3.2. Reagents

The Leucoperm Kit (Reagents A and B, both not defined) and final fixation solution (1% paraformaldehyde) were the same as in Chapter 3 and 4. Diethylpyrocarbonate (DEPC, Sigma) was used to render solutions RNase free. Four TRIM5α-probes were tested versus one keyhole limpet hemocyanin (isotype-)probe (Table 1). Background is studied with the help of the isotype-probe. These fluorescein-labelled single stranded DNA (ssDNA)-probes

[677,692,693] were designed with the help of the Vector NTI software considering the gene's mRNA structure (*mfold* of Zuker and Turner) and were purchased from Eurogentec.

Table 1 - Characteristics of ssDNA-probes used in the mRNA ICS experiments.

	Tm	Primer Sequence 5' to 3'	% GC content
TRIM5α-probe-1	40.6	GTA-TGG-AAG-GAA-CTA-TCC	44
TRIM5α-probe-2	40.6	GGC-ACA-ATG-AAA-GGA-ACA	44
TRIM5α-probe-3	40.6	TGC-AAG-CCT-CAT-AGT-CTA	44
TRIM5α-probe-4	40.6	GGA-AAT-ACA-GGC-TGA-GAA	44
Isotype-probe	40.6	TCT-TGC-CCT-TGA-ACT-TCT	44

Tm: Melting temperature considering the %GC.

3.3. Protocol 1

3.3.1. Buffers

The pre-hybridization buffer from **protocol 1** was composed of 0.5% SDS (sodium dodecyl sulphate, Sigma), 0.1% Triton X-100 (Merck), or Leucoperm reagent B; 2 mM $CaCl_2$ (Merck); 20 mM Tris (Sigma); and DEPC (0.1%) treated PBS pH7.4 (Invitrogen). The hybridization buffer contained 0.5% SDS, or reagent B; 50% deionized formamide (Sigma); 40 µg/ml E. coli tRNA (Sigma); 100 µg/ml of sheared salmon sperm DNA (Invitrogen); 10% NaCl (Merck); 20 mM Tris; and DEPC (0.1%) in PBS. As washing buffer, protocol 1 used 50% deionized formamide; 0.9 M NaCl; 20 mM Tris; and DEPC (0.1%) in PBS with or without 0.1% SDS. The protocol was investigated with and without addition of proteinase K (Invitrogen).

3.3.2. Procedure

The global design of protocol 1 exists of the following subsequent steps. First, cells were transferred sterilely into sterile 96-microtiterplate wells, and washed once with sterile RNase free PBS, pH7.4 (630 g, 10 min, RT). Next, cells were fixed with Leucoperm reagent A (10 min, RT), and washed twice with RNase free PBS. Then, cells were incubated in pre-hybridization buffer (10 min, RT; followed by 630 g, 10 min, RT), washed in RNase free PBS, and incubated in hybridization cocktail containing the probes of interest. This latter incubation occurred at 37°C $\pm$ 1°C (24 h) in the CO_2-incubator, or at 50°C $\pm$ 1°C in a plate heater (24 h). Next, the hybridization buffer with probe was removed (630 g, 10 min, RT), and pre-warmed hybridization buffer without probe was added (30 min at the same temperature; followed by 630 g, 10 min, RT). Finally, cells were washed twice with the formamide-containing washing buffer (4°C, 15 min; followed by 630 g, 10 min, RT), prior to re-suspension in 1% paraformaldehyde. Data acquisition and analysis occurred with the FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences). Median fluorescence intensity (MFI) values were used to quantify the mRNA expression level.

Aside from the mRNA staining, we attempted to simultaneously stain the cell membrane with a mix of CD3-APC, CD4-PE, and CD8-PerCP antibodies (4°C, 20 min) in RNase free PBS. This step as well as the two subsequent washing steps with RNase free PBS were applied prior to or directly after cell fixation.

3.4. Protocol 2

3.4.1. Buffers

The pre-hybridization buffer from **protocol 2** was composed of 30% 20x SSC (standard sodium citrate containing NaCl, $NaH_2C_6H_5O_7$, and HCl all from Merck); 10% 50x Denhardt's solution (Sigma); 1% sheared salmon sperm DNA (10 mg/ml); 2M sodium phosphate (pH6.5, Merck); 50% deionized formamide; and RNase free water (Ambion). The hybridization buffer

contained 23% 26x SSC; 10% 50x Denhardt's solution; 1% sheared salmon sperm DNA; 2M sodium phospate (pH6.5); 40% deionized formamide; 20% dextran sulphate (Sigma); and RNase free water. Two washing buffers were used in protocol 2. The first buffer was composed of 30% 20x SSC; 50% deionized formamide; and 20% RNase free water. The second, less stringent, buffer contained 0.01% Tween (Fluka) in 1x PBS.

3.4.2. Procedure

Protocol 2 includes additional in situ hybridization settings as obtained from literature [668–675]. Here too, cells were transferred sterilely into sterile 96-microtiterplate wells, and washed once with sterile RNase free PBS, pH7.4 (630 g, 10 min, RT). Next, cells were fixed with 4% paraformaldehyde in RNase free PBS (30 min, RT), and washed once with RNase free PBS. Then, cells were incubated in pre-hybridization buffer (10 min, RT; followed by 630 g, 10 min, RT), and directly incubated in hybridization cocktail containing the probes of interest at 37°C $\pm$ 1°C (24 h) in the CO_2-incubator. Next, the hybridization buffer with probe was removed (630 g, 10 min, RT), and pre-warmed hybridization buffer without probe was added (30 min at 37°C; followed by 630 g, 10 min, RT). Finally, cells were washed twice. Firstly, cells were washed stringently with 37°C washing buffer for 30 min at 37°C prior to centrifugation (630 g, 10 min, RT). Secondly, cells were washed with the less stringent buffer (0.01% Tween in PBS, 630 g, 10 min, RT), prior to re-suspension in 1% paraformaldehyde. The FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences) were used to acquire and analyze the data. Aside from the mRNA staining, we attempted to simultaneously stain the cell membrane as also described for protocol 1. Here, this step was included prior to cell fixation.

3.5. Protocol 3

3.5.1. Buffers

For **protocol 3**, the ViroTect^{TM}Plus kit (Invirion) was used. Buffers were not defined by the manufacturer.

3.5.2. Procedure

The ViroTect™Plus kit (Invirion) is a flow cytometry-based test designed for the detection of HIV-1 mRNA in intact human cells [694–698]. The protocol was applied according to the manufacturer's instructions. However, we also performed experiments in which we replaced reagent 5 of the kit with our fluorescein-labelled TRIM5α- or isotype-probe mixture with or without 10-fold excess of unlabelled probe to "block" the staining. Data acquisition and analysis occurred with the FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences).

3.6. "Blocking" experiments

To verify the binding specificity of TRIM5α-probe-4, unlabelled TRIM5α-probe-4 and unlabelled isotype-probe were purchased (Eurogentec). Upon cell incubation with the fluorescently labelled probe, the unlabelled probe was co-incubated at a 10-fold and/or a 100-fold excess to compete for the specific binding sites of interest.

3.7. Stimulation experiments

PBMC were cultured with or without the addition of stimulation agents like PMA/Ionomycin (P/I, both from Sigma), Interferon-beta (IFNβ, Peprotech) and staphylococcus enterotoxin B (SEB, Sigma). The stimulation conditions were 0.02 µg/ml of PMA and 1 µg/ml of ionomycin for 3 h, 1000 U/ml of IFNβ for 16 h, and 5 µg/ml of SEB for 16 h, 24 h, and 40 h.

3.8. Cell line experiment

HEK293T cell lines were transiently transfected without vector, with an empty pcDNA3.1-vector, or with a human TRIM5α-vector. All vectors were obtained by the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH).

4. Results

4.1. Preliminary results of the three mRNA staining protocols

In first instance, we set up **protocol 1**, which is very much alike to our protein ICS assay. PBMC were subjected to distinct proteinase K, permeabilization, and buffer preparation (sterile, RNase free) conditions. Addition of proteinase K, a non-specific serine protease that removes nucleases [699], deteriorated the cell morphology (data not shown). Consequently, we omitted further use of proteinase K in any of the three protocols. Cell morphology was best maintained when permeabilizing cells with reagent B (data not shown), explaining the further use of reagent B in protocol 1. TRIM5α- and isotype-probe concentrations of 50, 500, 5000, 50000 and 300000 ng/ml were tested in the mRNA ICS protocol. Elevated TRIM5α relative to isotype MFI-values were obtained when stained with probe concentrations of 50000 ng/ml or more. This observation is particularly true for TRIM5α-probe-4 (Figure 1A). MFI-shifts were limited for the other TRIM5α-probes when compared to the isotype-probe (data not shown). Therefore, the three protocols were investigated with TRIM5α-probe-4.

Next, we attempted to improve the mRNA staining using specific in situ hybridization buffers. In **protocol 2**, we included the use of solutions like 4% paraformaldehyde, SSC (standard sodium citrate) buffer, dextran sulphate, and Denhardt's solution. In situ hybridization experiments often use 4% paraformaldehyde solution to fix and simultaneously permeabilize the cells [675,679]. Dextran sulphate is added to increase probe binding to target mRNA, although excess amounts of dextran sulphate should be avoided as these can induce hard to remove background staining. Next, Denhardt's solution is a mixture of BSA (bovine serum albumin), ficoll and polyvinylpyrrolidone. The herein contained polymers saturate non-specific binding sites. We tested the staining potential of protocol 2 with the TRIM5α-probe-4 and isotype-probe (both applied at 50000 ng/ml) (Figure 1B).

Finally, we used the ViroTect^{TM}Plus kit that is normally used for the detection of HIV-1 mRNA levels. In **protocol 3**, we adopted this kit to study TRIM5α mRNA levels. Instead of measuring the viral gag-pol levels, we tried to measure cellular TRIM5α mRNA levels. The kit contains different solutions of which the composition is unknown. We tested the kit on PBMC as well as on whole blood of healthy and HIV-1-infected subjects. PBMC experiments appeared more specific (data not shown). However, the protocol resulted in higher isotype than TRIM5α MFI-values (Figure 1C).

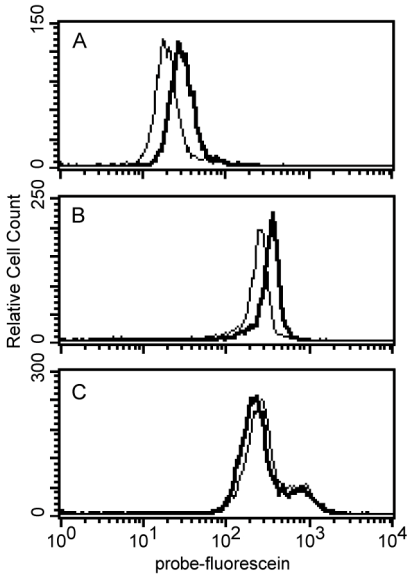

Figure 1 – TRIM5α mRNA versus isotype staining. The histogram depicts the TRIM5α and isotype mRNA staining of lymphocytes obtained according to protocol 1 (A), protocol 2 (B), and protocol 3 (C). The two peaks highlight the median fluorescence intensity (probe-fluorescein) obtained for the TRIM5α-probe-4 (**bold peak**) and the isotype-probe (regular peak); both added at the concentration of 50000 ng/ml.

Of note, when using the same acquisition and analysis settings as for protocol 1, protocol 2 and 3 led to higher MFI-values for both TRIM5α and isotype compared to protocol 1 (Figure 1 and 2A).

4.2. Blocking experiments

To analyse TRIM5α-probe-4 specificity, we purchased both TRIM5α-probe-4 and isotype-probe without fluorescein label. We stained the TRIM5α mRNA of the cells with a mixture of fluorescein-labelled TRIM5α-probe-4 and a 10-fold molar excess of unlabelled TRIM5α-probe-4. Both probes are expected to compete for the same target mRNA, so that reduced MFI-values are expected upon specific interaction of the TRIM5α-probe-4. We also combined

fluorescein-labelled TRIM5α-probe-4 with an excess of unlabelled isotype-probe, after which the TRIM5α MFI-value should remain constant. Regardless the protocol applied, we could not block the TRIM5α mRNA staining when a 10-fold excess of unlabelled TRIM5α-probe-4 was added (Figure 2A). Notably, addition of an excess of unlabelled isotype-probe resulted in a decrease in TRIM5α MFI-value for protocol 2 and 3. Considering these latter observations, a 100-fold excess of unlabelled TRIM5α-probe-4 was tested solely with **protocol 1** applying two different hybridization conditions (Figure 2B and C). Upon hybridization at 37°C for 24 h (Figure 2B), we succeeded in reducing the mRNA staining with 18.7%. Since TRIM5α MFI-values increased following hybridization at 50°C for 24 h, we also used the latter hybridization condition to test blocking with a 100-fold excess of unlabelled TRIM5α-probe-4. Remarkable, we observed increased MFI-values following incubation with a 100-fold excess of unlabelled TRIM5α-probe-4 (19.7%, Figure 2C).

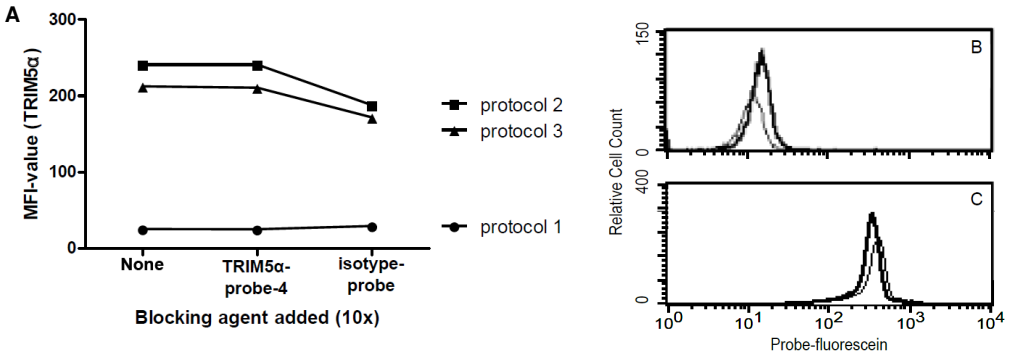

Figure 2 – Blocking experiments. (A) The graph depicts the TRIM5α mRNA expression (MFI-value, y-axis) upon staining with TRIM5α-probe-4 (50000 ng/ml) with or without the addition of a 10-fold excess of unlabelled TRIM5α-probe-4 or isotype-probe. Each protocol was performed once to test the TRIM5α-probe-4 specificity. (B-C) The two histograms depict blocking of TRIM5α mRNA staining (bold black line) with 100-fold excess of unlabelled TRIM5α-probe-4 (thin black line) when hybridizing according to protocol 1 at 37°C (B) or 50°C (C).

4.3. Stimulation experiments (protocol 1)

Next to general staining and blocking experiments performed for all three protocols, we attempted to detect increased mRNA levels (using protocol 1) by inducing TRIM5α mRNA expression levels. To achieve this, we subjected PBMC to different stimulation conditions

prior to hybridization at 37°C for 24 h with TRIM5α-probe-4 or isotype-probe. Three hours of stimulation with P/I resulted in increased TRIM5α (60%) and isotype (12%) MFI-values compared to non-stimulated cells (Figure 3A). Sixteen hours of stimulation with IFNβ or SEB led to enhanced MFI-values of respectively 13% and 18% for TRIM5α-probe-4 or 8% and 30% for the isotype-probe (Figure 3B).

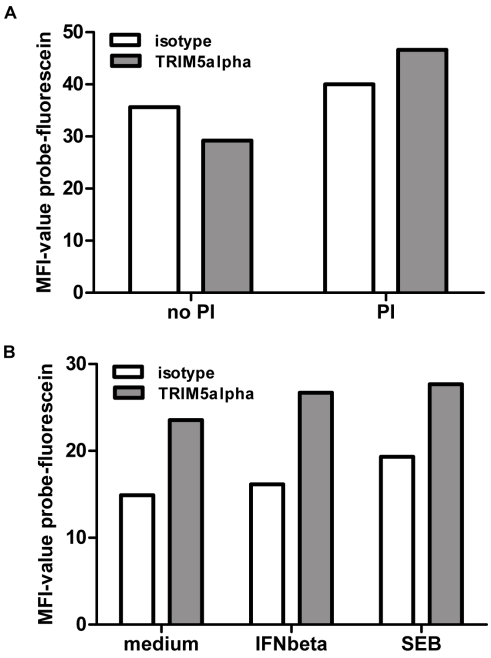

Figure 3 – stimulation experiments. (A) After 3 hours of stimulation with PMA/Ionomycin (P/I), TRIM5α as well as isotype levels increased. Of note, the isotype level was higher than the TRIM5α level when cells were not stimulated. (B) Both isotype and TRIM5α levels increased upon 16 hours of stimulation with either Interferon-beta (IFNβ) or Staphylococcus enterotoxin B (SEB).

