Evaluation of type I interferon responses to HIV infection in CD4+ T cells

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Thesis submitted in fulfillment of the requirements for the degree of Doctor in Medical Sciences – Faculty of Medicine and Health Sciences, Ghent University

Promoter: Prof. Dr. Bruno Verhasselt - Department of Clinical Chemistry, Microbiology and Immunology - Ghent University

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List of abbreviations

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
AP 1	Activator protein 1
APC	Antigen-presenting cell
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3
ART	Antiretroviral therapy
AZT	Azidothymidine
BST2	Bone marrow stromal cell antigen 2
CA	Capsid
cART	Combination antiretroviral therapy
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GAMP synthase
CKI	Cyclin-dependent kinase inhibitors
CpG	Cytidine-phosphate-guanosine
CPSFE6	Cleavage and Polyadenylation Specificity Factor subunit 6
CRISPR	Clustered regularly interspaced short palindromic repeats
Cyp A	Cyclophilin A
DAI	DNA-dependent activator of IRFs
DCs	Dendritic cells
DCAF 1	DDB1-cullin4-associated factor 1
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DDX41	DEAD box polypeptide 41
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleoside-triphosphate
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
ER	Endoplasmatic reticulum
ERAD	Endoplasmatic reticulum associated degradation pathway
ESCRT	Endosomal sorting complexes required for transport
GALT	Gut-associated lymphoid tissues
GAS	Gamma activating sequence
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony stimulating factor
gp	Glycoprotein precursor
HSA	Heat-stable antigen
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
IFI16	IFN-Y-inducible protein 16
IFN	Interferon
IFN-I	Type I IFN
IFNAR	IFN-α/β receptor
IKKβ	IκB kinase β
IN	Integrase
IRF	IFN-regulatory factor
ISG	IFN-stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
JAK1	Janus kinase 1
LGP2	Laboratory of genetics and physiology 2
LTR	Long-terminal repeat

MA	Matrix
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation–associated gene 5
MDDCs	Monocyte-derived dendritic cells
miRNA	microRNA
MLV	Mouse leukemia virus
MOI	Multiplicity of infection
Mre11	Meiotic recombination 11 homolog A
Mx	Myxovirus resistance protein
MyD88	Myeloid differentiation primary response gene 88
NC	Nucleocapsid
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κ light-chain-enhancer-of activated B-cells
NK	Natural killer
OAS	Oligoadenylate synthetase
PAMP	Pathogen-associated-molecular pattern
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid DCs
PERT	Product-enhanced RT
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PKR	Protein kinase R
PR	Protease
PRR	Pattern-recognition receptor
P-TEFb	Positive transcription elongation factor
qPCR	Real-time quantitative PCR
RNAi	RNA interference
RIG-I	Retinoic acid inducible gene-I
RISC	RNA-induced silencing complex
RLR	RIG-I-like receptor
RT	Reverse transcriptase
RTC	Reverse transcription complex
SAMHD1	Sterile alpha motif and HD-domain-containing protein 1
SCF	SKP1-cullin1-F-box
shRNA	Short hairpin RNA
Siglec	Sialic acid-binding immunoglobulin-type lectin
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
ssDNA	Single-stranded DNA
SSE	Structure-specific endonuclease
STAT1	Signal transducer and activator of transcription 1
STING	Stimulator of IFN genes
TAR	Transactivation-responsive region
TBK1	(TANK)-binding kinase 1
TGN	Trans-Golgi network
TLR	Toll-like receptor
TREX1	Three prime repair exonuclease 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	Tyrosine kinase 2
UNG2	Uracil DNA glycosylase
VSV	Vesicular Stomatitis Virus
ZFN	Zinc-finger nucleases
β-TrCP	β-transducin repeat-containing protein

Summary

The human immunodeficiency virus type 1 (HIV-1) has infected over 70 million people (and killed 36 million) since the start of the epidemic. This makes it one of the most successful pathogens in human history. Characteristic for the HIV-1 pathogenesis is a long-lasting battle between the virus and the human immune system, which eventually leads to immune exhaustion and onset of AIDS. A considerable part of HIV-1 research has focused on both defining the immune responses against the virus and understanding the mechanisms that drive immune dysfunction, in the hope to artificially boost these responses or to prevent/repair their deterioration. Type 1 interferon (IFN-I) responses are an interesting target in this regard. They have extremely potent antiviral effects and are a key part of the immune response against multiple viral pathogens. During chronic viral infections, such as HIV-1, the role of IFN-I is however dual: while early responses initially limit viral spread, prolonged exposure to IFN-I is associated with hyper-immune activation and dysfunction, both hallmarks of pathogenic HIV-1 infection. Our primary goal was to investigate mechanisms that could potentially drive and regulate IFN-I production during HIV-1 pathogenesis.

This mechanistic evaluation required the use of a large set of HIV variants and lentiviral vectors to perform shRNA knock-down of host genes. This introduced early-on the need for a method to evaluate lentiviral productions, to normalize infection experiments and quantify HIV replication. We found that most standard methods for retroviral quantification were not compatible with such high-throughput production, as they are usually quite labor-intensive or expensive. This led to optimization of a real-time PCR based technique for measurement of retroviral reverse transcriptase (RT) activity. The method outperformed the current gold standard for *in vitro* quantification (p24 ELISA) by a lower inter-run variation, lower cost and higher linear range. Furthermore, RT activity correlated well with levels of transducing or infectious viral particles.

To subsequently pursue our main goal, we evaluated the ability of HIV to induce IFN-I responses in its main target cells, primary CD4+ T cells. By employing several assays, we show that infection of these cells leads to a bioactive IFN-I response, characterized by induction of IFN-I and several interferon stimulated genes (ISGs). This demonstrates that CD4+ T cells are able to sense HIV and indicates them as a potential source of elevated IFN-I levels in HIV-1 patients.

In a second part, we investigated host and viral mechanisms that regulate the IFN-I induction by HIV-1 in primary CD4+ T cells. This led to three important findings: 1) the cytosolic DNA receptor cGAS and its downstream signaling molecules are required for IFN-I induction; 2) IFN-I induction only occurs upon successful integration and protein expression by the virus; 3) the IFN-I response is regulated by two HIV-1 accessory proteins:

it is potentiated by Vpr, but counteracted by Vpu. Together, these results indicate a model in which HIV DNA is sensed by cGAS upon productive infection and this requires assistance of newly expressed Vpr and possibly other newly formed viral replication products. It also suggests that HIV-1 may have evolved partial counteraction of this process through Vpu.

In summary, this work has led to new insights into innate immunity against HIV-1. We identify HIV-infected CD4^{+} T cells as a possible new source of IFN-I and delineate virus and host components that mediate IFN-I responses in these cells. Given the extensive viral regulation of this process and the potential harmful role of IFN-I in HIV-1 pathogenesis, these findings might form the basis for novel therapeutic strategies.

Samenvatting

Het humaan immunodeficiëntie virus 1 (HIV-1) is één van de belangrijkste ziekteverwekkers in de geschiedenis van de mensheid. Sinds de start van de epidemie werden al meer dan 70 miljoen mensen besmet en zijn meer dan 36 miljoen mensen overleden aan de gevolgen van AIDS. Karakteristiek voor het HIV-1 ziekteproces is een langdurig gevecht tussen het virus en ons immuunsysteem. Dit gevecht leidt na vele jaren tot een volledige uitputting van het immuunsysteem en de ontwikkeling van AIDS. Een aanzienlijk deel van HIV-1 onderzoek richt zich daarom op het beter begrijpen van de immuunreacties die ontwikkeld worden tegen het virus en het begrijpen van de mechanismen die leiden tot immuun-uitputting. De hoop is om zo methodes te vinden om sterkere immuunreacties op te wekken en immuun-uitputting tegen te gaan. In dit kader vormen de type I interferon (IFN-I) responsen een interessant doelwit. Dit type van immuunrespons wekt van nature zeer sterke antivirale effecten op en speelt bijgevolg een belangrijke rol in de afweer van vele virale ziekteverwekkers. Tijdens chronische virale infecties zoals HIV-1 heeft IFN-I echter een dubbele rol: vroege responsen zijn belangrijk om de initiële virale spreiding tegen te gaan, maar langdurige blootstelling aan IFN-I kan leiden tot hyper-immuunactivatie en dysfunctie, beiden kenmerkend voor het HIV-1 ziekteproces. Het doel van deze thesis was om mogelijke processen te identificeren die IFN-I productie veroorzaken en reguleren tijdens de HIV-1 infectie.

Dergelijk mechanistisch onderzoek vergde het gebruik van vele HIV varianten alsook van lentivirale vectoren voor shRNA knockdown van cellulaire eiwitten. Bijgevolg was al snel een methode nodig om op grote schaal o.a. lentivirale producties te evalueren, infectie te normaliseren en HIV replicatie te kwantificeren. Standaardmethodes voor retrovirale kwantificatie bleken niet te voldoen aan deze eisen, aangezien ze vaak te duur of arbeidsintensief zijn. Dit leidde tot optimalisatie van een real-time PCR gebaseerde techniek om retrovirale reverse transcriptase (RT) activiteit te meten. Deze assay bleek de huidige standaardmethode (p24 ELISA) te overtreffen met een lagere inter-run variabiliteit, lagere kost en groter lineair bereik. RT activiteit toonde bovendien een goede correlatie met het aantal transducerende of infectieuze partikels.

In het kader van onze eigenlijke doelstelling, werd vervolgens de capaciteit van HIV geëvalueerd om IFN-I te induceren in zijn belangrijkste doelwitcellen, primaire CD4$^+$ T cellen. Door gebruik te maken van verschillende assays, demonstreren we dat infectie van deze cellen leidt tot ontwikkeling van een bioactieve IFN-I respons. Dit gaat gepaard met inductie van zowel IFN-I alsook verschillende interferon-geïnduceerde genen. Dit toont aan dat CD4$^+$ T cellen in staat zijn om HIV-1 replicatieproducten te detecteren en een mogelijke bron kunnen zijn van de verhoogde IFN-I niveaus in HIV-1 patiënten.

In een tweede deel werden vervolgens gastheer en virale mechanismen onderzocht die de IFN-I inductie door HIV-1 reguleren in primaire CD4$^+$ T cellen. Dit leidde tot drie belangrijke bevindingen: 1) de cytosolische DNA receptor cGAS alsook signalisatie eiwitten in de cGAS pathway zijn noodzakelijk voor IFN-I inductie; 2) IFN-I inductie treedt enkel op wanneer succesvolle integratie en eiwitproductie door het virus plaatsvindt; 3) de IFN-I respons wordt gereguleerd door twee HIV-1 accessorische eiwitten: Vpr stimuleert de respons en Vpu onderdrukt de respons. Deze bevindingen suggereren een mechanistisch model waarin HIV DNA gedetecteerd wordt door cGAS tijdens productieve infectie, door toedoen van nieuw gevormd Vpr en eventueel andere nieuw gevormde replicatie producten. Mogelijks heeft het virus een mechanisme ontwikkeld om dit proces deels te onderdrukken via Vpu.

Samenvattend heeft dit werk geleid tot verschillende nieuwe inzichten in onze aangeboren immuunrespons tegen HIV-1. We identificeren HIV geïnfecteerde CD4$^+$ T cellen als een mogelijke nieuwe bron van IFN-I en determineren virale en gastheer factoren die de IFN-I respons in deze cellen mediëren. De sterke virale regulatie van IFN-I productie en de schadelijke rol van chronisch IFN-I in HIV-1 pathogenese, maken dit proces tot een mogelijk interessant doelwit voor nieuwe therapeutische strategieën.

Chapter I :
General Introduction

Chapter I-1. Human immunodeficiency virus 1

In 1983, a new retrovirus was isolated and identified as the causative agents of a rapidly spreading, deadly new disease called AIDS (acquired immunodeficiency syndrome) [1-3]. The Human Immunodeficiency Virus type 1 (HIV-1) has been one of the most successful pathogens in human history, having killed over 35 million people since the start of the epidemic [4]. In the thirty years since its discovery, a lot has been learned about the emergence, structure, life cycle and pathogenic properties of HIV-1. The purpose of this section is to introduce the virus and its disease as a foundation for the more complex virus - host innate immune interactions discussed in the next section and the original research work discussed in Chapter III.

1.1 Back to the roots of HIV-1

1.1.1 Classification

HIV-1 is a lentivirus from the Retroviridae family and further comprises 4 different virus lineages or groups: M, N, O and P. The HIV-1 group M viruses are responsible for more than 95% of all HIV-1 infections and as such for the global HIV pandemic. HIV-1 group O, on the other hand, is much less prevalent and caused only a few tens of thousands of infections, mainly in West-Central Africa. HIV-1 group N and P infections are extremely rare (respectively 15 and 2 identified cases) and were mainly detected in people from Cameroonian origin [5, 6]. Viruses within the different groups have further diversified over time and group M viruses can be classified in 9 additional subgroups or clades (A-D, F-H, J-K), each with their own geographic spread [7]. Soon after the discovery of HIV-1, a morphologically similar but genetically distinct virus was found to also cause AIDS in patients in West Africa and named HIV-2 [8, 9]. However, this virus seems to have a lower transmission rate and less pathogenic course compared to HIV-1 and infections mainly remained restricted to Western Africa or countries with colonial links to the region [10].

1.1.2 Origin

AIDS was first recognized as a disease in 1981 in the United States [11]. However, retrospective analysis revealed the presence of HIV-1 sequences in African blood and tissue samples that were collected as early as 1959 [12, 13]. Based on the high level of diversity among HIV-1 group M strains from that period, the onset of the group M epidemics was dated back to the early 1920s and most likely originated in the region of Kinshasa (in what is now the Democratic Republic of Congo) [13-15]. Soon after the isolation of HIV-1 and HIV-2, different African monkey and ape species were found to carry lentiviruses with a close phylogenetic relationship to HIV (Figure 1). These viruses were collectively named simian