4.4. Cell line experiment (protocol 2)

In addition, we included an experiment in which Trim5α-, empty vector- or non-transfected HEK293T cell lines were used. Cells were stained according to protocol 2 with TRIM5α-probe-4 or isotype-probe (data not shown). TRIM5α MFI-values were similar for non-transfected and empty vector-transfected cells, whereas the staining was slightly increased (2.7%) for TRIM5α-transfected cells. However, although background staining with the isotype-probe was similar for non-transfected and TRIM5α-transfected cells, it was increased for the empty vector-transfected cells with 15%. This indicates that the 2.7% increase in staining of Trim5α-transfected cells is not relevant and rather a result of technical variation.

5. Discussion

In literature, host mRNA levels are mostly quantified with real-time qPCR [73,97,100,208–210,362,416,505,700,701] or micro-array [417,604] analyses. In the past, several research groups described the use of in situ hybridization in combination with flow cytometry to study e.g. telomere length [685,689], viral RNA [681,687], and mRNA [680,686]. In this PhD-thesis, three mRNA ICS protocols were tested to study TRIM5α mRNA expression with flow cytometry. The three protocols applied the same staining procedure and varied mainly in the buffers used. Overall, our results suggested that the TRIM5α-probe-4 could hybridize with TRIM5α mRNA. The hybridization appeared specific since TRIM5α mRNA staining with TRIM5α-probe-4 could be blocked when 100-fold excess of unlabelled TRIM5α-probe-4 was used, and could be slightly induced upon stimulation. However, deeper analyses pointed to a number of inconsistencies. Primarily, the isotype staining often resulted in higher MFI-values than observed for TRIM5α suggesting intolerable background staining. Consequently, as we selected the TRIM5α-probe-4 based on a peak shift compared to background staining, this TRIM5α-probe may not be well chosen either. Secondly, TRIM5α staining specificity was doubtful as blocking with a 10-fold excess of unlabelled isotype-probe – which should not influence TRIM5α staining – did result in reduced MFI-values, while unlabelled TRIM5α-probe-4 did not influence the staining. Finally, protocol 1 (hybridization at 50°C) as well as protocol 2 resulted in apparently improved TRIM5α mRNA detection which was concluded based on elevated MFI-values. However, further investigation showed that blocking experiments according to protocol 1 (hybridization at 50°C) resulted in enhanced TRIM5α MFI-values (Figure 2C) whereas decreased values were expected. The cell line experiment in which TRIM5α was stained according to protocol 2 led to slightly increased (2.7%) mRNA levels in TRIM5α-transfected cells. However, isotype staining and a western blotting experiment (data not shown) suggest that the increased staining observed in the TRIM5α-transfected cells is not relevant and rather a result of technical variation (data not shown).

In conclusion, to quantify low-level cell-specific mRNA levels, a very specific and sensitive mRNA staining method is needed. Many challenges like probe design, fixation, permeabilization, and (post-)hybridization conditions need to be overcome to allow for mRNA quantification by flow cytometry at high sensitivity and specificity. Similar to the limitations in antibody availability for the protein ICS method, probe design is of utmost importance for the mRNA ICS method. In 2009, Robertson et al [686] described an LNA flow-FISH procedure to quantify mRNA using locked nucleic acid (LNA) probes which would bind DNA and RNA with higher affinity than any of the probes previously described. Today, we consider that the oligodeoxynucleotide probes that we used may have been insufficiently appropriate for hybridization purposes. As we obtained inconsistent results with this mRNA ICS for which only a limited number of (recent) similar publications were observed in literature, we decided to cease our efforts and to apply the often used real-time qPCR technique instead.

Chapter 6: Expression analysis of LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin in a Senegalese cohort

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Part I: Expression analysis of LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin in a Senegalese cohort of HIV-1-exposed seronegative individuals

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Adapted from:

Mous, K., Jennes, W., Camara, M., Seydi, M., Daneau,G., Mboup,S., Kestens, L. and Van Ostade, X. *PLoS ONE* 2012;**7**:e33934. Epub 2012 Mar 27

1. Abstract

Objective: HIV-1 replication depends on a delicate balance between cellular co-factors and antiviral restriction factors. Lens epithelium-derived growth factor (LEDGF/p75) benefits HIV, whereas apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G), tripartite motif 5alpha (TRIM5α), and tetherin exert anti-HIV activity. Expression levels of these proteins possibly contribute to HIV-1 resistance in HIV-1-exposed populations.

Methods: We used real-time PCR and flow cytometry to study mRNA and protein levels respectively in PBMC and PBMC subsets.

Results: We observed significantly reduced LEDGF/p75 protein levels in CD4+ lymphocytes of HIV-1-exposed seronegative subjects relative to healthy controls, whereas we found no differences in APOBEC3G, TRIM5α, or tetherin expression. Untreated HIV-1-infected patients generally expressed higher mRNA and protein levels than healthy controls. Increased tetherin levels, in particular, correlated with markers of disease progression: directly with the viral load and T cell activation and inversely with the CD4 count.

Conclusions: Our data suggest that reduced LEDGF/p75 levels may play a role in resistance to HIV-1 infection, while increased tetherin levels could be a marker of advanced HIV disease. Host factors that influence HIV-1 infection and disease could be important targets for new antiviral therapies.

2. Introduction

Human immunodeficiency virus type 1 (HIV-1) interacts with many cellular host proteins during its replication cycle [702–704]. Some of these proteins are required for HIV-1 replication while others exhibit antiviral activity. Lens epithelium-derived growth factor p75 (LEDGF/p75) [212] is a cellular co-factor, whereas apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G, A3G) [611], tripartite motif 5alpha (TRIM5α) [705], and tetherin (BST2, CD317, HM1.24) [520] are cellular factors with distinct antiviral activities. The main working mechanisms of these factors can be summarized as follows. LEDGF/p75 assists in the integration of viral cDNA into specific regions of the host's genetic material [218,620]. APOBEC3G inhibits HIV replication by inducing G-to-A hypermutation of the viral HIV-1 genome during reverse transcription [611,706]. TRIM5α structurally disorders the retroviral capsid, leading to the interruption of the natural “uncoating” process in a species-specific manner [618,707]; and was recently described to promote innate immune signalling [433]. Tetherin, finally, inhibits HIV release by tethering newly formed retrovirus particles to the cell membrane [520].

The significance of these four proteins as possible correlates of protection against HIV-1 remains to be determined. Several studies on peripheral blood mononuclear cells (PBMC) have found that APOBEC3G mRNA levels correlate positively with the CD4 count and negatively with the viral load of untreated HIV-1-infected subjects (HIV-UT) [209,411,412], suggesting that APOBEC3G contributes to the control of HIV-1 infection. However, other studies did not find such correlations for APOBEC3G [208,413,414] or TRIM5α [503] mRNA. In addition, certain studies compared the expression of APOBEC3G or TRIM5α between distinct study populations like untreated HIV-1-infected subjects, healthy controls, and HIV-1-exposed seronegative individuals. Some studies observed lower mRNA expression levels of APOBEC3G [208,209,414] or TRIM5α [503] in untreated HIV-1-infected subjects compared to healthy controls, although the opposite was also found for APOBEC3G [412]. APOBEC3G mRNA and protein expression levels were also studied respectively in PBMC and PBMC-subsets (CD4+ and CD8+ lymphocytes, and CD14+ monocytes) of HIV-1-exposed seronegative subjects

relative to healthy controls [411,414,621]. Here too, conflicting data were obtained, showing either similar [414] or higher [411,621] levels of antiviral APOBEC3G in HIV-1-exposed seronegative subjects relative to healthy controls.

In the present study, we investigated mRNA and protein expression levels of the 4 HIV-1-related factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in HIV-1-exposed seronegative subjects, healthy controls, untreated HIV-1-infected subjects, and antiretroviral therapy-treated HIV-1-infected subjects. HIV-1-exposed seronegative subjects are individuals who remain HIV-1 seronegative despite frequent and unprotected exposure to HIV, and are observed in cohorts of commercial sex workers, men having sex with men, intravenous drug users, and discordant couples [55]. In this study, HIV-1-exposed seronegative subjects were enrolled from a Senegalese cohort of HIV-1 serodiscordant couples. Real-time quantitative polymerase chain reaction (qPCR) was applied to study mRNA expression in PBMC. Protein expression levels were determined simultaneously in different PBMC subsets by flow cytometry. We hypothesized that expression levels of the above-mentioned proteins may contribute to the putative *in vivo* HIV-1 restriction in HIV-1-exposed seronegative subjects.

3. Materials and Methods

3.1. Ethics statement

The study was approved by the Internal Review Board of the Institute of Tropical Medicine (Antwerp, Belgium) and by the Ethical Committees of the Senegalese Ministry of Health (Dakar, Senegal) and the University Hospital of Antwerp (Belgium). All study subjects gave written informed consent prior to enrolment.

3.2. Study population

This study was conducted on a cohort of heterosexual couples recruited at the Department of Infectious Diseases at the Fann University Teaching Hospital, Dakar, Senegal, as previously

described [59]. Twenty three HIV-1 exposed seronegative subjects (HESN) in HIV-1 serodiscordant couples, 23 healthy controls (HC) in HIV-1-negative seroconcordant monogamous couples, and 45 HIV-1-infected patients in HIV-1 serodiscordant or HIV-1-positive seroconcordant couples were studied. Within the group of 45 HIV-1-infected patients, 9 patients were untreated HIV-1-infected subjects (HIV-UT) and 36 patients were antiretroviral therapy-treated HIV-1-infected subjects (HIV-ART). All couples had a sexual relationship of at least 7.5 years. HESN and HC were matched for gender. Blood samples and standard questionnaires with information on socio-demographics and sexual behaviour were collected. Male condom provision and sexual risk reduction counselling were provided during every visit.

3.3. Sample collection and processing

Whole blood samples were collected in EDTA tubes. CD4+ T cell counts and T cell activation levels were determined in fresh whole blood using CD3, CD4, CD8, and CD38 fluorochrome-labelled antibodies and a FACSCalibur flow cytometer (BD Biosciences) like previously reported [59]. Plasma and PBMC were separated from fresh whole blood, frozen down at -80°C and in liquid nitrogen, respectively, and shipped to Belgium. HIV and HSV-2 (Herpes simplex virus type 2) status were determined in plasma by ELISA. HIV-1 viral loads were quantified in plasma by the Amplicor HIV-1 Monitor assay, version 1.5 (Roche Diagnostics GmbH). PBMC were thawed, washed twice, and counted prior to mRNA and protein expression analysis.

3.4. RNA extraction, DNase treatment and reverse transcription

PBMC samples were dissolved in TRIzol® Reagent (Invitrogen) and stored at -80°C for less than three months. Total RNA was isolated according to the manufacturer's instructions (Invitrogen). Removal of genomic DNA was performed with the DNA-free Kit (Ambion) following the manufacturer's protocol. Additional wash steps, once with 200 µl isopropanol (in the presence of 0.7 µl of glycogen) and twice with 75% ethanol, were performed to

remove traces of contaminants. The NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was used to quantify total RNA levels. Total DNase-treated RNA was stored at -80°C for less than one month. The iScript cDNA synthesis Kit (Bio-Rad Laboratories NV-SA) was applied to 400 ng of total DNase-treated RNA following the manufacturer's instructions. In parallel, 100 ng of the same RNA was dissolved in RNase/DNase-free water (no-RT control) at the same final concentration (20 ng/10.5 µl) as the RT (reverse transcribed) samples. Both RT and no-RT samples were stored at -80°C for less than one month. For each sample, the difference in quantification cycle (Cq)-value between RT and no-RT samples was higher than 10. During optimization of the qPCR protocol, RNA quality was evaluated by RNA gel electrophoresis (1% agarose gel). The 18S and 28S fragments were visualized with GelRed (VWR) versus a 1-kb DNA ladder (Invitrogen) and were observed in the expected 1:2 ratio on representative samples.

3.5. Real-time PCR

Messenger RNA (mRNA) expression levels were quantified by real-time PCR using the Bio-Rad CFX96 system and SYBR Green mix (Bio-Rad). Target-specific primers were designed using the Harvard website: http://pga.mgh.harvard.edu/primerbank/, except for *TRIM5α* and *TBP*, for which Primer3 software (v.0.4.0) was used. Specificity was tested using BLAST (NCBI). All primer pairs, except for *TRIM5α*, could be designed to span at least one intron. Gene name, GeneID, primer sequences, exon localization and amplicon length are listed in Table 1.

The primer mixes were prepared by combining Forward (Fw) and Reverse (Re) Primers (Eurogentec), both at a concentration of 2 pmol/µl. Each optimized 25 µl reaction contained 12.5 µl of iQ SYBR Green Supermix (Bio-Rad), 2 µl of the primer mix and 5, 10 or 20 ng of the cDNA template at a volume of 10.5 µl. Each assay included, in triplicate, a standard curve, a no-template control (water), the RT samples, and, in the case of *TRIM5α*, the no-RT samples. The following subsequent heating steps were included as PCR cycling conditions: 50°C (10 min), 95°C (3 min), 40 cycles of 95°C (10 s) and 60°C (30 s) with fluorescence capturing following each cycle, 95°C (10 s), and melting curve acquisition with fluorescence capturing

every 5 s during a temperature increase in 0.5°C increments from 65°C to 95°C. The amplicon length of the PCR product was validated by 2% agarose DNA gelelectrophoresis. PCR products were visualized with GelRed (VWR) versus a 100-bp DNA ladder (Invitrogen).

Table 1 - Primers used for reverse transcriptase quantitative polymerase chain reaction.

	GeneID		Primer Sequence 5' to 3'	Exon localization	Amplicon length
Genes of interest					
LEDGF/p75	11168	Fw*	TCGACTTCAAAGGATACATGCTG	12	122
		Re†	GAGCTTGTTGCATTGTGACCT	13	
APOBEC3G	60489	Fw	TCAGAGGACGGCATGAGACTT	5	107
		Re	TGGAGCCTGGTTGCATAGAAA	5 and 6	
TRIM5α	85363	Fw	TGCTGGCTTCCAACCTGAT	8	165
		Re	ACAGAGAGGGGCACAATGAA	8	
Tetherin (BST2)	684	Fw	GAGTGTCGCAATGTCACCCAT	1	120
		Re	GGAAGCCATTAGGGCCATCAC	1 and 2	
Reference genes					
UBC‡	7316	Fw	ATTTGGGTCGCAGTTCTTG	1	123
		Re	TGCCTTGACATTCTCGATGGT	2	
B2M§	567	Fw	GGCTATCCAGCGTACTCCAAA	1 and 2	246
		Re	CGGCAGGCATACTCATCTTTTT	2	
GAPDHǁ	2597	Fw	TGTTGCCATCAATGACCCCTT	3	202
		Re	CTCCACGACGTACTCAGCG	5	
RPL13A¶	23521	Fw	CGAGGTTGGCTGGAAGTACC	7	121
		Re	CTTCTCGGCCTGTTTCCGTAG	8	
TBP**	6908	Fw	ACCCAGCAGCATCACTGTTT	1 and 2	200
		Re	CCAAGCCCTGAGCGTAAG	2	

* Forward primer, † Reverse primer, ‡ Ubiquitin C, § Beta-2-Microglobulin, ǁ Glyceraldehyde 3-phosphate dehydrogenase, ¶ 60S ribosomal protein L13a, and ** TATA-binding protein.

3.6. mRNA data analysis

The obtained results (Cq-values) were exported from the Bio-Rad CFX Manager version 1.5 and imported into qbase^{PLUS} software, version 1.5, from BioGazelle (Ghent, Belgium) following baseline subtraction and manual threshold matching. The GeNorm applet selected the reference genes *B2M* and *GAPDH* out of five genes (*UBC, B2M, GAPDH, RPL13A, TBP*) as the

most stably expressed across the different study populations (25 samples, all applied in triplicate). Both reference genes were used to normalize target gene expression in the qbase^{PLUS} software. For the standard curve, a 2-fold serial dilution of cDNA of one Caucasian healthy control was used. Cq-values were converted into relative expression values taking into account amplification efficiencies, inter-run variations, and normalization factors. Outliers were excluded when a difference of more than 1.0 was registered in the Cq-values. Finally, CNRQ (Calibrated Normalized Relative Quantity) values were exported from the qbase^{PLUS} software and statistically investigated.