immunodeficiency viruses (SIVs) because of their genetic resemblance to the human AIDS viruses, although they usually do not cause an immunodeficiency in their natural hosts. Interestingly, HIV-1 and HIV-2 viruses seemed more closely related to different types of SIVs than to each other. This provided a first indication that both viruses had emerged from direct cross-species transmissions of SIV to humans [16, 17]. Based on phylogenetic analyses of different SIVs and HIVs, it is now assumed that the four different HIV-1 groups and HIV-2 all originated from independent zoonotic transmission events from chimpanzees, gorilla or sooty mangabeys, with SIVcpz from the chimpanzee *Pan troglodytes troglodytes* being the ancestor of the pandemic HIV-1 group M strains (Figure 1). Although many "wild" theories exist regarding the circumstances of transmission, it most likely occurred in the process of hunting and butchering of primates for bushmeat or through scratches and bites of monkeys that were kept as pets. The subsequent events that fueled the emergence of a global HIV-1 group M pandemic remain speculative. The rapid growth of the city populations, the increase in sexually transmitted diseases and the use of unsterile needles during large-scale injection campaigns could all have contributed to the explosive spread of HIV (reviewed in [6, 8]).
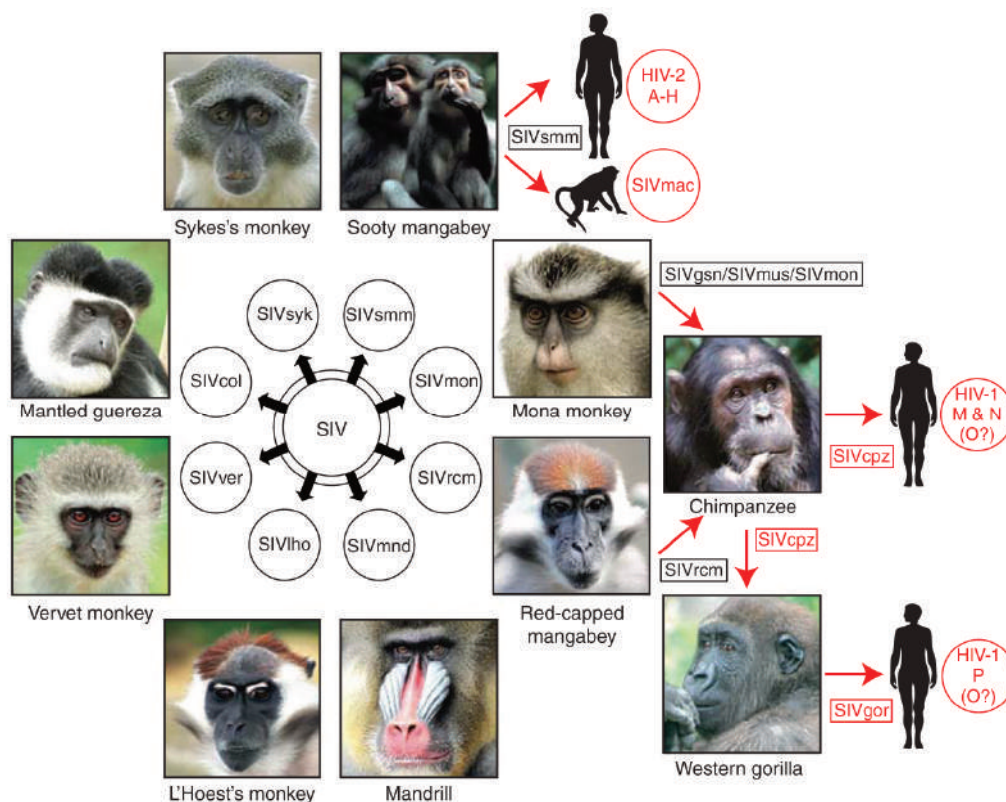
Figure 1.1: Origin of HIV-1. Over 40 different lentiviruses have been detected in African monkey and ape species and they have collectively been named simian immunodeficiency viruses (SIV). Transmission of these viruses across the species barrier, eg. to humans, has occurred on multiple independent occasions and has given rise to the different HIV-1 and HIV-2 groups. *Reprint from Sharp et al. 2011 [8] with permission from the authors.*

1.2 Structure of HIV-1

HIV-1 viral particles have a spherical shape of approximately 100-150 nm diameter. They consist of an outer lipid bilayer that is of cellular origin and an inner cone-shaped core that is formed by structural proteins of viral origin. Like all retroviruses, HIV-1 has a positive sense, single-stranded RNA genome of which two copies are packed into the viral particles. This genome encodes for 15 final proteins that are synthesized from one primary alternatively spliced transcript. An envelope glycoprotein precursor (gp160), encoded by the *env* gene, is cleaved by cellular proteases, such as furin, into surface (gp120) and transmembrane (gp41) moieties. Complexes of these glycoproteins are inserted into the lipid bilayer of the virus and mediate interaction with the viral target cells. The *gag-pol* gene gives rise to two polyprotein precursors (Gag and Gag-Pol), which are both cleaved into smaller proteins by a virus encoded protease during maturation of the viral particle (see also 1.3). In this process, the Gag polyprotein (p55) is transformed in several structural proteins: matrix (MA or p17), capsid (CA or p24) and nucleocapsid (NC or p7), as well as a p6 protein and two small spacer peptides SP1 and SP2. Matrix proteins mainly associate with the inner part of the lipid bilayer, while capsid proteins assemble in a conical core structure that surrounds the viral genomic material. The Pol fragment of the Gag-Pol polyprotein on the other hand is further processed into three viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN) and these proteins are also contained within the viral particles (Figure 1.2, reviewed in [18, 19]). The HIV-1 genome furthermore encodes two regulatory proteins, Tat and Rev, which are required for optimal viral protein production, as well as four accessory proteins (Vif, Vpr, Vpu and Nef). Based on *in vitro* cell-culture systems, the latter were initially thought to be dispensable for viral replication. However, they seem to be essential for *in vivo* infection and pathogenesis (see also 1.6). Vif, Nef and large quantities of the Vpr proteins are also incorporated into the viral particles (reviewed in [19, 20]). Finally, virions contain a multitude of host proteins that are incorporated because of their presence at the sites of viral budding or through interaction with HIV proteins (eg. cyclophilin A – capsid interaction) [21]. HIV-2 and different SIV viruses have in general a similar genomic and structural organization as HIV-1. However, they clearly differ by the types of accessory genes they encode. While HIV-2 and certain SIV viruses do not encode a Vpu protein, some of them acquired an extra accessory protein, called Vpx, which is not encoded by HIV-1 (Figure 1.2) [22, 23].
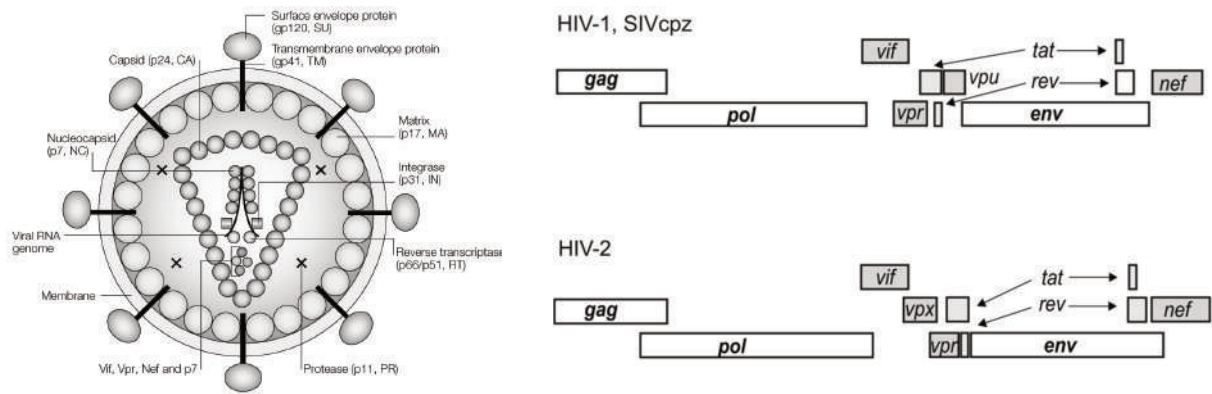
Figure 1.2: Structure and genome of HIV. Left: organization of HIV-1 mature viral particles with indication of the different viral components. *Reprint from Robinson 2002 [24] with permission from Macmillan Publishers Ltd: Nature Reviews Immunology, copyright © 1998, and with permission from Elsevier, copyright © 1998.* Right: genomic organization of HIV-1, HIV-2 and SIVcpz viruses. *Adapted from Kirchhoff 2010 [23] with permission from Elsevier, copyright © 2010.*

1.3 HIV-1 replication cycle

HIV-1 infects different types of immune cells *in vivo*, predominantly CD4$^+$ T helper cells, monocytes/macrophages and to a lesser extent also dendritic cells [25-27] (see also 1.4.1). This viral tropism is largely determined by the presence of the primary HIV receptor CD4 and a secondary co-receptor on the surface of these cells. Although alternative co-receptors have been proposed, most HIV-1 isolates use CXCR4 or CCR5 and the affinity for these receptors is mainly determined by the V3 region of the HIV envelope (Env). As such, viruses are termed X4-, R5- or R5X4-tropic based on their usage of CXCR4, CCR5 or both respectively. The sequence of events during a productive HIV-1 replication cycle are outlined in Figure 1.3 and described below.

Entry of HIV in the target cells is initiated by binding of a gp120 subunit of HIV Env to CD4 (see **(1)** on Figure 1.3). This induces a conformational change in gp120 that allows its subsequent binding to the co-receptor. This results in exposure and insertion of the hydrophobic fusion peptide of gp41 in the membrane of the cell, which in turn induces a conformational change that brings the viral and cellular membranes in closer proximity, allowing their fusion (reviewed in [28, 29]) (**2**). The actual site of HIV fusion is however still a topic of debate : both direct fusion at the plasma membrane and fusion after endocytotic uptake of the virus have been suggested as the predominant route of viral entry [30, 31]. Following fusion, the HIV capsid core is released into the cytoplasm of the cell and reverse transcription of the HIV RNA genome into double-stranded DNA (dsDNA) is initiated (**3,4**). Rearrangements of the virion core lead to formation of a mature reverse transcription complex (RTC), in which multiple viral and cellular proteins assist the HIV RT protein to

complete reverse transcription. During this process, the RTC is simultaneously transported towards the nucleus through interactions with the host cytoskeleton. The complex is gradually transformed into an integration-competent complex, termed pre-integration complex (PIC). Before this complex can enter the nucleus through the nuclear pores, disassembly of the viral capsid that surrounds the genomic material has to occur, a process known as uncoating (**3**). Initially, this was thought to take place immediately after viral entry in the cell. However, more recent data suggest that uncoating occurs several hours later and that the virion core serves important functions during transport towards the nucleus, reverse transcription and nuclear import (reviewed in [32-34]). After entry into the nucleus (**5**), linear dsDNA in the PIC is inserted into the host chromosomal DNA. This process is mediated by the HIV IN protein, again assisted by other proteins, and involves three subsequent steps. First, two nucleotides are removed from the 3' end of both viral DNA strands by the IN protein. Subsequently, the two 3' ends attack phosphodiester bonds on opposite strands of the target DNA, resulting in a covalent linkage between the host and viral DNA. Finally, the nucleotide overhang at the 5' ends and the remaining gaps are repaired by cellular enzymes to complete viral integration (reviewed in [35]) (**6**).
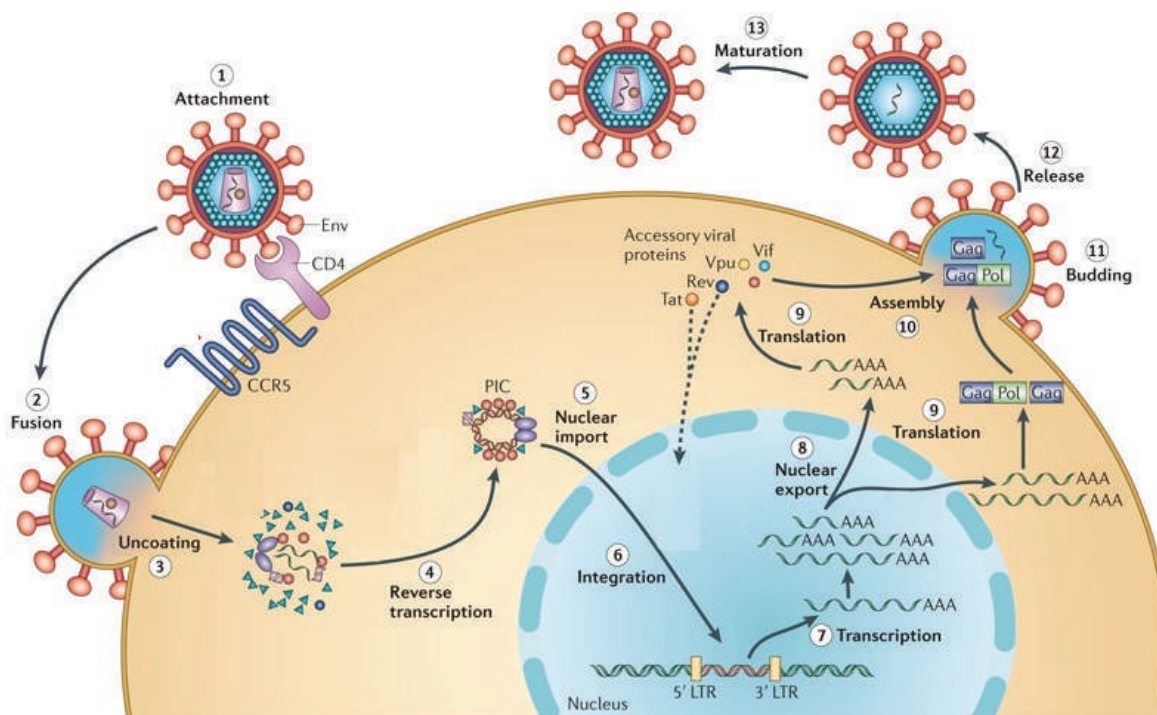
Figure 1.3 Overview of the HIV life cycle. Sequential steps in the HIV-1 life cycle, from initial attachment to the target cell (1) to release and maturation (12, 13) of the newly formed viral particle are depicted. *Adapted from Engelman et al. 2012* [36] *with permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, copyright © 2012*

The integrated provirus then serves as a template for viral gene expression (**7**). The long-terminal repeat (LTR) located in the 5' region of the provirus, contains enhancer and

promoter elements and these are recognized by the host transcription machinery. Viral transcription is initially activated by cellular transcription factors, such as NF-κB, and this results in a suboptimal production of early viral proteins: Tat, Rev and Nef. Once sufficient levels of Tat have accumulated, transcription is drastically enhanced through a complex process that involves recruitment of the positive transcription elongation factor (P-TEFb) to the HIV transactivation-responsive region (TAR) by Tat and which relieves a block in transcription elongation of the viral RNA. Furthermore, the synthesized Rev proteins will mediate transport of incompletely spliced and unspliced viral RNA products to the cytoplasm (**8**) and thereby allow their translation into Env, Vpu, Vpr, Vif and the Gag-Pol polyprotein (reviewed in [37]) (**9**). Newly formed proteins, viral RNA and different host proteins subsequently assemble at the cell membrane under control of the Gag protein (**10**). These complexes bud through the cell membrane, thereby acquiring the host cell-derived lipid membrane that enwraps the viral core (**11**, **12**). Budding activates the HIV-1 protease through an incompletely understood mechanism, which will cleave Gag and Gag-Pol polyproteins into fully processed structural and enzymatical viral proteins. These proteins rearrange within the virion core and transform the immature virion into a mature infectious virus [38] (**13**).