3.7. Intracellular and surface protein staining

The protein expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin were investigated in CD4+ T cell and CD14+ monocyte subsets based on simultaneous intracellular and/or cell surface staining of PBMC. The following reagents were used: 0.1% bovine serum albumin (Acros Organics) and 0.05% sodium azide (Merck) in phosphate buffered saline (PBS, Lonza) as washing buffer; Reagents A and B to fix and permeabilize cells, respectively (Leucoperm kit, AbD Serotec); primary mouse unlabeled antibody (isotype IgG1 and IgG2a) and anti-LEDGF/p75 (IgG1) from BD Biosciences, anti-APOBEC3G and anti-TRIM5α (both IgG1) from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH), and anti-tetherin (IgG2a) as kindly provided by Chugai Pharmaceutical Co., Japan; secondary FITC-labelled antibody (BD Biosciences); normal mouse serum (eBioscience); membrane antibodies anti-CD4-PerCP, anti-CD45RO-APC, anti-CD38-PE, anti-CD14-APC, and anti-CD16-PE from BD Biosciences; and 1% paraformaldehyde (Sigma-Aldrich). LEDGF/p75, APOBEC3G, TRIM5α, and tetherin were analyzed using an in-house optimized intracellular staining (ICS) protocol [24,25]. Cells were washed (630 x g, 10 min, RT), fixed (10 min, RT), washed twice, permeabilized (30 min, 4°C), washed, incubated with primary antibody (24 h, 4°C), washed twice, incubated with secondary antibody supplemented with reagent B (30 min, 4°C), washed twice, incubated with normal mouse serum (10 min, 4°C), incubated with a membrane antibody cocktail (CD4/CD45RO/CD38 or CD4/CD14/CD16 to study lymphocytes or monocytes, respectively, 20 min, 4°C), washed twice, and dissolved in 1% paraformaldehyde

prior to data acquisition (FACSCalibur flow cytometer, BD Biosciences). To study membrane tetherin levels, PBMC were processed according to the protocol above with a 30-min anti-tetherin incubation at 4°C, without fixation or permeabilization steps. In general, at least 25000 and 50000 events were respectively measured to study expression patterns in CD4+ lymphocytes and CD14+ monocytes. Data analysis with CellQuest Pro Software (BD Biosciences) produced median fluorescence intensity (MFI) values, reflecting the median amount of bound antibody per cell and thus, the protein's abundance. Host protein MFI-values were corrected for non-specific background staining by subtracting isotype MFI-values.

3.8. Statistical analyses

Differences in expression levels between individuals of different study groups were investigated with non-parametric Mann-Whitney *U* tests. Correlation analyses were performed using non-parametric Spearman's rank correlation tests. Observations were considered statistically significant when $p < 0.05$. Statistical analyses were performed with SPSS version 17.0, and graphs were drawn with GraphPad Prism, version 5.

4. Results

4.1. Study population

Twenty three HIV-1-exposed seronegative subjects (HESN), 23 healthy controls (HC), and 45 HIV-1-infected patients were studied. Nine HIV-1-infected patients were untreated (HIV-UT) and 36 were antiretroviral therapy-treated (HIV-ART). Several parameters like age, gender, duration of the sexual relationship, CD4 count, number of sexual contacts per month, condom use, and HSV-2 serostatus were compared between HESN and HC, HIV-UT and HC, and HIV-ART and HIV-UT (Table 2). The HC subjects were in a monogamous relationship for at least 7.5 years at the time of enrolment. They reported a significantly lower percentage of condom use. HIV-1-infected subjects were more often male individuals. Therapy-naïve HIV-1 infected

subjects (HIV-UT) showed significantly lower CD4 count levels than HC, and a higher prevalence of HSV-2 seropositivity.

Table 2 - Characteristics of HIV-exposed seronegative, HIV-1-infected, and healthy control subjects included in the study.

	HC* (n = 23)	HESN† (n = 23)	HIV-UT‡ (n = 9)	HIV-ART§ (n = 36)	p-value		
					HESN vs. HC	HIV-UT vs. HC	HIV-ART vs. HIV-UT
Age, years	43 (37-46)	41 (33-47)	46 (37-51)	44 (36-51)	0.409	0.449	0.955
Gender (% male)	48	44	89	72	0.770	**0.036**	0.303
CD4 count (cells/µl)	789 (703-1090)	738 (548-891)	228 (133-337)	334 (195-412)	0.215	**< 0.001**	0.294
Number of sexual contacts per month	8 (3-12)	5 (4-8)	5 (4-11)	7 (4-8)	0.164	0.831	0.831
Duration of sexual relation, years	12 (8-19)	11 (8-18)	11 (9-18)	10 (8-16)	0.938	0.866	0.540
Condom use (% always)	0	61	44	50	**< 0.001**	**0.001**	0.768
HSV-2 serostatus (% HSV-2 positive)	22	45	78	59	0.095	**0.004**	0.301

Data are median (interquartile range) values or n (%) if indicated. * Healthy controls, † HIV-exposed seronegatives, ‡ therapy-naïve HIV-1-infected subjects, § antiretroviral therapy-treated HIV-1-infected subjects. P-values (Mann Whitney *U*) below 0.05 are in bold.

4.2. Differences in mRNA expression in PBMC

The mRNA expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin were analyzed for all study subjects in PBMC by real-time qPCR (Table 3). None of the genes displayed a significant difference in mRNA expression between HESN and HC. HIV-UT showed significantly lower levels of LEDGF/p75 ($p = 0.008$) and higher levels of tetherin ($p = 0.027$) than HC. Additionally, higher expression levels of APOBEC3G ($p = 0.078$), TRIM5α ($p = 0.016$), and tetherin ($p < 0.001$) were observed in HIV-UT relative to HIV-ART subjects.

Table 3 - mRNA expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in PBMC of HIV-1-exposed seronegative, HIV-infected, and healthy control subjects.

mRNA expression	HC* (n = 20)	HESN† (n = 20)	HIV-UT‡ (n = 9)	HIV-ART§ (n = 31)	p-value		
					HESN vs. HC	HIV-UT vs. HC	HIV-ART vs. HIV-UT
LEDGF/p75	1.22 (0.88-1.50)	1.03 (0.83-1.36)	0.72 (0.53-1.11)	0.92 (0.70-1.10)	0.387	**0.008**	0.391
APOBEC3G	0.98 (0.79-1.23)	0.99 (0.86-1.25)	1.29 (1.00-1.38)	0.96 (0.83-1.22)	1	0.172	0.078
TRIM5α	1.08 (0.79-1.42)	0.85 (0.74-1.27)	1.22 (0.92-1.89)	0.78 (0.65-1.18)	0.372	0.370	**0.016**
Tetherin	1.12 (0.88-1.36)	1.10 (0.96-1.29)	1.46 (1.14-1.85)	0.99 (0.78-1.06)	1	**0.027**	**< 0.001**

Data are median (interquartile range) values depicting Calibrated Normalized Relative Quantity (CNRQ-values). * Healthy controls, † HIV-exposed seronegatives, ‡ therapy-naïve HIV-1-infected subjects, § antiretroviral therapy-treated HIV-1-infected subjects. P-values (Mann Whitney *U*) below 0.05 are in bold. Of note, due to sample loss during RNA extraction, data on mRNA expression were gathered for 20 HESN, 20 HC, 9 HIV-UT, and 31 HIV-ART.

4.3. Differences in protein expression in lymphocytes and monocytes

Next, we analyzed protein expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in CD4+ lymphocytes and CD14+ monocytes by a novel intracellular staining assay [456]. Expression of LEDGF/p75 was found to be significantly reduced (p = 0.022) in CD4+ lymphocytes of HESN compared to HC (Table 4). APOBEC3G, TRIM5α, and tetherin protein levels were similar for HESN and HC. HIV-UT showed significantly higher tetherin levels in CD4+ lymphocytes and significantly higher levels of all four proteins in CD14+ monocytes compared to HC and HIV-ART.

Table 4 - Protein expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in PBMC of HIV-1-exposed seronegative, HIV-infected, and healthy control subjects.

Protein expression	HC* (n = 23)	HESN† (n = 23)	HIV-UT‡ (n = 9)	HIV-ART§ (n = 36)	p-value		
					HESN vs. HC	HIV-UT vs. HC	HIV-ART vs. HIV-UT
CD4+ lymphocytes							
LEDGF/p75	113.10 (97.62-126.05)	98.80 (77.95-110.13)	121.91 (85.58-153.40)	106.85 (94.77-122.80)	**0.022**	0.516	0.371
APOBEC3G	39.61 (35.48-42.93)	38.01 (31.08-44.88)	39.68 (36.58-47.88)	40.64 (35.53-47.08)	0.613	0.572	0.691
TRIM5α	107.57 (96.87-123.01)	112.80 (93.67-125.22)	126.13 (100.47-156.08)	115.15 (100.26-134.41)	0.939	0.148	0.427
Membrane tetherin	4.16 (3.71-4.68)	4.35 (3.94-4.81)	6.55 (5.34-9.29)	4.77 (4.03-5.19)	0.733	**< 0.001**	**< 0.001**
Total Tetherin	5.32 (4.64-6.74)	5.33 (4.43-6.54)	10.73 (7.20-18.52)	5.88 (5.43-6.90)	0.809	**0.003**	**0.004**
CD14+ monocytes							
LEDGF/p75	49.26 (40.55-70.39)	45.51 (36.73-71.27)	77.05 (62.22-86.85)	45.65 (40.94-59.45)	0.750	**0.005**	**0.002**
APOBEC3G	102.09 (81.37-128.12)	98.62 (80.19-113.40)	123.28 (111.52-172.55)	105.30 (90.89-116.28)	0.328	**0.031**	**0.002**
TRIM5α	280.38 (234.04-335.57)	283.79 (235.40-332.42)	398.98 (317.44-513.08)	287.94 (257.72-331.20)	0.869	**0.005**	**0.002**
Membrane tetherin	1.62 (0.00-6.67)	2.42 (0.00-4.95)	11.77 (6.38-21.86)	4.78 (1.19-7.17)	0.894	**0.002**	**0.005**
Total tetherin	49.28 (40.00-65.42)	49.84 (40.99-55.70)	79.48 (59.27-113.97)	46.13 (38.27-50.87)	0.956	**0.003**	**< 0.001**

Data are median (interquartile range) values depicting Median Fluorescence Intensity (MFI-) values. * Healthy controls, † HIV-exposed seronegatives, ‡ therapy-naïve HIV-1-infected subjects, § antiretroviral therapy-treated HIV-1-infected subjects. P-values (Mann Whitney *U*) below 0.05 are in bold.

4.4. Correlations between mRNA and protein levels

Subsequently, we correlated mRNA levels in PBMC with protein levels specifically in lymphocytes and monocytes within the entire study population including HESN, HC, HIV-UT, and HIV-ART subjects (Figure 1). For most HIV-related host factors, we observed a significant positive correlation between mRNA and protein level as was most prominent for total tetherin, whereas no significant correlations were observed for LEDGF/p75.

4.5. Correlates of reduced LEDGF/p75 in HESN

To obtain insight into the possible role played by LEDGF/p75 in resistance to HIV-1 infection, we studied the reduced LEDGF/p75 expression levels in HESN considering the duration of the sexual relation, the number of sexual contacts per month, the frequency of condom use, and the HSV-2 serostatus (Table 5). We found that LEDGF/p75 expression levels were most reduced among HESN with the highest number of sexual contacts per month ($p = 0.065$), whereas no difference in LEDGF/p75 expression was found in the HIV-1-negative control group (Table 5).

Legend Figure 1 (see next page): Correlation patterns were studied in all study subjects between mRNA (CNRQ-values, x-axis) and protein (MFI-values, y-axis) levels of (A) LEDGF/p75 (LGF), (B) APOBEC3G (A3G), (C) TRIM5α (T5a), (D) membrane tetherin (memTeth), and (E) total tetherin (totTeth). mRNA levels were studied in PBMC while protein levels were analyzed in lymphocytes (Ly, left), CD4+ lymphocytes (CD4Ly, middle), and CD14+ monocytes (CD14Mo, right). CNRQ-values refer to Calibrated Normalized Relative Quantity of mRNA expression levels and MFI-values refer to Median Fluorescence Intensity of protein expression levels. Spearman's Rho (Rho) and p-values were obtained by non-parametric Spearman's rank correlation tests. P-values below 0.05 are in bold.

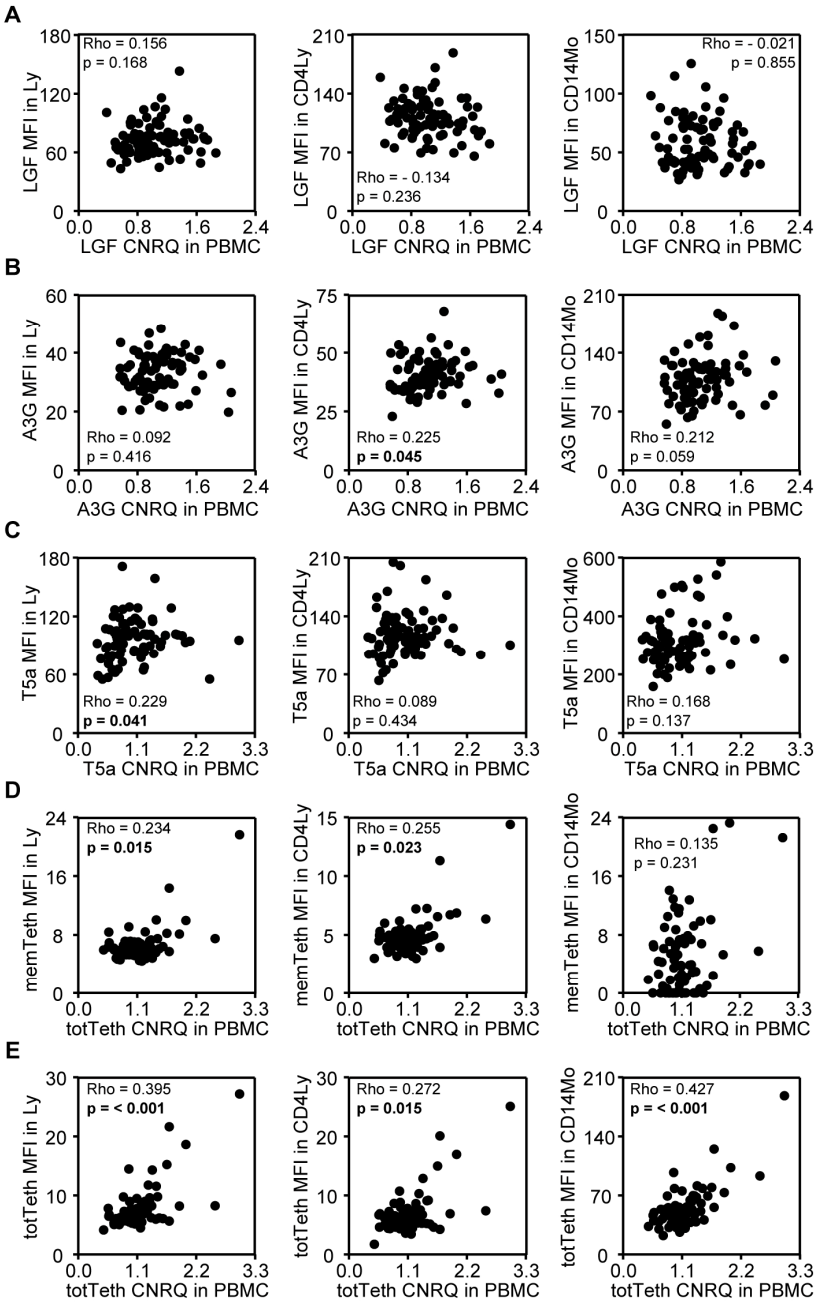

Figure 1 - Correlations between mRNA and protein levels. **(legend see previous page)**

Table 5 - Expression levels of LEDGF/p75 versus HIV-1 risk/exposure parameters within different study populations of interest.

	HC*			HESN†		
	subgroup 1[a]	subgroup 2[b]	**P-value**	subgroup 1	subgroup 2	**P-value**
Sexual contacts/month (<[a] vs ≥[b] median value)	109.47 (98.59-124.73)	114.26 (94.84-137.76)	0.537	105.63 (95.04-112.09)	85.37 (74.88-104.96)	0.065
Duration of relation, years (<[a] vs ≥[b] median value)	113.10 (99.57-141.30)	110.90 (89.88-124.04)	0.284	104.58 (73.50-111.30)	95.46 (81.66-112.71)	1.000
Condom use (always[a] vs not always[b])	-	110.23 (96.24-126.13)	ND	98.30 (69.33-111.37)	98.80 (81.66-112.66)	0.571
HSV-2 status (negative[a] vs positive[b])	110.23 (95.70-124.73)	123.96 (96.80-132.58)	0.502	98.30 (76.20-110.11)	101.50 (85.31-126.40)	0.468

* Healthy controls, † HIV-exposed seronegatives. Each HIV-1 risk /exposure parameter (left column) was used to split the study population in two subgroups ([a] subgroup 1 versus [b] subgroup 2) based on a categorical or on a numerical parameter. Numerical parameters use median values (see Table 2) to split the study group in two subgroups comparable in size. LEDGF/p75 expression levels are median (interquartile range) MFI-values. Within each study population, the P-value (Mann Whitney *U*) was calculated for each HIV-1 risk/exposure parameter. ND: not determined as none of the subjects used a condom.

4.6. Correlations between expression levels and T cell activation

Finally, we investigated whether the observed differences in mRNA and/or protein expression level could be correlated to the subject's T cell activation and/or disease status (viral load and CD4 count). Whereas no significant difference was observed in CD4+ T cell activation level (% CD38+ cells among the CD4+ T cells, p = 0.297), HESN showed a trend towards lower CD8+ T cell activation levels than HC (p = 0.095) in Figure 2A. Reduced T cell activation levels among HESN did not correlate with their reduced LEDGF/p75 protein expression levels (p = 0.970, Figure 2B). Neither did we observe differences in LEDGF/p75 expression between distinct CD4+ lymphocytes based on CD45RO (memory) or CD38 (activation) staining among HESN (data not shown). In HIV-UT, T cell activation levels correlated directly with TRIM5α (p = 0.002) and tetherin (p = 0.086) mRNA levels, and inversely with LEDGF/p75 mRNA levels (p = 0.037) (data not shown). Membrane and total expression levels of tetherin, in particular, correlated directly with T cell activation and the viral load, and inversely with the CD4 count. These correlations were most prominent for membrane tetherin as is depicted in Figure 2C-E.

Figure 2 – Statistical analyses within HESN and untreated HIV-1-infected subjects (see next page): (A) Difference in CD4 (left) and CD8 (right) T cell activation status (y-axis) between HESN and HC (x-axis). P-values were obtained through the Mann Whitney *U* test. (B) Correlation pattern in HESN subjects between LEDGF/p75 protein levels (LGF, y-axis) in CD4+ lymphocytes (CD4 Ly, left) and CD14+ monocytes (Mo, right) and T cell activation status (x-axis). (C-E) The graphs depict correlation patterns in CD4+ lymphocytes (CD4 Ly, left) and CD14+ monocytes (Mo, right) of HIV-UT subjects between membrane tetherin protein levels (memTeth, y-axis) and T cell activation status (x-axis, C), CD4 count (cells/mm³) (x-axis, D) and log viral load (x-axis, E). The subject's general T cell activation status is described by the percentage of CD38+ cells among the CD8+ T cells (B,C). Spearman's Rho (Rho) and p-values were obtained by non-parametric Spearman's rank correlation tests. P-values below 0.05 are in bold. MFI = Median Fluorescence Intensity.

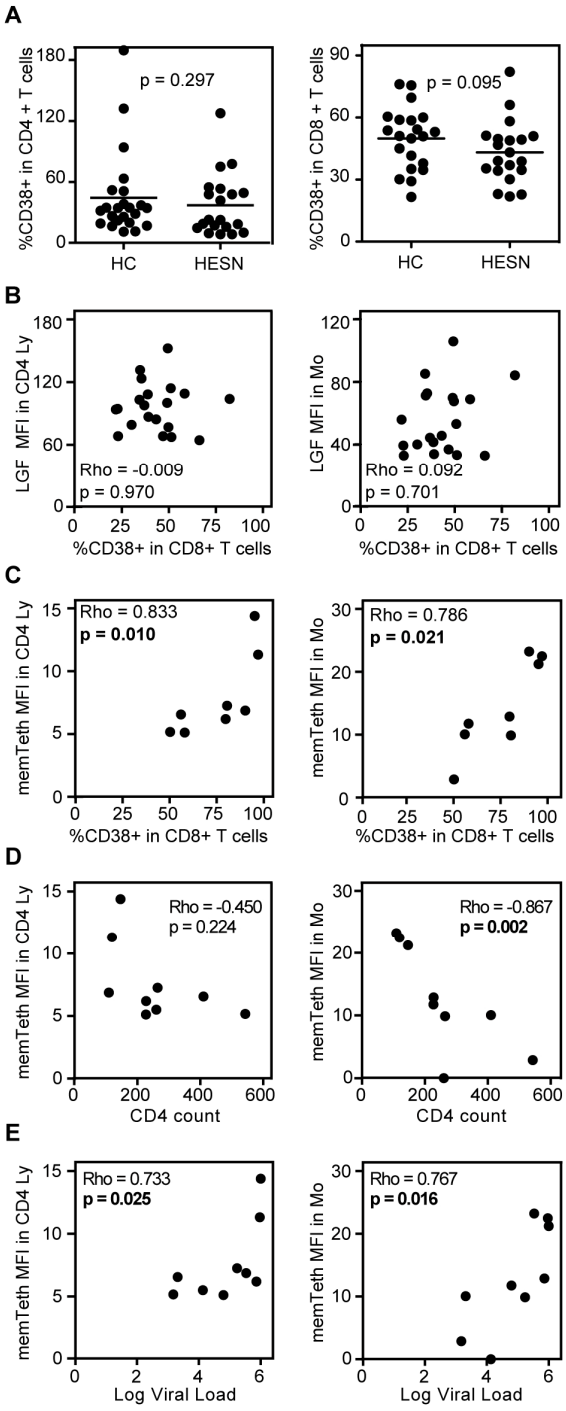

Figure 2 - Statistical analyses within HESN and untreated HIV-1-infected subjects. **(legend see previous page)**

5. Discussion

In this study, we explored whether the expression of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin could play a role in HIV-1 resistance. We studied mRNA levels in PBMC and protein levels in CD4+ lymphocyte and monocyte subsets. We observed significantly reduced levels of LEDGF/p75 protein expression in the CD4+ T cells of HESN compared to those of HC, whereas similar mRNA levels were found for both HESN and HC. Our findings of lower levels of LEDGF/p75 in CD4+ lymphocytes of HESN are in line with two recent HESN-studies showing reduced gene expression levels of several host genes important for HIV replication [708,709]. Lower LEDGF/p75 protein levels may cause HIV-1 integration to occur less efficiently, thus contributing to HIV-1 resistance in HESN subjects. This hypothesis is supported by an *in vitro* experiment that found a direct correlation between LEDGF/p75 expression levels and the efficiency of HIV-1 integration in the host genome [710]. Other studies, however, reported that efficient knockdown or knock-out of LEDGF/p75 is not sufficient to completely abolish HIV-1 replication [306,332,711,712], suggesting that other factors may be needed in concert with LEDGF/p75 to limit HIV integration in HESN subjects.

We observed no differences in mRNA or protein levels of APOBEC3G, TRIM5α, or tetherin between HESN and HC. These findings are at variance with previous reports showing elevated APOBEC3G expression levels in HESN compared with HC [411,621]. On the other hand, it has been suggested that reduced exposure to HIV-1 results in comparable APOBEC3G mRNA levels for HESN and HC over time [411]. In our HESN population, most HIV-1-infected partners were treated with antiretroviral therapy, and all couples received counselling to promote condom use. Therefore, the lack of distinct expression patterns may indeed result from the relatively low levels of HIV exposure in our population. Moreover, Reddy et al [414] observed similar levels of APOBEC3G mRNA between pre-infection values of seroconverters and exposed persistently negative individuals, suggesting that APOBEC3G mRNA levels would not contribute to protection against HIV-1.

Untreated HIV-1-infected patients (HIV-UT) showed significantly lower LEDGF/p75 and higher tetherin mRNA levels than HC, and higher APOBEC3G, TRIM5α, and tetherin mRNA levels than HIV-ART. At protein level, HIV-UT expressed significantly enhanced levels of tetherin in the lymphocytes and of all four proteins in the monocytes. These data suggest that HIV-1-infected subjects try to curb HIV-1 replication by increasing the expression level of the antiviral restriction factors.

Interestingly, increased levels of tetherin in HIV-UT correlated directly with T cell activation and the viral load and inversely with the CD4 count. This finding suggests that tetherin is not capable of controlling viral replication; instead it appears to be a marker of advanced HIV disease in therapy-naïve HIV-1-infected patients. Recently, Coleman et al [553] also reported increased tetherin expression levels upon exposure to HIV-1 without it being capable of restricting cell-to-cell spread of the virus. At least two other studies support our observations by suggesting that the tetherin-induced accumulation of unreleased virions at the cell membrane could actually promote cell-to-cell spread, e.g., by enhancing fusogenicity or regulating the integrity of the viral synapse [563,564]. In turn, accumulation of virus particles at the cell membrane may trigger further increases in immune activation [344,713]. Thus, tetherin may have a dual role during HIV-1 infection and disease. As an innate mediator, tetherin may be involved in the control of early or acute virus replication but it could become ineffective as the infection progresses.

T-cell activation levels also correlated directly with mRNA levels of TRIM5α but not with those of APOBEC3G; nevertheless we expect that similar mechanisms like those observed for tetherin are involved here. Indeed, type I interferons which are induced upon viral replication are known to upregulate APOBEC3G [412,639,714,715], TRIM5α [97,101], and tetherin [99,713]. High-level interferon receptor expression on monocytes [621] may explain why our data showed relatively higher increases of APOBEC3G, TRIM5α, and tetherin in monocytes than in lymphocytes.

Intriguingly, HIV-UT expressed reduced mRNA levels in PBMC and elevated protein levels in monocytes for LEDGF/p75, relative to HC. The mRNA levels correlated inversely ($p = 0.05$; Rho = -0.667) and the protein levels tended to correlate directly ($p = 0.170$; Rho = 0.5) with the viral load of these patients (data not shown). These correlations suggest that LEDGF/p75 expression may also account for disease progression in HIV-UT, supporting recently described observations by Madlala et al [709]. Parallel to type I interferon induction, viral replication induces the production of several cytokines and chemokines amongst which Tumor Necrosis Factor alpha (TNF-α), which can induce LEDGF/p75 levels [716,717]. Of note, long period or high level exposure to TNF-α makes LEDGF/p75 levels decrease again [717] which may play a role in the difference seen between LEDGF/p75 mRNA and protein levels.

Apart from the reasoning in the previous paragraph, mRNA and protein levels may differ as mRNA is not necessarily translated into (functional) protein [656], and external factors may act differently upon protein and mRNA levels [362,718]. Our data e.g. also show a difference in mRNA versus protein expression for LEDGF/p75 in HESN (Figure 1A). Nevertheless, we did observe some correlations between protein levels in (CD4+) lymphocytes, and mRNA levels in PBMC which are predominantly composed of lymphocytes (Figure 1B-E). Of note, due to the limited number of CD4+ cells in HIV-1-infected subjects, we were obliged to work with PBMC for quantification of mRNA levels. On the other hand, the flow cytometry based method allowed us to investigate protein levels in PBMC subsets. As earlier studies also focused on mRNA levels in PBMC, we could easily compare our results with these results previously obtained. For example, our data supported that APOBEC3G mRNA levels are similar for HESN and HC [414]. On the other hand, in literature, limited information is forehand regarding protein expression in particular cell-types while proteins are considered to be of greater relevance in the “direct” fight against HIV-1 infection as they are the “work horses” of the cell.

In summary, we observed significantly lower levels of LEDGF/p75 in CD4+ lymphocytes of HESN subjects, suggesting that LEDGF/p75 may play a role in the *in vivo* resistance to HIV-1 infection. Untreated HIV-1-infected patients showed increased levels of the antiviral proteins APOBEC3G, TRIM5α, and tetherin, with tetherin in particular as a possible marker of

advanced HIV disease. Future studies of LEDGF/p75 expression in other populations of HESN subject will be required to confirm our findings. Understanding the role of HIV-1-related host factors in HIV-1 susceptibility and disease could be of great interest for the development of future antiviral therapies.

6. Acknowledgements

We thank the individuals who kindly participated in the study; Dr Ndeye Fatou Ngom Gueye, Dr Ibrahima Ndiaye, and Dr Khady Ba Fall for recruiting and Marianne Ndiaye for counselling and follow-up of the Senegalese participants; Dr Aïssatou Gaye Ndiaye for HIV serological testing; and Katrien Fransen for HIV-1 viral load and HSV-2 testing. We acknowledge Chugai Pharmaceutical Co. (Japan) for kindly providing the anti-tetherin antibody and the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) for providing the anti-APOBEC3G and anti-TRIM5α antibodies.

This work is supported by the fund for Scientific Research (FWO) Flanders (grant G.0660.06) and an FWO doctoral fellowship to KM.

Part II: Impact of LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin expression levels on HIV-1 transmission capacity

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(Unpublished data)

1. Abstract

Objective: The phenomenon of HIV-1 restriction is mostly studied in subjects who are frequently exposed to HIV-1 but remain seronegative. Yet, in HIV-discordant couples, one could also investigate HIV-1 restriction from another point of view thereby focussing on the HIV-1-infected partner who may be incapable to transmit the virus.

Methods: We used real-time PCR and flow cytometry to study mRNA and protein levels respectively in PBMC and PBMC subsets from HIV-1 transmitters and non-transmitters.

Results: We found no differences in mRNA or protein expression for LEDGF/p75, APOBEC3G, TRIM5α, and tetherin between HIV-1 transmitters and non-transmitters. Instead, especially within HIV-1 transmitters, differential host factor levels correlated with clinical markers of HIV-1 disease progression.

Conclusions: Our data suggest that expression levels of the HIV-1-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin are not involved in HIV-1 transmission.

2. Introduction

To date, HIV-1 restriction has mostly been studied in HIV-1-exposed seronegative subjects (HESN) including commercial sex workers, men having sex with men, intravenous drug users, and HIV-negative partners in HIV-discordant couples. However, instead of focussing solely on the frequently HIV-1-exposed seronegative subject (HESN), the HIV-1-infected subject to who the HESN is exposed is of equal interest since HIV-1 restriction may also come forth from limited HIV-1 transmission capacity. In general, sexual HIV-1 transmission occurs at a low efficiency rate [79,719]. Nevertheless, 75 to 85% of the HIV-1 infections reported worldwide are caused by unprotected sexual intercourse [79]. Sexual HIV-1 transmission is influenced by several determinants including the HIV-1 strain, viral load, antiviral treatment, and the presence of other sexually transmitted diseases or genital ulcerations [75,720–723]. During sexual intercourse, the virus "travels" via human mucosal tissues from the HIV-1-infected person to his/her partner. We hypothesize that host factors of HIV-1-infected subjects may restrict cellular viral replication and/or virus shedding into the mucosa e.g. by hyper-mutating the viral genome or by tethering newly formed viral particles to the cell membrane. In this amendment, we broaden our research question to address the hypothesis that expression levels of HIV-1-related host factors like LEDGF/p75, APOBEC3G, TRIM5α, and tetherin are involved in onward HIV-1 transmission capacity. Therefore, we extended our analyses on HIV-1-infected subjects selected from Senegalese HIV-1-discordant and HIV-1-infected concordant heterosexual couples.

3. Materials and Methods

The "Materials and Methods" section of part I also describes all experimental settings as applied in this part II.

4. Results

4.1. Study population

In Chapter 6, forty five HIV-1-infected subjects were selected from 20 HIV-1-discordant and 25 HIV-1-concordant heterosexual couples and grouped based on antiretroviral treatment. In this amendment, we regrouped these 45 HIV-1-infected subjects based on their capacity to transmit the HIV-1 virus. Within discordant couples, we refer to the HIV-1-infected partners as HIV-1 non-transmitters (NT). Within HIV-1-infected concordant couples, we selected the HIV-1-infected partners who transmitted the virus to their partner and now refer to them as HIV-1 transmitters (T). The genetic linkage and direction of HIV-1 transmission within HIV-1-infected concordant couples was determined by Jennes et al [724]. First, true intra-couple transmission was determined via phylogenetic analysis of viral gp41 sequences from both partners. Once intra-couple transmission was confirmed, the HIV-1 transmission direction was unravelled based on the standard questionnaires filled in by both HIV-1-infected partners.

For each individual, the parameters age, gender, duration of the sexual relationship, CD4 count, number of sexual contacts per month, condom use, immune activation status, HSV-2 serostatus, antiretroviral therapy treatment status, and viral load in untreated HIV-1-infected subjects were investigated. Non-transmitters were more frequently women and reported a higher frequency of condom use (Table 1).