1.4 HIV-1 Pathogenesis

1.4.1 HIV-1 target cells

As mentioned above, HIV-1 mainly infects CD4+ T cells and cells of the monocyte/macrophage lineage as these cells express the necessary receptors for HIV-1 entry. CD4+ T cells are the primary targets *in vivo* and their ability to support productive infection is highly determined by their activation state. *In vitro* experiments in the early days of HIV research already revealed that HIV-1 efficiently replicates in activated, proliferating CD4+ T cells, while resting cells are largely non-permissive [39, 40]. It is now known that HIV-1 replication in the latter is blocked at the level of reverse transcription due to the actions of the host restriction factor SAMHD1 (see also 1.6). Furthermore, transcription of viral genes might be less efficient in resting T cells, since transcription factors required for initiation of LTR activity (eg. NF-κB and NFAT (nuclear factor of activated T cells)) are usually inactive [41]. The latter also contributes to establishment of a latent state of the virus in resting CD4+ T cells. HIV-1 DNA-containing resting T cells are indeed found in infected patients and serve as a latent reservoir, i.e. cells harbouring transcriptionally silent but replication-competent proviruses, from which virus replication can be initiated upon activation. These cells may arise from infection of activated CD4+ T cells that subsequently revert to a resting state and survive as long-lived memory T cells. However, HIV-1 DNA has also been detected in naive T cells *in vivo*, implying direct infection of resting cells. It is therefore thought that the presence of certain signals *in vivo*, such as chemokines or cell-to-

cell signaling, might be sufficient to temporarily relieve the block to infection without completely activating the cells [41, 42].

Macrophages are non-dividing, terminally differentiated cells. Although they generally resist HIV-1 infection better than activated CD4+ T cells, productively infected macrophages are detected *in vivo* and seem to contribute to the viral burden. The susceptibility of macrophages to HIV-1 is highly dependent on their activation and polarization state. Interestingly, macrophages are much more resistant to cytopathic effects of HIV-1 replication compared to CD4+ T cells. Especially long-lived macrophages may therefore carry the virus for long periods of time and form a chronically infected reservoir for HIV-1 [43, 44].

Myeloid dendritic cells (mDCs) are usually inefficiently infected by HIV-1, due to high expression levels of SAMHD1 [45]. However, they may play an important role in HIV-1 dissemination through *trans*-infection of CD4+ T cells. mDCs are able to capture and internalize intact HIV viral particles through interaction of gp120 with DC cell surface receptors, such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) and Siglec-1 (sialic acid binding Ig-like lectin 1), and subsequently transmit these virions to contacting CD4+ T cells with high efficiency [46]. Langerhans cells, a subset of mDCs that line the epithelial layer of mucosal surfaces, can also interact with HIV through the C-type lectin langerin and dependent on their maturation status either destroy internalized viral particles or transmit them to CD4+ T cells [47]. The germinal centers in lymphoid tissue furthermore contain a unique population of cells, called follicular DCs. These cells can trap large amounts of HIV at their surface and retained virus can be transferred to surrounding CD4+ T cells. Since captured viral particles remain infectious for long periods of time, these cells may provide a reservoir for continuing viral dissemination [48, 49].

1.4.2 Course of natural HIV-1 infection

HIV-1 transmission results from exposure to the virus at mucosal surfaces (eg. by sexual contact, mother-to-child) or from percutaneous inoculation (eg. needlesticks). Especially in the case of mucosal transmission, infection is usually established by a single founder virus [50]. The course of natural, untreated HIV-1 infection is characterized by different stages (Figure 1.4).

The acute phase of HIV-1 infection is the most critical stage, during which most events determining the clinical course of chronic infection are established. Following transmission, there is an initial period of 1-3 weeks in which HIV RNA remains undetectable in the plasma, known as the "eclipse phase". During this period, small foci of infection are formed at the transmission site and the virus can subsequently disseminate to the local draining lymph nodes. This process is facilitated by DCs, that capture the virus and transport it to

local lymph nodes for transmission to CD4+ T cells. Furthermore, inflammatory responses orchestrated by DCs and macrophages lead to recruitment of CD4+ T cells to the infection site and amplify the pool of available target cells (although they also counteract viral replication by eg. secretion of type I interferon (IFN-I)). The virus subsequently spreads to other lymphoid tissues, particularly to the gut-associated lymphoid tissues (GALT) where the majority of activated CD4+ CCR5+ T cells reside. This is followed by a rapid increase in virus replication, which results in a massive depletion of CD4+ T cells in the GALT and an exponential rise in plasma viremia. In this phase, patients sometimes experience transient "flu-like" symptoms (eg. fever, enlarged lymph nodes,...). A peak in plasma viremia is usually reached within 2 to 4 weeks after infection. The viral load subsequently declines to a more stable level, which is known as the viral set-point. This is due to exhaustion of activated target cells and partial control of viral replication by the immune system. The latter is largely mediated by HIV-1 specific CD8+ T cell responses and at a later stage by the appearance of neutralizing antibodies. Innate immune responses, in particular those mediated by natural killer (NK) cells, might also contribute to viral suppression at this point. However, under pressure of the anti-HIV immune responses the viral population starts to diversify and viral escape mutants can be readily detected following peak viremia. Furthermore, a pool of latently infected cells is probably formed within days of infection and provide a long-lasting reservoir that remains invisible to the immune system [51-54].
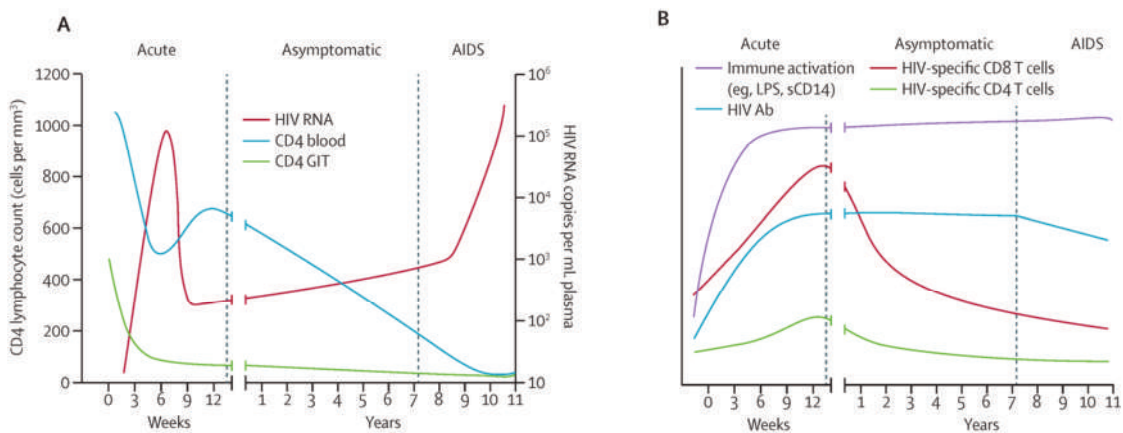