Table 1 - Characteristics of non-transmitters (NT) and transmitters (T).

	NT (n = 20)	T (n = 25)	**p-value** NT vs. T
Age, years	39 (35-50)	47 (41-52)	0.052
Gender, n (% male)	11 (55)	23 (92)	**0.005**
Duration of sexual relation, years	11 (8-17)	11 (6-14)	0.563
CD4 count (cells/µl)	350 (197-488)	240 (186-387)	0.337
Number of sexual contacts per month	7 (4-8)	7 (4-10)	0.810
Condom use, n (% always)	14 (70)	8 (32)	**0.012**
Immune activation status (%CD38+ CD8+ T cells)	52 (43-75)	56 (31-77)	0.654
HSV-2 serostatus, n (% HSV-2 seropositive)	11 (55)	16 (64)	0.848
Therapy naïve, n (%)	3 (15)	6 (24)	0.458
Log viral load within therapy naïve subjects	4.1 (3.2-6.0)	5.4 (4.4-5.9)	0.606

Data are median (interquartile range) values or n (%) if indicated. NT, HIV-1 non-transmitter; T, HIV-1 transmitter. P-values (Mann Whitney *U*) below 0.05 are in bold.

4.2. Differences in mRNA and protein expression levels between non-transmitters and transmitters

The expression of four HIV-1-related host factors – LEDGF/p75, APOBEC3G, TRIM5α, and tetherin – was compared between non-transmitters and transmitters. Real-time qPCR was used to study mRNA levels in PBMC, whereas the flow cytometry-based intracellular staining assay [456] was applied to study protein levels in CD4+ lymphocytes and CD14+ monocytes. No significant differences in mRNA or protein level were observed as is depicted in Table 2.

Table 2 – HIV-1-related host factor expression levels compared between HIV-1 non-transmitters and transmitters.

	NT (n = 20)	T (n = 25)	p-value NT vs. T
mRNA expression in PBMC			
LEDGF/p75	0.84 (0.65-1.06)	0.93 (0.70-1.22)	0.280
APOBEC3G	0.93 (0.77-1.27)	1.05 (0.89-1.29)	0.404
TRIM5α	0.81 (0.67-1.06)	1.05 (0.66-1.40)	0.436
Total tetherin	1.04 (0.94-1.21)	0.99 (0.78-1.26)	0.538
Protein expression in CD4+ lymphocytes			
LEDGF/p75	106.17 (93.35-128.36)	114.18 (95.11-129.68)	0.599
APOBEC3G	40.00 (35.59-46.00)	40.00 (36.37-47.57)	0.723
TRIM5α	112.25 (99.96-136.34)	116.40 (100.68-136.98)	0.493
Membrane tetherin	4.89 (4.45-5.32)	4.83 (4.11-5.88)	0.900
Total tetherin	6.14 (5.60-7.19)	6.01 (5.15-9.04)	0.962
Protein expression in CD14+ monocytes			
LEDGF/p75	50.15 (41.22-63.66)	51.38 (42.38-77.47)	0.584
APOBEC3G	108.01 (94.21-116.40)	111.03 (92.00-124.79)	0.299
TRIM5α	307.43 (257.72-335.61)	315.09 (262.11-360.41)	0.553
Membrane tetherin	3.38 (0.00-7.33)	6.16 (3.08-9.46)	0.162
Total tetherin	49.20 (39.23-55.65)	47.38 (39.49-69.48)	0.891

Data are median (interquartile range) CNRQ- or MFI-values. For mRNA expression, CNRQ-values refer to Calibrated Normalized Relative Quantity values. For protein expression, MFI-values refer to Median Fluorescence Intensity values. NT, HIV-1 non-transmitters; T, HIV-1 transmitters. P-values (Mann Whitney *U*) below 0.05 are in bold.

4.3. HIV-1 transmissibility parameters versus host factor expression within non-transmitters and transmitters

Next, we investigated the expression pattern of the four HIV-1-related host factors within immune cells of either HIV-1 non-transmitters or transmitters. Whereas several HIV-1 risk/exposure parameters were included in the study of HESN subjects, we now address host factor expression levels within both HIV-1 non-transmitters and transmitters while considering particular HIV-1 transmissibility parameters. Each study group (NT or T) was divided in two groups of equal size based on the median of those HIV-1 transmissibility parameters. The parameters studied are HSV-2 serostatus, therapy treatment status, log viral load in the untreated subjects, and immune activation status. We observed that the here-studied host factor levels were clearly enhanced in CD4+ lymphocytes and CD14+ monocytes of HIV-1 transmitters who were therapy naïve (untreated) as well as of those who showed higher immune activation levels (Table 3a). Mostly, no significant differences in HIV-1-related host protein level were observed within the HIV-1 non-transmitters; except for membrane tetherin which was elevated in CD4+ lymphocytes of therapy naïve non-transmitters, and for APOBEC3G and total tetherin levels which were both elevated in CD4+ lymphocytes of HSV-2 co-infected non-transmitters (Table 3b).

Table 3a – Expression of HIV-1-related host factors within HIV-1 transmitters considering HIV-1 transmissibility parameters.

		HIV-1 transmitters					
		CD4+ lymphocytes			CD14+ monocytes		
		subgroup 1[a]	subgroup 2[b]	**P-value**	subgroup 1	subgroup 2	**P-value**
HSV-2 status (negative[a] vs positive[b]) (n = 9 vs n = 16)	LEDGF/p75	96.83 (92.50-117.99)	121.53 (97.52-131.76)	0.213	46.53 (36.28-74.84)	60.72 (43.76-77.67)	0.336
	APOBEC3G	40.00 (36.37-46.38)	40.26 (36.44-49.75)	0.777	105.15 (91.02-114.46)	122.89 (95.91-145.34)	0.126
	TRIM5α	113.72 (97.66-130.69)	121.35 (103.82-158.80)	0.258	261.96 (244.92-351.86)	318.72 (275.56-444.44)	0.141
	Total tetherin	5.74 (4.69-6.66)	7.03 (5.42-9.16)	0.245	42.11 (38.45-46.29)	51.31 (41.97-78.44)	0.113
	Membrane tetherin	4.51 (3.73-5.86)	5.01 (4.31-6.09)	0.428	6.00 (1.94-7.91)	7.35 (2.86-10.02)	0.428
Therapy status (UT[a] vs ART[b]) (n = 6 vs n = 19)	LEDGF/p75	134.64 (109.14-162.36)	106.60 (94.76-121.91)	0.080	81.36 (72.85-91.22)	45.33 (40.98-61.19)	**0.008**
	APOBEC3G	41.63 (38.88-56.97)	39.10 (36.20-47.41)	0.203	145.37 (117.22-185.41)	102.86 (90.76-118.92)	**0.006**
	TRIM5α	140.56 (118.89-174.12)	115.14 (99.85-131.92)	0.075	449.16 (318.61-541.46)	276.10 (260.50-330.59)	**0.006**
	Total tetherin	12.86 (8.08-17.74)	5.76 (5.02-6.94)	**0.009**	88.34 (59.93-108.47)	44.69 (38.08-49.24)	**0.001**
	Membrane tetherin	6.71 (5.93-8.28)	4.56 (3.70-5.04)	**0.001**	12.33 (10.02-22.67)	5.21 (1.87-6.83)	**< 0.0001**

Log viral load in HIV-UT (<[a] vs ≥[b] median value) (n = 2 vs n = 4)	LEDGF/p75	110.26 (73.15-147.36)	140.67 (121.33-168.22)	0.355	69.06 (60.24-77.88)	86.85 (79.00-95.93)	0.165
	APOBEC3G	54.36 (40.83-67.89)	41.05 (37.28-50.60)	0.355	159.08 (130.22-187.93)	140.88 (109.17-178.56)	0.355
	TRIM5α	148.80 (97.17-200.42)	140.56 (128.18-160.71)	1.000	407.21 (315.09-499.33)	462.91 (339.58-570.73)	0.355
	Total tetherin	9.98 (4.98-14.98)	13.85 (9.52-19.30)	0.355	68.71 (57.93-79.48)	100.08 (69.75-119.48)	0.165
	Membrane tetherin	5.84 (5.12-6.55)	7.07 (6.37-10.31)	0.165	10.92 (10.06-11.77)	17.68 (10.63-23.04)	0.355
Immune activation status (<[a] vs ≥[b] median value) (n = 12 vs n = 13)	LEDGF/p75	105.86 (94.94-119.63)	121.91 (91.77-146.84)	0.242	45.10 (42.28-50.69)	72.41 (46.48-86.85)	0.082
	APOBEC3G	39.30 (36.35-44.49)	40.83 (35.41-51.93)	0.446	101.28 (90.89-115.86)	124.53 (102.23-155.45)	**0.044**
	TRIM5α	113.78 (96.56-128.99)	126.31 (112.25-164.08)	0.057	262.11 (242.45-319.47)	332.12 (304.42-487.76)	**0.004**
	Total tetherin	5.80 (4.88-6.77)	8.83 (5.25-12.86)	0.060	41.54 (38.27-48.37)	60.60 (47.10-88.34)	**0.014**
	Membrane tetherin	4.44 (3.53-5.24)	5.12 (4.80-6.71)	**0.022**	5.65 (4.01-7.63)	9.03 (1.81-12.33)	0.276

Each HIV-1 infection/transmission parameter (left column) was used to split the study population in two subgroups ([a] subgroup 1 versus [b] subgroup 2) based on a categorical or on a numerical (median) value. The table summarizes all median (interquartile range) MFI-values and p-values to compare protein levels within the HIV-1 transmitter study populations based on HIV-1 infection/transmission characteristics. P-values (Mann Whitney U) below 0.05 are in bold. UT: untreated; ART: antiretroviral treated.

Table 3b – Expression of HIV-1-related host factors within non-transmitters considering HIV-1 transmissibility parameters.

		HIV-1 non-transmitters					
		CD4+ lymphocytes			CD14+ monocytes		
		subgroup 1[a]	subgroup 2[b]	**P-value**	subgroup 1	subgroup 2	**P-value**
HSV-2 status (negative[a] vs positive[b]) (n = 7 vs n = 11)	LEDGF/p75	105.23 (93.26-111.14)	107.21 (93.62-134.87)	0.415	48.82 (42.11-54.23)	62.39 (40.92-81.07)	0.258
	APOBEC3G	35.30 (29.91-45.76)	43.64 (38.04-47.80)	**0.042**	97.14 (81.09-113.35)	108.88 (101.93-117.89)	0.258
	TRIM5α	104.76 (92.70-141.38)	119.65 (104.42-138.56)	0.298	314.81 (270.33-347.23)	304.19 (254.90-324.00)	0.497
	Total tetherin	5.59 (4.90-5.97)	6.90 (5.75-8.65)	**0.033**	40.60 (36.14-51.19)	49.96 (44.59-64.50)	0.189
	Membrane tetherin	5.03 (3.82-5.32)	4.86 (4.51-5.72)	0.497	5.45 (0.00-7.35)	2.88 (2.01-6.39)	0.963
Therapy status (UT[a] vs ART[b]) (n = 3 vs n = 17)	LEDGF/p75	90.58 (80.58-130.11)	107.10 (94.20-128.78)	0.223	62.39 (62.04-64.08)	45.97 (40.18-63.76)	0.153
	APOBEC3G	36.67 (36.47-38.04)	43.64 (34.76-46.94)	0.315	117.89 (103.75-123.28)	107.74 (91.00-112.87)	0.153
	TRIM5α	103.76 (93.66-119.65)	115.55 (101.56-139.97)	0.266	323.78 (314.81-411.88)	303.26 (256.10-334.21)	0.153
	Total tetherin	10.30 (5.28-25.08)	5.97 (5.61-7.00)	0.266	69.96 (38.77-188.16)	48.64 (39.18-52.81)	0.186
	Membrane tetherin	5.50 (5.17-14.39)	4.83 (4.29-5.27)	**0.050**	2.88 (0.00-21.23)	3.87 (0.00-7.32)	0.830

Log viral load in HIV-UT (<[a] vs ≥[b] median value) (n = 1 vs n = 2)	LEDGF/p75	130.11	85.58 (80.58-90.58)	0.221	62.39	63.06 (62.04-64.08)	1.000
	APOBEC3G	36.67	37.26 (36.47-38.04)	1.000	123.28	110.82 (103.75-117.89)	0.221
	TRIM5α	119.65	98.71 (93.66-103.76)	0.221	411.88	319.30 (314.81-323.78)	0.221
	Total tetherin	10.30	15.18 (5.28-25.08)	1.000	69.96	113.47 (38.77-188.16)	1.000
	Membrane tetherin	5.17	9.95 (5.50-14.39)	0.221	2.88	10.62 (0.00-21.23)	1.000
Immune activation status (<[a] vs ≥[b] median value) (n = 8 vs n = 9)	LEDGF/p75	109.12 (102.89-139.87)	107.21 (93.44-128.78)	0.500	50.15 (40.10-76.40)	52.58 (41.98-68.69)	0.773
	APOBEC3G	44.34 (35.64-48.47)	41.28 (37.39-46.78)	1.000	110.47 (98.54-121.81)	107.74 (95.50-115.62)	0.441
	TRIM5α	129.11 (105.40-160.87)	115.55 (99.44-126.70)	0.386	317.33 (257.76-368.94)	282.14 (254.93-330.40)	0.386
	Total tetherin	6.51 (5.81-7.32)	6.55 (5.61-7.94)	0.923	45.55 (34.37-55.02)	49.96 (44.62-60.28)	0.290
	Membrane tetherin	4.77 (3.96-5.14)	5.24 (4.58-6.47)	0.178	4.51 (2.23-6.82)	2.01 (0.00-9.59)	0.354

Each HIV-1 infection/transmission parameter (left column) was used to split the study population in two subgroups ([a] subgroup 1 versus [b] subgroup 2) based on a categorical or on a numerical (median) value. The table summarizes all median (interquartile range) MFI-values and p-values to compare protein levels within the HIV-1 non-transmitter study populations based on HIV-1 infection/transmission characteristics. P-values (Mann Whitney *U*) below 0.05 are in bold. UT: untreated; ART: antiretroviral treated.

5. Discussion

In this amendment, we explored whether the expression of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin could play a role in HIV-1 transmission. We studied mRNA levels in PBMC and protein levels in CD4+ lymphocyte and monocyte subsets of HIV-1 transmitters and non-transmitters. We found that the expression pattern of these four HIV-1-related host factors did not significantly vary between transmitters and non-transmitters (see Table 2). These data suggest that host factor expression levels of LEDGF/p75, APOBEC3G, TRIM5α, and tetherin do not influence HIV-1 transmission capacity in our study population.

Additional statistics were included to study host factor expression levels within HIV-1 transmitters and non-transmitters while taking into account several known factors that influence HIV-1 transmission (Table 3). Parameters like HSV-2 co-infection status, therapy status, and viral load have been described in literature to influence HIV-1 transmission capacity [725–731]. We observed that almost all HIV-1-related host factors under investigation (LEDGF/p75, APOBEC3G, TRIM5α, and tetherin) are significantly enhanced in HIV-1 transmitters who are therapy naïve and/or show an elevated immune activation status (%CD38+ cells within CD8+ cells). In general, therapy-naïve HIV-1 transmitters are expected to show a higher HIV-1 transmission capacity than therapy-treated transmitters. Whereas elevated levels of the co-factor LEDGF/p75 could eventually explain enhanced HIV-1 transmission capacity in the therapy-naïve HIV-1 transmitters, it is extremely unlikely that increased expression profiles of host restriction factors such as APOBEC3G, TRIM5α, and tetherin support HIV-1 transmission. Hence, we conclude that APOBEC3G, TRIM5α, and tetherin are not involved in HIV-1 transmission. Instead, we rather believe that the HIV-1 infection may have caused the host's immune activation status to increase, thereby inducing tumour necrosis factor and/or interferon levels which in turn induce the expression profile of the HIV-1-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin. Thus far, it remains unclear why this effect does not appear in HIV-1 non-transmitters.

Interestingly, we also observed enhanced protein levels of the host restriction factors APOBEC3G and tetherin in HIV-1 non-transmitters who were co-infected with HSV-2. These data could suggest that HSV-2 co-infection may have caused increased host restriction factor levels. It remains unclear though why this effect was observed solely in HIV-1 non-transmitters. Reduced HIV-1 transmission capacity could eventually be explained by the induced host restriction factor expression levels following HSV-2 co-infection, but we agree that this hypothesis is rather far fetched and certainly needs further investigation.