Figure 1.4. Natural course of untreated HIV infection. (A) Acute infection is associated with high levels of plasma viral load (red line). Following development of HIV-specific immune responses, partial viral control is reached and associated with a drop in plasma viral RNA levels. CD4+ T cells in the gastrointestinal tract (GIT) are rapidly depleted early on (green line), while blood CD4+ T cell levels progressively decline during the chronic phase of infection due to continued systemic repletion/depletion (blue line). Ongoing destruction of the immune system eventually leads to loss of immune control and AIDS. (B) Immune response to HIV infection is characterized by a dramatic increase in markers of immune activation, production of HIV-specific CD8+ (and CD4+) T cells and development of antibody (Ab) responses. *Reprinted from The Lancet, Maartens et al. 2014 [51] with permission from Elsevier, copyright © 2014.*

The ongoing struggle between active viral replication and immune control leads to a more chronic phase of infection in which the patient remains asymptomatic and which can last for many years. However, continued destruction of HIV-1 target cells and the inability to completely repopulate these cells results in a progressive depletion of CD4^{+} T cells. Loss of CD4^{+} T cells occurs due to direct killing by the virus, but also as a bystander effect of immune activation and senescence (see also 1.4.3) [55, 56]. Eventually, CD4^{+} T cell levels drop below a critical limit and immune control of HIV-1 and other infectious agents is lost. As a result, the level of viremia rises during the AIDS phase and new infectious [57] and malignant complications [58] are common. In untreated infection, this inevitably culminates in death of the vast majority of infected patients [52, 53].

1.4.3 Immune activation

A hallmark of HIV-1 infection is the chronic state of systemic immune activation. This is reflected by eg. increased levels of activation (CD38, HLA-DR) and proliferation (Ki-67) markers on CD4^{+} and CD8^{+} T cells and increased inflammatory markers in the plasma (eg. neopterin, β2-microglobulin) of HIV-1 infected individuals [59]. Interestingly, it was noted early on that such markers are highly predictive for disease progression [60-62], to an equal or even higher extent than viral load levels [63-65]. This has led to the idea that suicidal overdrive of the immune system, more than viral cytopathic effects, is the major cause of immune deficiency and pathogenesis. In support of this, naturally infected hosts of SIV, in which the infection rarely progresses to AIDS, do not show signs of chronic immune activation despite high levels of viral replication and early CD4^{+} T cell depletion [66, 67]. Different factors have been suggested to contribute to the hyper-immune activation. The most obvious one are the direct innate and adaptive immune responses against the virus. In this regard, IFN-I produced upon innate sensing of HIV-1 has been highly associated with immune activation levels [68, 69]. Responses against other agents, such as translocated microbial products in the gut and viral co-infections (eg. cytomegalo-virus and hepatitis C virus) are also likely to contribute [70, 71]. Furthermore, specific imbalances in levels and functions of certain CD4^{+} T-cell subsets, such as Th17 and Treg cells, arise during HIV-1 infection and may disturb immune homeostasis [59, 72, 73]. Finally, different viral proteins are able to directly activate or increase activation sensitivity of HIV-1 target cells [74-76].

Continuous immune stimulation creates a permissive environment for viral replication, leading to a vicious cycle in which infection stimulates activation and activation stimulates infection. Depletion of CD4^{+} T cells, by direct viral killing and activation-induced cell death, will in turn trigger homeostatic responses that induce T cell activation and proliferation to replenish the T cell pool, further supplying viral target cells. Chronic activation can further inhibit normal functions of other immune cells [77, 78], leading to less viral control and again more activation. Over time, the perpetual induction of T cell regeneration most likely

leads to premature aging of the immune system [79] and architectural disruption of tissues important for immune homeostasis [80, 81]. As such, through continued stimulation, the virus gradually drives the immune system to a point of complete exhaustion [59, 82].

1.5 Antiretroviral therapy

Over the last two decades, effective antiretroviral therapies have become available, which have transformed HIV infection from an inevitably lethal condition into a chronical manageable disease. They exist of combinations of drugs (combination antiretroviral therapy, cART) that are directed against viral enzymes (reverse transcriptase, protease or integrase) or target the viral entry or fusion process [83]. Although current treatment can suppress viral load to undetectable levels, the virus persists in a latent reservoir with a remarkably long half-life and viral rebound occurs when ART is discontinued. As such, ART is not curative and life-long treatment is required [42, 84, 85]. Futhermore, treatment does not fully restore health. Immune reconstitution is not always complete and markers of residual inflammation can be detected, even after many years of viral supression. Remaining elevated immune activation under cART is associated with increased mortality and co-morbidities (eg. cardiovascular disease, liver disease,...) and is thought to contribute to persistance of viral replication and the viral reservoir [86, 87]. Toxic effects from decades of ART exposure can furthermore culminate in metabolic disturbance and organ damage [88]. An additional problem with current therapy is emergence of drug resistance. HIV-1 has a highly error-prone enzymatic machinery and any cause of residual replication under ART can therefore result in selection of resistant variants [89].

Because of the reasons indicated above, new treatment strategies are highly pursued and actively being developed. These include the targeting of viral processes that are less prone to mutational escape, such as protein-protein interactions between viral and cellular proteins [90, 91]. Also approaches to counteract chronic immune activation and its associated effects are being evaluated, in order to improve immune reconstitution and decrease inflammation-associated morbidities in patients under cART [59, 92]. Other strategies are more ambitious and aim at curing the infection. In this regard, gene therapy approaches to target the HIV-1 coreceptor CCR5 are highly investigated. These are inspired by the apparent cure of the "Berlin patient" after receiving hematopoeitic stem cell transplantation from an CCR5Δ32 homozygous donor [93]. Another line of research is focused on elimination of the viral reservoir. A pursued approach in this regard is to reactivate latent proviral genomes in infected cells, leading to virus-producing cells that either die or are cleared by host immune mechanisms [94, 95]. In order to be effective such strategies will likely need to be combined with approaches to 1) suppress residual viral replication and to 2) enhance host immune functions to recognize virus-producing cells and eliminate rebound virus. The latter is addressed by the field of immunotherapy and pursues

strategies to artificially boost anti-HIV responses and counteract abberant immune activation, in order to obtain functional immunity and life-long remission of HIV-1 [86, 96-98].

1.6 HIV accessory proteins

One of the features that distinguishes HIV and SIV from other retroviruses, is the set of "accessory" proteins they encode. Although these factors are often dispensable for viral replication in *in vitro* cell culture systems, they are strongly conserved *in vivo* and functional mutations in these genes have been associated with slower disease progression [99]. In general, they seem to modify the cellular environment for optimal viral replication, which often involves regulation of the host immune defense. These proteins do not have an enzymatic activity, but mediate their effects by interacting with cellular proteins and hijacking them into aberrant functions [100]. Here, we will discuss the known functions of the different accessory proteins, with a focus on Vpu and Vpr (see also Figure 1.5).

NEF

Nef enhances viral dissemination and protects infected cells from immune surveillance, through a remarkable number of activities in the infected cells. For example, Nef regulates cell surface expression of a wide range of host proteins, including CD4 and MHC-I. Down-regulation of CD4 could eg. enhance viral release and prevent viral superinfection (see also Vpu), while selective counteraction of MHC-I by Nef protects infected cells from lysis by cytotoxic T cells, without rendering them susceptible to NK cell-mediated responses [101-103]. Furthermore, by interacting with cellular kinases and signaling pathways Nef alters the activation threshold of infected T cells and affects cellular motility and migration [102, 104]. Nef also enhances infectivity of progeny virions through an incompletely understood mechanism [105, 106].

VIF

The main function of Vif is to counteract members of the APOBEC3 (apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3) family of retroviral restriction factors, especially APOBEC3G. These host proteins can mutate single-stranded DNA (ssDNA) by catalyzing deamination of cytosines into uracils. Although they are constitutively present in a wide variety of cells, their expression is often further induced by IFN-I. In absence of Vif, APOBEC3G is packaged into budding viral particles and induces C to U transitions during synthesis of the first (minus) HIV DNA strand. This leads to degradation of HIV DNA genome or fixed G to A mutations in the proviral sequences. Although alternative mechanisms have been proposed, Vif mainly prevents incorporation of APOBEC3G in the virion by targeting APOBEC3G for polyubiquitination and subsequent proteasomal degradation. This is

achieved by simultaneous binding of Vif to APOBEC3G and the EloB/C subunit of the cullin 5 E3 ligase complex [107, 108].

VPU

Vpu contributes to viral egress by counteracting host antiviral immunity and promoting efficient release of newly formed viral particles. This is mainly achieved by down-regulation of two molecules from the surface of the cell: CD4 and tetherin (also known as bone marrow stromal cell antigen 2 (BST2)) [109-111]. Targeting of CD4 and tetherin by Vpu relies, at least in part, on induction of their ubiquitination and thereby targeting them for degradation. This is accomplished by connecting both molecules with the SKP1-cullin1-F-box (SCF) E3 ubiquitin ligase complex through interaction of Vpu with the SCF subunit, β-transducin repeat-containing protein (β-TrCP) [112, 113].

Vpu is mainly localized in the endoplasmatic reticulum (ER), the trans-Golgi network (TGN) and the endosomes [114, 115]. In the ER, Vpu interacts with newly synthesized CD4 molecules [109]. Subsequent poly-ubiquitination of CD4 induces both retention of CD4 in the ER as well as its delivery to the ER-associated degradation pathway (ERAD) for proteasomal degradation [112, 113, 116]. In this way, vpu might prevent sequestration of newly produced Env molecules by CD4 in the ER and decrease surface levels of CD4. The latter is also mediated by two other HIV-1 proteins, by Nef and to a lower extent also by Env, and seems therefore important for optimal viral spread. Several possible viral advantages of CD4 down-regulation have been proposed: enhancing release of viral particles by preventing the binding of newly formed virions to CD4, preventing lethal superinfection or inducing immune dysfunction of infected cells [117-119].

Tetherin on the other hand is a protein induced by IFN-I that can physically trap newly formed viral particles at the cell surface and thereby inhibit their release. This is due to the unusual structure of tetherin: it can insert both its C-terminal transmembrane domain as well as its N-terminal glycophosphatidylinositol anchor into membranes. By simultaneously binding to both cellular and viral membranes, it tethers viral particles to the cell [110, 111, 120]. Although the mechanism of tetherin down-regulation by Vpu remains somewhat controversial, Vpu is thought to interact with recycling or newly synthesized tetherin molecules in the TGN and inhibit their transport to the plasma membrane [114, 121]. This interaction traps tetherin molecules in the cell, but also results in tetherin ubiquitination which most likely enhances its lysosomal degradation through an Endosomal Sorting Complexes Required for Transport (ESCRT)-mediated pathway and is thought to be required for full tetherin counteraction [119, 122-124].

While the ability to down-regulate CD4 is conserved among most primate lentiviruses encoding Vpu [125], counteraction of tetherin is more lineage- and species-specific. Most

SIVs, including the immediate ancestors of HIV-1 and HIV-2, use Nef rather than Vpu to target tetherin. However, human tetherin is resistant to SIV Nef due to a deletion in its cytoplasmic tail [125-127]. It is therefore believed that successful cross-species transmission to humans required the acquisition of other means to counteract tetherin. The pandemic HIV-1 M strains and (to a lesser extent) the non-pandemic HIV-1 N strains overcame this species barrier by evolving a mechanism of counteraction through Vpu [125, 128], while HIV-1 O strains adapted their Nef protein [129]. HIV-2 strains, which do not encode a Vpu protein, most likely switched from Nef to Env to counteract tetherin [130].

Vpu further contributes to innate immune evasion by interfering with function of natural killer (NK) and NKT cells. Vpu down-regulates several NK(T) cell activating proteins from the surface of infected cells, such as CD1d, CD155 and NTB-A [131]. In addition, by preventing accumulation of viral particles on the surface of the cell through tetherin and CD4 down-regulation, Vpu protects infected cells from NK cell elimination by antibody-dependent cell-mediated cytotoxicity (ADCC) [132-134]. Recent studies also indicate that tetherin can induce activation of nuclear factor-κ light-chain-enhancer-of activated B-cells (NF-κB) upon restriction of viral particles [135, 136]. By targeting tetherin or by directly interfering with innate signaling pathways [137, 138], Vpu might counteract expression of pro-inflammatory cytokines or IFN-I (see also 2.4).
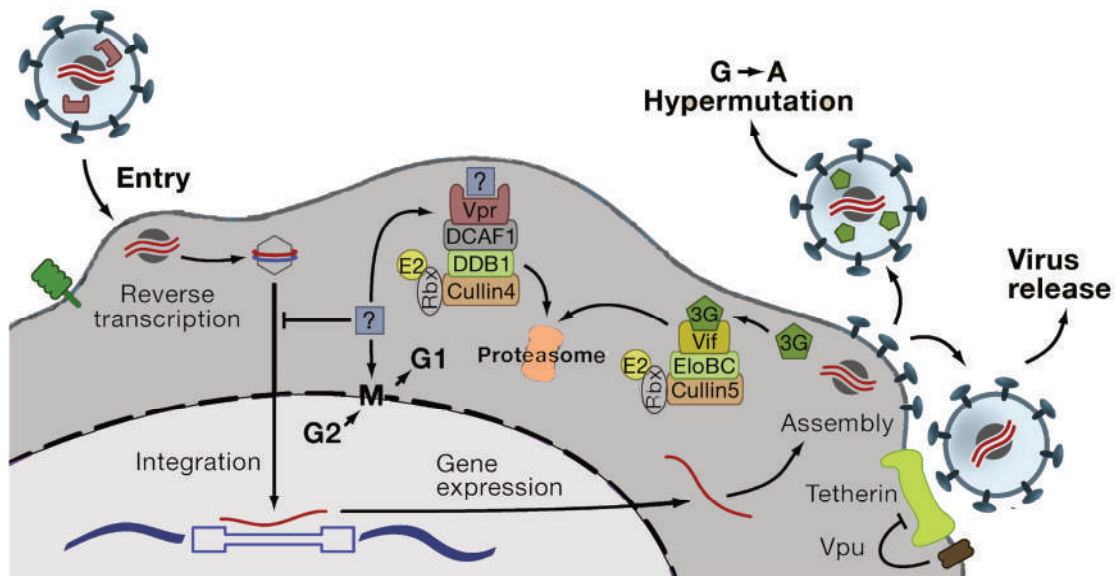
Figure 1.5 Counteraction of host restriction factors by HIV-1 accessory proteins Vpr, Vif and Vpu. Counteraction of APOBEC3G (3G) by Vif and tetherin by Vpu is depicted. These viral proteins can induce proteasomal degradation of their target through recruitment of E3 ubiquitin ligase complexes and subsequent ubiquitination of the target. Vpr is also able to recruit such complexes and induces degradation of certain cellular proteins, although its main target remains elusive. *Adapted from Malim et al. 2008 [139] with permission from Elsevier, copyright © 2008.*