Consequently, as was also discussed in Chapter 6 part I, we believe that the HIV-1 infection alters host factor expression profiles rather than that host factor levels significantly influence HIV-1 replication and/or transmission capacity in HIV-1-infected individuals. Of note, one should be careful not to over-interpret these data since the sample size of the therapy-naïve (HIV-UT) study populations was low and condom use – especially in the HIV-1 non-transmitter study population – was high. Further investigation may be required to confirm/expand our findings.

Chapter 7: General Discussion & Future Perspectives

1. General Discussion

The Human Immunodeficiency Virus (HIV) needs the human host to replicate. Many human host factors – cellular co-factors as well as host restriction factors – are involved in the HIV-1 replication cycle [153,732,733]. In this PhD-thesis, we focused on the expression pattern of four of such HIV-1-related host factors namely the cellular co-factor LEDGF/p75 and the three putative host restriction factors APOBEC3G, TRIM5α, and tetherin. Real-time qPCR was used to analyze mRNA levels in PBMC. Protein levels were studied specifically in HIV-susceptible cell-types like CD4+ lymphocytes and CD14+ monocytes with an in-house optimized flow cytometry-based intracellular protein staining method. Both real-time qPCR and intracellular protein staining experiments allowed for comparative expression analyses of specific HIV-1-related host factors between different study populations and cell-types which may be of interest for the development of potentially new antiviral therapies.

As can be deduced from the title, the **aim** of this PhD-thesis was to study the impact of cellular HIV-related host factor expression in frequently HIV-1-exposed seronegative (HESN) individuals. This scientific question can be examined from different angles focusing e.g. on expression patterns, working mechanisms, or polymorphisms of HIV-1-related host factors. We explored the quantitative impact of four HIV-1-related host factors that may be involved in HIV-1 restriction. Our hypothesis is that enhanced restriction factor (APOBEC3G, TRIM5α, or tetherin) and/or reduced co-factor (LEDGF/p75) expression levels may result in protection against HIV-1 infection. This hypothesis is supported by previous findings on LEDGF/p75, APOBEC3G, and TRIM5α expression. Madlala et al [330] reported higher pre-infection mRNA levels of LEDGF/p75 in seroconverters suggesting a link between enhanced LEDGF/p75 levels and enhanced HIV-1 infectivity. Enhanced APOBEC3G mRNA levels have been described to block HIV-1 entry to the central nervous system [418], and were observed in two different study groups of HESN [73,411]. Sewram et al [503] detected higher TRIM5α mRNA levels in non-seroconverters when compared to seroconverters. Moreover, TRIM5α levels were similar pre- and post-infection. Based on the latter data, higher TRIM5α mRNA levels are suggested being involved in reduced susceptibility to HIV-1 infection.

Currently, the most common method to study the expression of APOBEC3G and TRIM5α is the mRNA quantifying real-time qPCR method [73,97,100,208,209,416,505,701]. However, as proteins can be seen as the actual "work horses" of living cells, **protein expression** may be considered of greater relevance than mRNA expression. Although mRNA molecules are translated into proteins, mRNA and protein expression levels do not necessarily correlate. Possible explanations are that mRNA may not be translated into (functional) protein [665], and that external factors may influence mRNA and protein levels differently [362,718]. In literature, mainly two protein quantification methods (western blotting and ELISA) are described that address APOBEC3G and TRIM5α expression [73,97,100,210,362,416,505,700]. Unfortunately, to study mRNA or protein expression levels in particular cell-types, labor intensive and sample/material consuming cell purification steps are required. Therefore, an alternative method to study specific host protein intracellular expression at the level of a single cell and in different cell-types simultaneously was looked for. We based ourselves on a common intracellular cytokine staining protocol combined with flow cytometry [734,735]. While performing a thorough optimization procedure of this intracellular protein staining (ICS) method for of LEDGF/p75, APOBEC3G, and TRIM5α as fully described in Chapter 3 and [456], several study groups published total and membrane tetherin expression results applying a similar flow cytometry-based quantification method [213–217]. Considering the interest of tetherin in HIV-1 replication, we incorporated tetherin in our panel of HIV-1-related host factors as documented in Chapter 4. Total and membrane tetherin levels were addressed respectively with an intracellular and extracellular protein staining method. The biggest hurdle in optimizing these protein staining methods was finding appropriate antibodies that stain each of the HIV-1-related host proteins specifically. Preferentially, monoclonal antibodies (MoAbs) are used since MoAbs recognize one single antigen-epitope and thus stain proteins specifically in a 1:1 ratio, whereas polyclonal antibodies (PoAbs) often recognize multiple antigen-epitopes leading to inaccurate quantitative results and protein family recognition [736,737]. Therefore, MoAbs usually stain proteins in a highly specific and reproducible manner with low background staining via their Fab-fragment, whereas the Fc-fragment can serve as target for indirect staining. Potential background staining due to binding of MoAbs via their Fc-fragments is avoided through the addition of the blocking agent

bovine serum albumin (BSA). Next, the selected MoAbs require binding capacity for the specific antigen in its folded and fixed state [738], which is opposed to e.g. western blotting experiments in which (partially) denatured proteins are targeted. We tested several antibodies and selected one for each HIV-1-related protein which was further used for thorough optimization (see Chapter 3 and 4). Of note, for LEDGF/p75, we were obligated to use an anti-LEDGF antibody that interacts with both splice variants p52 and p75 as no specific anti-LEDGF/p75 antibody was available. In conclusion, the major bottleneck in this type of protein staining experiments is the availability of appropriate antibodies. Therefore, further investment is warranted on method- and antigen-specific antibody development.

Throughout the PhD-thesis, two study populations were enrolled to address HIV-1-related host factor expression. In first instance, we applied the ICS methodology on a small **Belgian study population** of healthy controls (HC), untreated HIV-1-infected subjects (HIV-UT), and antiretroviral therapy-treated HIV-1-infected subjects (HIV-ART) (for each group, n=10). In this study population, LEDGF/p75, APOBEC3G, and TRIM5α protein expression was studied in CD4+ lymphocytes (naïve and memory) and CD14+ monocytes (CD16- and CD16+) (see Chapter 3 and [456]). This pilot study confirmed the capability of the ICS method to identify differential protein expression patterns between cell types and study populations. Of note, tetherin analyses were not performed on the Belgian study population. Secondly, both real-time qPCR and ICS/ECS methodology were applied to study the four HIV-1-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin in a **Senegalese study population**, which was enrolled in function of the COPRIM study (see Chapter 6 and [724,739] for more details). The COPRIM study investigates the correlates of protective immunity against HIV and is a collaborative project between the Institute of Tropical Medicine (ITM) in Antwerp (Belgium), the “Hôpital de Fann” and the “Hôpital Le Dantec” both in Dakar (Senegal). The study population included healthy controls (HC, n = 23), HIV-1-exposed seronegative subjects (HESN, n = 23), and HIV-1-infected subjects (n = 45). This latter group can be split into either untreated HIV-1-infected subjects (HIV-UT, n = 9) and antiretroviral therapy-treated HIV-1-infected subjects (HIV-ART, n = 36), or into HIV-1 transmitters (n = 25) and non-transmitters (n = 20) (see Chapter 6).

Of note, although real-time qPCR is used to study **mRNA expression** levels in the Senegalese study population, we had first attempted to develop an mRNA intracellular staining method based on hybridization and flow cytometry methodology. As described in Chapter 5, three approaches were investigated without success, for which several explanations can be formulated. One major obstacle was to find a probe that can specifically interact with a particular mRNA sequence within a fixed cell. Moreover, the detection method should be highly sensitive to allow for trustworthy quantification of the mRNA sequence of interest without prior amplification. A similar approach of mRNA hybridization followed by flow cytometry is described in the ViroTect^{TM}Plus kit (Invirion) which is designed to quantify HIV-1 mRNA in host cells. When we applied the kit according to the manual, we did not obtain satisfactory results. The disappointing data that we obtained with the ViroTect^{TM}Plus kit (Invirion) were discussed with the company without result improvement. The difficulty to develop an mRNA hybridization method combined with flow cytometry appears confirmed in literature where we found mostly older references to a limited number of articles on either flow cytometry based mRNA quantification methods [676–684] or on the ViroTect^{TM}Plus kit (Invirion) [694–697]. In 2009, Robertson et al [686] described an LNA flow-FISH procedure to quantify mRNA using locked nucleic acid (LNA) probes which would bind DNA and RNA with higher affinity than the oligodeoxynucleotide probes that we used. The sum of disappointing results lead to the decision to cease our efforts and to use the commonly used real-time qPCR method preceded by a reversed-transcription step instead. The mRNA of unfixed cells is then reverse transcribed into DNA after which the real-time qPCR method allows for quantification of a small amount of template DNA through amplification. As also mentioned above, to study mRNA levels specifically in particular cell-types, cell purification is required. Hence, limitations in the available number of peripheral blood mononuclear cells (PBMC) and thus in the number of CD4+ cells specifically in HIV-1-infected subjects made us decide to study mRNA levels in PBMC. In Table 1, our expression data are summarized based on four scientific questions. Below, we further discuss obtained results, thereby including the comparison between the Belgian and Senegalese study population.

Table 1 - Differential protein expression results from the Belgian and Senegalese study population.

Belgian Study population (n = 30) (LEDGF/p75, APOBEC3G, TRIM5α)		**Senegalese Study population (n = 91)** (LEDGF/p75, APOBEC3G, TRIM5α, tetherin)	
Expression analysis between different cell-types			
• LGF/A3G/T5α	➢ in memory > naïve CD4+ Ly	• LGF/A3G/T5α/Teth	➢ in memory > naïve CD4+ Ly
• A3G/T5α	➢ in CD16- ≈ CD16+ Mo	• A3G/T5α/totTeth	➢ in CD16- > CD16+ Mo
• LGF	➢ in CD16- < CD16+ Mo	• LGF/memTeth	➢ in CD16- < CD16+ Mo
Expression analysis between different study populations			
• A3G/T5α	➢ Weak trend HIV-UT < HC	• LGF in CD4+ Ly	➢ HESN < HC
		• LGF/A3G/T5α/Teth	➢ NT ≈ T
		• Teth in CD4+ Ly	➢ HIV-UT > HC and HIV-UT > HIV-ART
		• LGF/A3G/T5α/Teth in Mo	➢ HIV-UT > HC and HIV-UT > HIV-ART
Correlation statistics in HIV-UT between host factor expression and T cell activation levels or parameters of HIV disease status			
• A3G/T5α	➢ Neg. correlation with viral load ➢ Pos. correlation with CD4 count	• Teth	➢ Pos. correlation with T cell activation ➢ Pos. correlation with viral load ➢ Neg. correlation with CD4 count
Correlation statistics between mRNA and protein expression			
		In 10 HC:	
		• LGF/A3G/T5α	➢ No correlation
		In all subjects:	
		• LGF	➢ No correlation
		• A3G/T5α/Teth; mainly totTeth	➢ Pos. correlation between mRNA and protein expression

LGF: LEDGF/p75; A3G: APOBEC3G; T5α: TRIM5α; Teth: Tetherin; totTeth: total Tetherin; memTeth: membrane Tetherin; Ly: lymphocytes; Mo: monocytes; HC: HIV-1 seronegative individuals; HESN: HIV-1-exposed seronegative subjects; HIV-UT: Therapy-naïve HIV-1 seropositive patients; HIV-ART: Antiretroviral Therapy-treated HIV-1 seropositive patients; NT: HIV-1-infected non-transmitters; T: HIV-1-infected transmitters; Neg.: negative; Pos.: positive. T cell activation levels refer to the percentage of CD38+ cells among the CD8+ T cells.

A) Expression analysis between different cell-types

We observed that LEDGF/p75, APOBEC3G, TRIM5α, and tetherin expression levels were generally enhanced in **memory versus naïve CD4+ lymphocytes**. Higher virus susceptibility has been noted for memory than naïve CD4+ lymphocytes [740–742]. Thus, these data suggest that the expression pattern of the restriction factors may not be involved in the preferential tropism of HIV-1 for memory CD4+ T cells. Alternatively, it is possible that the putative antiviral effects of increased APOBEC3G, TRIM5α, and tetherin levels were masked by concomitantly higher levels of LEDGF/p75. In the monocytes, CD16+ monocytes expressed higher levels of the co-factor LEDGF/p75 and lower levels of the restriction factors APOBEC3G, TRIM5α, and total tetherin, as compared to CD16- monocytes, which may support the greater HIV-1 susceptibility of **CD16+ monocytes relative to CD16- monocytes** [377,743,744]. However and in contrast with this observation, we found membrane tetherin protein expression to be enhanced in the CD16+ monocyte subset. It may be of interest to investigate whether membrane tetherin may act as an HIV-receptor, as was recently suggested by Kuhl et al [562]. Of note, in the Belgian study population, APOBEC3G and TRIM5α levels did not differ between CD16- and CD16+ monocytes suggesting that the contribution of host restriction factor levels to the greater HIV-1 susceptibility of CD16+ monocytes may be overvalued based on the Senegalese study population results or, that differences in ethnicity or demography may be involved. Ethnicity may influence HIV-1 transmission and/or replication through genetic diversity and varying socio-behavioral and/or environmental factors, whereas demography may be involved e.g. in access to appropriate health care and degree of exposure to more virulent HIV strains.

B) Expression analysis between different study populations

- HESN versus HC subjects within the Senegalese study population

We observed significantly reduced levels of LEDGF/p75 in the CD4+ lymphocytes of the HESN subjects, suggesting that LEDGF/p75 may play a role in resistance to HIV-1 infection. This observation is supported by the correlation that was found between reduced LEDGF/p75 levels and decreased efficiency in HIV-1 integration [299]. Conversely, several *in vitro* studies reported that efficient knockdown or knock-out of LEDGF/p75 is not sufficient to completely

abolish HIV-1 replication [240,332,745]. Consequently, we hypothesize that other factors may be needed in concert with LEDGF/p75 to limit HIV-1 integration in HESN subjects. Moreover, we observed that reduced LEDGF/p75 levels occurred mainly in those HESN subjects who showed a higher frequency of sexual exposure defined by the average number of sexual contacts per month. HIV-1 risk/exposure information was collected during a standard interview by a trained social assistant. However, none of the other parameters studied (Chapter 6 part I, Table 2) seemed to correlate with the reduced LEDGF/p75 levels in the HESN study population. Nevertheless, we carefully hypothesize that frequent sexual exposure to HIV-1 might trigger HESN subjects more to adapt their immune system to resist HIV-1 infection. This hypothesis is supported by the observation that the frequency of sexual exposure did not influence LEDGF/p75 levels in study populations other than the HESN study group. Of note, since no appropriate antibody specific for the HIV-1-related co-factor LEDGF/p75 was available when we initiated optimization of the ICS method, we used an anti-LEDGF antibody that recognizes both splice variants p75 and p52. In 1998, Ge et al [220] described that p75 is the more abundant splice variant, implying that the observed reduction in LEDGF most likely concerns LEDGF/p75. LEDGF/p75 expression can be induced by stress-related conditions like oxidative stress, tumour necrosis factor alpha (TNF-α), heat shock, and UV irradiation [326,746], whereas the opposite has been observed upon stimulation with transforming growth factor beta (TGF-β) [324]. In 2010, the post-translational modification sumoylation has been described to regulate the half-life of LEDGF/p75 [328]. In 2011, Desfarges et al [327] identified specific regulatory elements that control the expression of one or both of the splice variants p75 and p52. In conclusion, a lot of work remains to be done to elucidate the contribution of LEDGF/p75 expression patterns in HIV-1 infection.

- HIV-1 transmitters versus non-transmitters in the Senegalese study population

The phenomenon of HIV-1 restriction is mostly studied in HIV-1-exposed seronegative subjects. However, it may be interesting to study HIV-1 restriction from another perspective. In discordant couples, one could also investigate the HIV-1-infected partner for virus transmitting capacity and/or virulence of the virus. Whereas the efficiency of sexual HIV-1 transmission is generally low [79,719], still 75 to 85% of the HIV-1 infections reported

worldwide are caused by unprotected sexual intercourse [79]. Here, HIV-1 transmission is influenced by many determinants amongst which the HIV-1 infection phase, the HIV-1 strain, viral load, antiviral treatment, and the presence of other sexually transmitted diseases or genital ulcerations [75,720–723]. Therefore, if available, HIV-1 transmissibility parameters should be taken into account when analyzing possible correlates of protection in HIV-1 infection. In the Senegalese study population, all couples were screened to divide HIV-1-infected subjects in **HIV-1 transmitters (T) and non-transmitters (NT)** based on phylogenetic analysis of viral gp41 and questionnaire data [724]. We observed no significant differences in LEDGF/p75, APOBEC3G, TRIM5α, or tetherin expression between HIV-1 transmitters and HIV-1 non-transmitters (see Chapter 6 part II). These results suggest that the four HIV-1-related host factors are not involved in HIV-1 transmission. Of note, additional statistical analyses showed that the therapy status of the HIV-1-infected subject seemed to influence host factor expression profiles. Therefore, it would be of interest to repeat this experiment on a much larger study population thereby taking into account transmissibility parameters like the therapy status of the HIV-1-infected subjects.

- HIV-UT versus HC or HIV-ART subjects in the Belgian and Senegalese study populations

In the Belgian study population, no significant differences in LEDGF/p75, APOBEC3G and TRIM5α expression were observed between HIV-UT and HC or HIV-ART subjects. However, in the Senegalese study population, tetherin was significantly enhanced in CD4+ lymphocytes of the HIV-UT subjects, and all four HIV-1-related host factors were enhanced in the CD14+ monocytes of the HIV-UT subjects. A possible explanation for the higher LEDGF/p75, APOBEC3G and TRIM5α levels observed in monocytes of African versus Belgian HIV-1-infected patients may be that monocytes of the African study population show a higher activation state induced by factors like more advanced disease progression, co-infections, and environmental induced immune stimulation. The enhanced tetherin levels in the Senegalese HIV-UT subjects could suggest that, after cellular infection, tetherin may be induced to tether virus particles in order to control viral replication, or that tetherin may become induced during HIV disease progression. Additional experiments are required to further elucidate the role of tetherin in HIV-1 replication. As for the higher expression levels seen in monocytes

relative to lymphocytes, this observation can be explained by the fact that a) monocytes express high levels of interferon receptor [73], b) HIV-1 infection induces type I interferon expression, and c) APOBEC3G, TRIM5α, and tetherin are IFN-inducible [97,98,101,210,214,362,412,540]. Still, due to the limited number of HIV-UT subjects in the Belgian as well as the Senegalese study population (respectively 10 and 9 subjects) we should be careful not to over-interpret these results.

C) Correlation statistics in HIV-UT between host factor expression and parameters of HIV disease status or T cell activation levels

Correlation statistics were performed to verify whether HIV-1-related host factor levels were associated with the HIV-1 disease status (CD4 count and viral load) and/or T cell activation. Whereas statistical correlations were obtained between levels of APOBEC3G/TRIM5α and HIV-1 disease parameters in HIV-UT subjects of the Belgian study population, we could not reproduce this result in the Senegalese study population. Since the statistical correlations found in the Belgian study population were rather weak, we conclude that the disease status is most likely independent of the expression pattern of APOBEC3G and TRIM5α. As for tetherin, we observed that protein levels were highly correlated with viral load (positively) and CD4 count (negatively). Although tetherin is described to control viral replication by preventing the release of newly formed viral particles [520] or by impairing cell-to-cell transmission of functionally active viral particles [561], our data suggest that tetherin may be a marker of advanced HIV disease instead. The enhanced tetherin levels may be the result of induced type I interferon expression following HIV-1 infection [92,93,553] which is also supported by the positive correlation that we observed between T-cell activation and tetherin levels. The incapability of tetherin to restrict cell-to-cell spread of the virus after induction by HIV-1 infection is supported by at least three studies. In 2000, Gummuluru et al [563] observed that even though ΔVpu mutant virus particles remained more easily attached to the plasma membrane than wild-type virus, the presence of these mature virus particles led to enhanced cell-to-cell transfer of the virus. In 2010, Jolly et al [564] reported that tetherin may promote cell-to-cell transfer by virion accumulation onto the cell membrane, or by regulation of viral synapse integrity. In 2011, Coleman et al [553] added that high levels of tetherin do

not significantly restrict dendritic cell-mediated HIV-1 transmission. In turn, tetherin may trigger further increases in immune activation as it causes accumulation of virus particles at the cell membrane [344,540]. Consequently, we hypothesize that tetherin may have two functionalities during HIV-1 infection and disease. As an innate host restriction factor, tetherin may be involved in the prevention of HIV-1 acquisition; but once a threshold (e.g. by systemic infection) is established it could become ineffective or even promote viral replication. Recently, Erikson et al [747] added that the widespread expression profile of tetherin renders this host factor potentially less suitable as target for immunotherapy.

D) Correlation statistics between mRNA and protein expression in the Senegalese study population

Even though mRNA is translated into proteins, expression levels do not necessarily coincide (see also Chapter 6 and [739]). Indeed, we observed no correlations between mRNA and protein levels of APOBEC3G, TRIM5α or LEDGF/p75 in CD4+ T cells of 10 Senegalese healthy control subjects [456]. The discordance between mRNA and protein levels may be explained e.g. by external factors. For example, in HIV-1-infected subjects, the external factor Vif has been described to act differently upon protein and mRNA levels of APOBEC3G [362,718]. The small sample size may also have contributed to the discrepancy since positive correlations were detected for the three host restriction factors when studying the entire Senegalese study population (Figure 1 in Chapter 6 and [739]). APOBEC3G and TRIM5α mRNA versus protein levels were shown to correlate weakly, whereas strong correlations were observed for tetherin. These correlations are confirmed by the effect of type I interferons whereby both induction of mRNA and protein levels of APOBEC3G, TRIM5α, and tetherin have been observed [97,98,101,210,214,362,412,540]. We observed that stimulation with IFNα could clearly induce tetherin levels (Chapter 4), whereas its effects on APOBEC3G and TRIM5α levels are rather limited (Chapter 3 and [456]). These latter findings support the weak correlations that we observed for APOBEC3G and TRIM5α mRNA versus protein levels, and the strong correlation for tetherin. On the other hand, we detected no correlations between LEDGF/p75 mRNA and protein levels in the Senegalese study population. This observation may come

forth from the observation that factors like cytokine TNFα can either induce or decrease LEDGF/p75 levels depending on the time or level of exposure [326].

In **conclusion**, we found that expression patterns of APOBEC3G and TRIM5α did not play a role in HIV-1 replication and/or resistance to HIV-1 infection in our Belgian and/or Senegalese study populations. For LEDGF/p75, reduced protein levels were observed in HESN subjects. Although no hard evidence could be provided on whether LEDGF/p75 actually plays a role in *in vivo* resistance to HIV-1 infection, we do believe that further research on LEDGF/p75 expression in larger and/or other populations of HESN subjects may be of interest to further investigate the potential contribution of LEDGF/p75 to HIV-1 resistance upon sexual and/or non-sexual exposure to HIV-1. Additionally, we included the recently described HIV-1-related host factor tetherin in the expression analyses of the Senegalese study population. Similar to our observations for APOBEC3G and TRIM5α in the HESN population, we found no evidence that tetherin would be involved in HIV-1 resistance. However, considering the capability of tetherin to tether newly formed viral particles to the cell membrane [214,538,542,550] and to internalize virions [538,542,551,552], we hypothesized that both membrane and total tetherin levels would be induced in HIV-1-infected subjects. This assumption was in correspondence with the data we obtained. However, further investigation showed that enhanced levels of tetherin correlated directly with T cell activation and viral load, and indirectly with CD4 count. Based on these findings, tetherin does not seem to be capable of controlling viral replication; instead, it appears to be a marker of advanced HIV disease in therapy-naïve HIV-1-infected patients. Therefore, we now conclude that tetherin may be involved in early or acute virus replication to control local infection, whereas it becomes ineffective when the virus bursts and the disease progresses.

2. Future Perspectives

Since the beginning of the epidemic, HIV has infected almost 60 million people worldwide of whom about 25 million people have died of HIV-related causes. Many research projects are ongoing to find an effective therapy with the ultimate goal to exterminate the HIV virus. Today, HIV-1 replication can be suppressed for an indefinite period of time with highly active anti-retroviral (HAART) therapy. The latter treatment applies a cocktail of three or four antiretroviral drugs [748–752] from different classes to prevent multi-drug resistance. The drugs can be entry inhibitors, nucleoside and nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, integrase inhibitors, or maturation inhibitors. Apart from the entry inhibitor Maraviroc which targets the cellular co-factor CCR5 [753], currently approved anti-retroviral drugs mostly target viral proteins. However, to prevent that the rapidly mutating virus escapes from therapy treatment, one should not focus solely on viral proteins [319]. Instead, new approaches should be explored. As the virus needs the host to replicate, cellular host proteins and/or virus-host interactions are of particular interest in the development of new anti-HIV therapeutics. As a result, research is needed to elucidate which cellular pathways or host factors are mandatory for the virus to survive, yet are redundant for host survival.

In the introduction of this PhD-thesis, a summary can be found on the potential therapeutic importance of the HIV-1-related host factors LEDGF/p75, APOBEC3G, TRIM5α, and tetherin and corresponding virus-host interactions being integrase-LEDGF/p75, Vif-APOBEC3G, capsid-TRIM5α, and Vpu-tetherin. Recently, the integrase-LEDGF/p75 interaction was demonstrated of particular interest for the development of viral integrase inhibitors [313,319,754,755]. Successful data have been obtained for the integrase inhibitor raltegravir [309,756,757]; and elvitegravir is under further investigation [758,759]. In the meanwhile, Christ et al [320] reported on LEDGINs, a new class of anti-HIV agents. LEDGINs are allosteric inhibitors that interfere with the integrase binding domain (IBD) of the cellular host factors LEDGF/p75 and HRP-2 thereby preventing HIV-1 integration [306]. The latter findings support the interest to

study specific HIV-1-related host factors and virus-host interactions for the development of new therapeutic agents.

Apart from the four HIV-1-related host factors studied in this thesis, other potentially interesting HIV-1-related host factors later attracted attention. KAP1 and p21 have been demonstrated to interact with the HIV-1 integrase protein similar to LEDGF/p75. KAP1 (KRAB-associated protein-1) is suggested to restrict HIV-1 infection at the HIV-1 integration step by inducing integrase deacetylation [760], whereas the cyclin-dependent kinase inhibitor p21 (Waf1/Cip1/Sdi1) was shown to block viral infection in hematopoietic stem cells through complex-formation with HIV-1 integrase thereby preventing chromosomal integration [761]. Bergamaschi et al [762] added that induced p21 levels upon FcγR activation (i.e. aggregation of activating IgG Fc-receptors) restrict HIV-1 replication in macrophages. Moreover, enhanced p21 levels appear involved in the mechanism via which elite controllers suppress the HIV-1 replication cycle [763,764]. Early HIV-1, HIV-2, and SIV replication events (from reverse transcription to integration) are inhibited by the PAF1 (peroxisome assembly factor-1) complex [765,766]. SAMHD1 (sterile alpha motif- and metal-dependent phosphohydrolase domain and HD domain-containing protein-1) prevents efficient viral cDNA synthesis by depleting the intracellular pool of deoxynucleoside triphosphates and is counteracted by the Vpx accessory protein expressed in HIV-2 [767–771]; and HERC5 (HECT domain and RCC1-like domain-containing protein-5) blocks HIV-1 Gag particle production thereby acting at the later stage of virion assembly [766,772].

Whereas specific HIV-1-related host factors and/or virus-host interactions may be of potential interest for vaccine development, it has been demonstrated that the virus exploits at least 250 host-derived HIV dependency factors [138–140]. Therefore, it is unlikely that one key-host factor will explain the phenomenon of HIV-1 restriction as observed in frequently HIV-exposed but seronegative subjects (HESN). With the development of comprehensive virus-host protein interaction networks, it becomes more evident that viral proteomes target a wide functional range of proteins as well as highly interconnected (hubs) and bridging proteins within the human interactome [140,773–776]. Within the HIV proteome, some viral

proteins have been demonstrated to mimic motifs of cellular proteins that interact with host hub proteins like kinases and transcription factors (i.e. proteins involved in many protein-protein interactions) [777]. Consequently, the host requires multiple defence mechanisms to suppress viral replication efficiently. Thus far, several host factors like APOBEC3G, tetherin, SAMHD1, and p21 have been described to target the viral replication cycle. Each of these host factors has been demonstrated to restrict HIV-1 replication in their proper way. Still, many other host factors and related restriction mechanisms remain to be discovered. Recently, Jäger et al [775] determined 497 HIV-host interactions in two different human cell lines. Upon further investigation, they discovered eleven cellular host factors which resulted in increased HIV replication when knocked-down through siRNA technology, indicating their protective role against the virus. Unexpectedly, the host restriction factors APOBEC3G, TRIM5α, and tetherin did not come up in this list, whereas the co-factor LEDGF/p75 was mentioned to interact with HIV-1 integrase [775]. Even though co-factor LEDGF/p75 interacts with HIV-1 integrase, five large-scale siRNA studies on HIV-1-infected cells showed that LEDGF/p75 was not essential for HIV-1 replication [138,778–781]. These data are supported by previous publications on LEDGF/p75 knock-down reporting continued HIV-1 integration [240,306]. It was demonstrated that other host factors like HRP-2 also assist in HIV-1 integration [306]. The latter observation evidences that a combination of cellular host factors ("sub-network") instead of one single host factor is used by the virus to assure each step of the viral replication cycle. Consequently, further research is warranted to elucidate the entire HIV-host interaction network involved in HIV-1 infection. A novel strategy to study virus-host networks may be to identify neighbourhood networks [782]. These networks contain all host interaction-partners of known HIV-1-related host factors which are then further investigated for interaction with HIV-1 viral proteins. Programs like Cytoscape can be used to identify cellular interaction partners within existing human protein-protein interaction networks captured in databases like DIPS, MINT, and IntAct. This *in silico* procedure allows for the study of "sub-networks" within which often more protein-protein contacts and interactions in closer proximity to each other are observed as compared to random networks of the same topology. Application of the latter strategy to study HIV-host interactions is expected to also result in many proteins involved in IFN production and activity [140]. Of note, HIV-1-related host factors will not

necessarily interact with viral proteins to influence the HIV-1 replication cycle. This is exemplified e.g. by SAMHD1 which interferes with the HIV-1 viral cDNA synthesis step indirectly by depleting the intracellular pool of deoxynucleoside triphosphates.

As a complex virus-host interaction network exists within HIV-1-infected subjects to assure efficient HIV-1-replication, it is expected that a complex protein-protein interaction network will be present in HESN that allows for HIV-1 restriction. Whereas in HIV-1-infected subjects, protein interactions can be studies versus viral proteins, another strategy will need to be in place to study the phenomenon of intrinsic cell HIV-1 restriction. One possible approach may be to compare host factor expression profiles of HESN subjects versus healthy controls. Hence, expression studies with microarray analysis [783–790] and quantitative proteomic analysis thereby using e.g. SILAC, iTRAQ, or label-free quantification methodology [791,792] can be set up. Both microarray and quantitative proteomics are technologies that allow for the identification of a large array of potentially involved HIV-1 restriction-related host factors. For example, microarray analysis was used to study HIV-1 resistance in female HESN sex workers from Nairobi [173,783]. These researchers observed that down-regulation of genes in key signalling pathways on which HIV-1 viruses rely may contribute to HIV-1 resistance. These data support the reduced LEDGF/p75 levels that we observed in the Senegalese HESN study population [415]. Of note, similar studies can be designed to study mechanisms like HIV-1 latency, long-term non-progression, and HIV-1 replication control in elite controllers. Once specific proteins of interest are selected for further analysis, the flow cytometry-based intracellular staining (ICS) method could be considered as an interesting tool to study specific protein expression levels in particular cell-types like immune cells.

In addition to studying host factor networks and/or expression profiles, other mechanisms like immunity and genetics may (also) be involved in processes like HIV-1 restriction. Thus, further research is warranted thereby taking into account that distinct HESN cohorts (e.g. female sex workers, intravenous drug users) may significantly differ in the way HIV-1 infection is restricted. Moreover, HIV-1 infection risk factors like therapy treatment, viral load, condom use in case of sexual exposure, etc. should be well considered when interpreting results on

possible correlates of protection. One major constraint in studying HIV-1 restriction is that HESN subjects are only defined as HIV-1-resistant after being seronegative for several years. Therefore, early immunological and virological changes that may be critical for the HIV-1 restriction phenomenon cannot be investigated. On the other hand, as HESN subjects remain HIV-1 seronegative for several years (in the Senegalese study populations here described for more than 7 years already), it may well be that no trigger has taken place that rendered the host HIV-1 resistant. Instead – as also observed for the 32 base pair deletion in the *CCR5* gene – the host may have been inherently programmed to prevent HIV-1 infection. Therefore, we consider it well worth studying host factor-specific working mechanisms, expression profiles, and/or complex protein-protein interactions in an attempt to explain the phenomenon of HIV-1 restriction through intrinsic cell-restriction.

In conclusion, although inhibition of viral and/or host factors as currently applied in HIV-therapy is a valuable approach to suppress HIV infection, additional efforts are required not only to suppress but also to prevent and/or eradicate HIV infection. To study HIV restriction mechanisms that prevent HIV infection, study populations including frequently HIV-1-exposed seronegative subjects (HESN) are of particular interest. Revealing the mechanisms of HIV-1 transmission, replication, latency, and eventual progression towards AIDS as well as of non-progression and restriction will greatly contribute to the development of anti-HIV therapies with as **ultimate goal** to develop an effective therapy that can prevent and/or eradicate HIV-1 infection.

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Scientific Awards

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Peer reviewed papers

Mous K, Jennes W, Kestens L, Van Ostade X. Working mechanisms and therapeutic relevance of the HIV-related host factors LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin. ***Submitted for publication in "Current HIV research"***

Mous K, Jennes W, Camara M, Seydi M, Daneau G, Mboup S, Kestens L, Van Ostade X. Expression Analysis of LEDGF/p75, APOBEC3G, TRIM5alpha, and Tetherin in a Senegalese Cohort of HIV-1-Exposed Seronegative Individuals. PLoS One. 2012;7(3):e33934. Epub 2012 Mar 27.

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Meeting abstracts

Mous K, Jennes W, Camara M, Seydi M, Daneau G, Mboup S, Kestens L, Van Ostade X. "Reduced expression of LEDGF/p75, an essential host factor for HIV integration, in HIV exposed but seronegative individuals" for poster presentation at AIDS Vaccine 2011 in Bangkok, Thailand (2011). I obtained a full scholarship to attend this conference.

Mous K, Jennes W, Kestens L, Van Ostade X. "Intracellular detection of differential APOBEC3G, TRIM5alpha and LEDGF/p75 expression in peripheral blood from HIV-1 infected subjects by flow cytometry" for poster presentation at AIDS Vaccine 2009 in Paris, France (2009). I obtained a full scholarship to attend this conference.

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Educational activities

Scientist@work event (2008): Blood as an indefinite source of information

Scientific event for kids (2008): DNA extraction from a kiwi-fruit

Assistance Bachelor Thesis Biomedical Sciences (2007) of Lien Buelens

Assistance Bachelor Thesis Biomedical Sciences (2007) of Dorien Proost

Scientific event for kids (2006): DNA extraction from a kiwi-fruit

Dankwoord

Na het succesvol afronden van de opleiding Biomedische Wetenschappen aan de Universiteit Antwerpen (UA) kon ik niet wachten om mijn onderzoek op HIV resistentie aan te vatten. Nu, vele jaren later, heb ik een leerrijke weg zowel op wetenschappelijk als persoonlijk vlak afgelegd. Deze weg bewandelde ik echter niet alleen...

Allereerst wil ik mijn promotoren Prof. Dr. Xaveer Van Ostade en Prof. Dr. Luc Kestens en co-promotor Dr. Ir. Wim Jennes bedanken. Dankzij jullie kristische bedenkingen, ontelbare verbeterrondes, wetenschappelijk enthousiasme, aanhoudende geloof in het onderzoek, en meerdere motiveringsmomenten zijn we samen geraakt waar we vandaag staan. Xaveer en Luc, dankjewel voor de kansen die ik kreeg om dit onderzoek ondanks de tegenslagen tot een goed einde te brengen. Wim, naast de wetenschappelijke kennis die je met me deelde, leerde jij me tevens dat mijn onzekerheden niet altijd op zijn plaats zijn en dat de lat soms hoger gelegd mocht worden om het uitgevoerde onderzoek voldoende naar waarde te laten gelden zoals bv. bleek met de publicatie in PloS One. Verder appliceerde ik op jouw aanraden twee keer voor een beurs voor het AIDS Vaccine congres. Beide keren (eerst te Parijs en vervolgens te Bangkok) werd ik uitgenodigd voor een posterpresentatie. Bedankt voor je aanhoudende aanmoedigingen! Ik wens je nog veel succes met je verdere carrière.

Graag bedank ik ook Prof. Dr. Jurgen Del-Favero, Em. Prof. Dr. Luc Moens, Prof. Dr. Zeger Debyser, Prof. Dr. Gianfranco Pancino, en Prof. Dr. Viggo Van Tendeloo voor hun feedback ter verbetering van deze thesis en voor hun bereidheid om te zetelen in de doctoraatsjury.

In de loop van de jaren heb ik het geluk gehad met vele collega's de werkvloer te mogen delen. Allereerst waren daar de collega's van de Universiteit Antwerpen. Specifiek in "ons" labo een woordje van dank voor Ellen, Mops, Elke en Geert VR. Samen met jullie beleefde ik meerdere mooie momenten op alsook buiten het labo; en sportieve grenzen werden verlegd. Ellen, na 4 jaar BMW, leerden we elkaar maar echt kennen in ons buro'tje. Dankjewel voor je steun en voor het uitwisselen van ervaringen. Je bent een voorbeeld voor me op vele vlakken.

Ook verdienen alle collega's van T1 een woordje van dank voor de ongedwongen babbeltjes, het delen van kennis/producten/toestellen, de administratieve hulp, de momenten van stoom afblazen, etc. Elke, Liesbet, Roos en Indra, ik zag jullie één voor één jullie PhD-thesis succesvol afronden en beschouw jullie als sterke vrouwen die het leven in eigen heft nemen. Samen met Ellen, Kirsten en An spreken we van tijd tot tijd af om ervaringen te wisselen en elkaars kindjes te bewonderen. Stiekem hoop ik dat deze traditie verdergezet zal worden. Kambiz, although I did not obtain a Nobel Prize, I'm happy to announce that my time as a PhD-student has also come to an end. Thank you for allowing me to contribute to your PhD-work. I hope you are doing well. In de lijn van Kambiz zijn onderzoek wil ik ook het CEPROMA bedanken voor de tijd die ik bij hen mocht spenderen. Stef, merci voor je hulp bij de massaspectrometrie-experimenten en voor je steeds bemoedigende woorden. In een later stadium kwam ik ook terecht bij het labo Cel en Weefselleer waar ik enorm geholpen werd door Isabelle en Inge bij het maken van cytospins, het (nucleair) kleuren van de coupes en het maken van de confocale beelden. Dankjewel voor jullie hulp en de mooie beelden! Verder werd ik administratief bijgestaan door de mensen van het secretariaat BMW (in het bijzonder Evy Pluym), alsook door Lucky en Natacha van de mediadienst waarvoor dank.

Naast de UA werd het ITG al snel mijn "tweede" en naar het einde toe zelfs eerder mijn "eerste" thuis. Graag wil ik eenieder van jullie bedanken voor de hulp bij het hanteren van de flow cytometer, het uitdokteren van de intra/extracellulaire kleuringen, het opzetten van de cellijn/stimulatie-experimenten, het delen van producten zoals celcultuurmedia, het afstaan van bloedstalen, het ontelbare keren bloedprikken, het op punt stellen van de real-time qPCR experimenten, en het ter beschikking stellen van toestellen zoals flow cytometers, het real-time qPCR toestel en trekkasten voor celkweek en TRIZOL extracties. Naast de vele collega's die ik in het labo Virologie, in het cryo-lokaal en in de wandelgangen leerde kennen, bedank ik graag in het bijzonder alle collega's van het labo Immunologie. Ik heb steeds graag vertoefd in jullie aanwezigheid en kijk met een warm gevoel terug op de vele mooie momenten die we deelden in het labo, in de tuin van het ITG, aan de koffiekoek-tafel op vrijdagmorgen, etc. Dankjewel Wim, Ann, Chris, Luc B, Géraldine, Justine, Jordan, Evelyn, Julie, Annette, Dominique, Odin, Anali, Pascale, Véronique, Teresia en Joran.

Ondertussen leerde ik nieuwe fijne collega's kennen eerst bij Novartis en nu bij HistoGeneX. Dankjewel voor de kansen die ik krijg om mijn wetenschappelijk onderzoek af te ronden en me een weg te vinden in de wereld van het klinisch onderzoek.

Tijdens mijn studententijd kwam ik in contact met vele gedreven personen dankzij wie ik me gesterkt voelde deze doctoraatsthesis aan te vangen. Prof. Dr. Pierre Van Damme, dankzij uw hulp kreeg ik de kans mijn masterthesis te Genève te maken. Hoewel u me niet persoonlijk kende, bracht u me in contact met Prof. Dr. Claire-Anne Siegrist en haar team waar ik een ongelooflijke tijd beleefde. Dankuwel om deze unieke ervaring mee mogelijk te maken. Au labo de Prof. Dr. Claire-Anne Siegrist j'aimerais dire: Même que je vous ai déjà remercié pendant ma thèse de master, j'aimerais vous remercier encore une fois pour votre croissance dans ma capacité scientifique. Je porte profondément dans mon cœur tous les moments au labo avec mes chers collègues multiculturels. Et grâce aux bonnes mémoires, j'ai pu continuer la recherche scientifique aux moments plus durs. Je vous souhaite tous le meilleur! Ook aan alle kotgenoten die ik onderweg tegenkwam zeg ik graag dankjewel. Elk kot had zijn bijzonderheden en overal woonde ik even graag. Jullie weten wie jullie zijn en ik hoop op de één of andere manier van dichtbij of verder weg met éénieder van jullie in contact te kunnen blijven. Aussi merci aux co-habitants de la résidence Hugo-de-Senger! Je ne vous oublierai jamais! Et Rea, à part de mon aventure à Genève, j'ai bien savouré nos voyages en Suisse, ton séjour à Bruxelles et n'oubliez pas l'Australie. Je te dis volontairement "à bientôt"! Ook niet te vergeten zijn natuurlijk mijn mede-afstudeervriendinnetjes van de opleiding BMW. In het bijzonder een welgemeende merci aan Evi, Elke, Ellen T, en Karen. Ik kijk steeds enorm uit naar de momenten waarop we ondanks onze drukke agenda's en de afstand toch tijd voor elkaar vrijmaken om bij te praten. Als het aan mij ligt, dan blijven we deze traditie nog lang verder zetten.

Naast mijn leven aan de unief te Wilrijk, kon ik ook "thuis" te Kalmthout/Essen rekenen op een grote achterban van familie en vrienden op wie ik steeds (ten gepaste en ongepaste tijden) kan terugvallen.

Allereerst, een welgemeende merci aan mijn ouders en zus. Ma, pa, jullie zorgden ervoor dat ik alle kansen kreeg om verder te studeren en op mijn eigen benen te staan! Me loslaten om op kot te gaan was een grote stap en toen ik meldde dat ik op Erasmus wilde, bleven jullie me steunen zodat ik mijn dromen waar kon maken. Ik weet dat het niet altijd gemakkelijk geweest is, en apprecieer het enorm dat ik steeds op jullie steun kan rekenen. Zussie, al zijn onze levens nog zo verschillend, ik zou me geen betere zus kunnen wensen dan jou. Ik hoop dat jij, Jakke en kleine Amber heel gelukkig mogen zijn en kijk al uit naar alle tijd die nu vrij gaat komen waarop we vaker bij elkaar de deur kunnen platlopen.

Ook een grote dankjewel aan mijn vriendinnen/vrienden die me ondanks de moeilijke momenten zijn blijven steunen.

Joke, wat ben ik blij dat we elkaar tijdens het schooltoneel leerden kennen! Samen maakten we onvergetelijke reizen, jij ging me voor naar de unief en toonde het studentenleven ook te Leuven, en jij bent er nog steeds om vreugde, verdriet, frustaties en ga zo maar door te delen. Ondertussen ben je mama van een prachtdochter en hoop ik dat we nog vele mooie momenten samen gaan delen!

Lucy, per toeval ontdekten we dat we buurmeisjes waren en zo kwam het dat we samen onze eerste stappen zetten in het uitgaansleven. Gaandeweg ontdekten we dat we beiden graag bezig zijn met muziek en kunnen genieten van wandelen in de Heide. Ondertussen vele jaren later slagen we er nog steeds in om ondanks de grote afstand regelmatig een ontspannen dag samen door te brengen. Dankjewel hiervoor! Ik wens jou en Stéphane veel succes met jullie lopende projecten. Weet dat ik er ook voor jullie ben!

Sofie, het pad dat wij bewandelden is niet altijd een effen pad geweest. Samen deelden we al veel mooie, maar ook evenveel moeilijke momenten. Het leven is niet altijd eerlijk en hoewel we dit misschien als een verrijking dienen te zien, hoop ik toch dat er stilaan een minder gecompliceerde toekomst ook voor jou weggelegd is. Ik bewonder je doorzettingsvermogen en wens je alvast nog veel succes op alle vlakken inclusief je studies natuurlijk.

Veerle VO, de afgelopen jaren leerden we elkaar steeds beter kennen. Vele bewogen periodes volgden elkaar op. Ik zal nooit de appelmomenten te UA vergeten waarop we beiden onze weg voorwaarts zochten. Ik ben er zeker van dat de ontstane vriendschap niet meer stuk kan.

Caro, onze gezamelijke wetenschappelijke achtergrond bracht ons dichter bij elkaar. Ondertussen zitten we beiden in het klinisch onderzoek en “treinen” we dagelijks naar de fashion-stad Antwerpen. Dankjewel voor je vele lieve sms’jes en praatjes!

Joke, Guy, Veerle VO, Caro, Diaz, Senne, Geert, Annelies, Ine, Joris, Veerle S en Michael, dankjewel voor de vele mooie momenten die we deelden/delen in de Withoef, op de thuis/kotfeestjes, weekendjes weg, vrijgezellendagen, trouwfeesten, geboortes, etc. Jullie waren er steeds als ik wilde uitrazen en zorgden er mee voor dat ik ten gepaste tijden mijn gedachten eens goed kon verzetten. Zonder jullie zou er teveel doctoraat en te weinig ontspanning geweest zijn, dus dankjewel!

Karo, Kristof VC, Kelly, Kristof M, Ellen W en Paul, dankjewel om mij – die biomedische-vriendin van Joke – in jullie rechten-vriendengroepje op te nemen. Van galabals tot badmintonmaatje tot collega-doctoraatsstudent ben ik mede dankzij jullie geraakt waar ik nu sta. Merci!

Mario, An, Gwen, Bob, Valerie, Ellen M, Lander, Caroline, Peter, Siets, Bart, Dominique, Marko en Jorien, hoewel ons sociaal leven soms op “on hold” staat, is het steeds een plezier om samen met jullie tijd te spenderen en ons eens helemaal te laten gaan.

Aan de vrienden van de fotografie, Griet, Luc, Nathalie en Sikkie, zeg ik ook graag dankjewel voor de gezellige avonden die we van tijd tot tijd samen spenderen!

Al bleef er weinig tijd over voor hobby’s, mijn muzikaal ei kon ik steeds op de fanfare kwijt. Eens goed doorblazen op de bugel en nu de trompet lucht steeds op. Ook de fijne babbels tijdens de pauzes maken dat ik (bijna) steeds graag op zondagmorgen mijn bed uitkom.

Ad, Vera, Stefan, Stephanie, Evelien, Brian, Jodie en Jahson, bij jullie vond ik een tweede thuis waar ik reeds gezellige schoonfamilie-aangelegenheden beleefde op Kerstdag, verjaardagen, communies, etc. Bedankt dat ik van jullie grote gezin deel mag uitmaken!

Ook aan de rest van de (schoon)familie (grootouders, tantes/nonkels, neven/nichten) zeg ik dankjewel voor de regelmatig getoonde interesse in mijn onderzoeksperikelen.

En zo kom ik stilaan aan de laatste maar daarom niet minst belangrijke paragraaf van deze thesis... John, lieveling, ik prijs me nog elke dag gelukkig dat wij elkaar zijn tegengekomen. Samen met jou deelde ik de afgelopen jaren de ups en vele downs die gepaard gingen met het werken aan deze thesis. Ik prijs me gelukkig voor alle onvergetelijke momenten die we naast mijn PhD-onderzoek de voorbije jaren deelden. Nooit vergeet ik onze eerste reis naar Schotland en onze “kot”momenten. Mijn “Antwerps” avontuur blijft onbegrijpelijk, en ja, samenwonen in jouw appartement bleek een beter plan. Ondertussen kochten we ons eigen huis en startten we grote verbouwingen waardoor we zelfs enkele maanden bij mijn ouders moesten gaan wonen. Terwijl ik veel tijd aan de PC doorbracht, bouw(de) jij aan onze toekomst en hielp je me zelfs tussendoor met het verbeteren van figuren die van slechte kwaliteit bleken. Dankzij jouw oneindig begrip en aanhoudende steun is het moment eindelijk daar! Laten we dit hoofdstuk afsluiten en nieuwe gezamelijke dromen waarmaken. Ik hou van je schat, en kijk al uit naar alle mooie momenten die ons nog te wachten staan!

Dankjewel allemaal !!!

Kim Mous

Juni 2012