VPR

In contrast to other accessory proteins, the prime role of Vpr in natural HIV infection is still enigmatic. Although Vpr is expressed at a late stage in viral life cycle, it is significantly present during early infection, because large amounts of Vpr are incorporated into the viral particle and thereby carried into the target cell by the virus [140]. This argues for an important role of Vpr during early steps of viral replication. Based on *in vitro* observations, a multitude of functions and effects have been ascribed to Vpr, including transactivation of the HIV-1 LTR, induction of cell cycle arrest, enhancing the fidelity of reverse transcription and induction of cell death [141-143]. HIV-1 replication *in vitro* is especially dependent on Vpr in non-dividing cells, particularly in macrophages [144-147]. This was initially attributed to facilitation of nuclear import by Vpr. Interaction between Vpr, members of the nuclear pore complexes and importin-α would promote docking of the viral PIC to the nuclear membrane or facilitate its transport across the membrane [148-152]. However, more recent studies suggest that other components of the PIC (eg. integrase, MA or the central DNA flap) have redundant properties and that these properties are not an absolute requirement for infection of non-dividing cells [153-155].

Like Vpu and Vif, Vpr is known to engage an E3 ubiquitin ligase complex, in this case the DDB1-cullin4 complex, which is recruited by Vpr through interaction with DDB1-cullin4-associated factor 1 (DCAF1) [156]. One of the proteins targeted by Vpr for ubiquitination and subsequent proteasomal degradation is uracil DNA glycosylase (UNG2). However, the exact role of UNG2 during HIV infection is controversial, since both positive and negative effects on viral replication have been described [157-161]. In analogy with other ubiquitination-inducing HIV/SIV accessory proteins, it is likely that inducing degradation of a cellular host factor is a crucial function of Vpr. However, UNG2 and the few other suggested cellular Vpr targets [162, 163] do not fully explain the ability of Vpr to enhance *in vitro* replication in non-dividing cells nor its importance during natural infection *in vivo*. Therefore, other cellular targets of Vpr probably remain to be identified [100, 143]. Engagement of the DDB1-cullin4-DCAF1 E3 ligase complex is also essential for Vpr's ability to induce G2 arrest in dividing cells [164-167]. This Vpr function is highly conserved among primate lentiviruses, but its biological role and precise mechanism remain unclear. Vpr somehow activates the DNA damage sensor ATR and thereby hijacks the downstream host DNA damage response pathway to trigger G2 arrest. Given the necessity of the E3 ubiquitin ligase complex, a model has emerged in which Vpr-induced degradation of an unknown cell cycle regulating protein triggers ATR activation [139, 156]. Laguette et al. recently demonstrated that induction of G2 arrest is dependent on recruitment and premature activation of the specific endonuclease regulator SLX4 complex by Vpr, which may result in faulty cleavage of host DNA replication intermediates and replication stress [168]. However, it is not clear yet how Vpr-induced proteasomal degradation fits into this process. Also the functional relevance of G2 arrest during natural infection remains speculative. It was shown that the HIV LTR

promoter is more active in the G2 phase [169-171]. As such, induction of G2 arrest might therefore provide a way for the virus to enhance viral protein expression and replication. Alternatively, Laguette et al. suggested that the SLX4 complex is engaged by Vpr to degrade excess viral DNA, thereby preventing it's sensing and subsequent IFN-I induction. Accordingly, the biological endpoint of SLX4 complex activation would be to evade innate immune responses [168]. Finally, since Vpr has a limited effect on HIV-1 replication in dividing cells *in vitro*, induction of G2 arrest in these exact cells may also be an unavoidable consequence of Vpr-induced degradation of an unknown host protein that is unfavourable for viral replication *in vivo* (eg. in a specific cell type) [139, 156] .

VPX

Vpx is only encoded by HIV-2 strains and certain SIVs and is homologous to HIV/SIV Vpr [172]. Like Vpr, it is packaged into viral particles through interaction with the p6 domain of Gag [173]. The main function of Vpx is counteraction of the IFN-I induced retroviral restriction factor SAMHD1 (sterile alpha motif and HD-domain-containing protein 1). SAMHD1 is a deoxynucleoside-triphosphate (dNTP) triphosphohydrolase and reduces the pool of dNTPs available for reverse transcription in both resting T cells and non-cycling myeloid cells [45, 174-178]. A recent study also suggest that SAMHD1 is able to degrade viral RNA. This activity would be lost in activated cells due to phosphorylation of SAMHD1 [179]. Vpx targets SAMHD1 for poly-ubiquitination and proteasomal degradation by hijacking the same E3 ubiquiting ligase complex (DCAF1-DDB1-cullin4) employed by Vpr, through interaction with DCAF1 [174, 180]. Interestingly, HIV-1 did not evolve anti-SAMHD1 activity and replicates less efficient in myeloid cells and resting T cells. It was shown that the absence of such activity protects HIV-1 from innate immune recognition in myeloid dendritic cells and might therefore be beneficial for the virus as it prevents activation of dendritic cells and induction of antiviral responses [181-183].

1.7 Mimicking HIV-1 infection *in vitro*

Key to the emergence of HIV-1 was its adaptation to overcome human restriction factors and exploit cellular cofactors to support its replication. As a consequence, the virus is unable to replicate or cause disease in most other species besides humans. This species-specificity has seriously hampered the development of animal models for *in vivo* HIV-1 research. SIV infection of Asian macaques is commonly used to evaluate new therapeutics or vaccines prior to human trials, but often too expensive for use in basic research [184]. Therefore, a substantial part of HIV-1 research relies on *in vitro* models of HIV-1 infection, employing isolated human HIV-1 target cells. CD4 and CCR5/CXCR4 expressing T cell lines (eg. MT4 or Jurkat) and monocytic cell lines (eg. THP-1) are most commonly used in this regard. Alhtough they efficiently support HIV-1 replication and are very easy to culture and manipulate, such transformed cell lines do not always reflect the behavior of the virus in

primary cells [185-187]. In context of this thesis it is important to note that selection against the IFN-I system may occur during malignant transformation, given the anti-proliferative role of IFN-I [188, 189]. Primary CD4$^+$ T cells can be readily isolated in substantial amounts from human peripheral blood. However, since most peripheral cells are in a resting state and will therefore not support efficient HIV-1 replication, activation of the cells is usually performed prior to infection by eg. stimulation with phytohaemagglutinin (PHA)/IL-2 or anti-CD3/CD28 beads. Primary macrophages and DCs, on the other hand, are difficult to obtain in sufficient amounts from human blood or tissue. A commonly used alternative is therefore to isolate monocytes from peripheral blood and differentiate them into macrophages or DCs *in vitro* by providing appropriate cytokine cocktails. Monocyte-derived dendritic cells (MDDCs) can for instance be generated upon culture with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 and currently represent the most wide-spread used model to study HIV biology in DCs [190].

Chapter I-2. HIV and type I interferon response

In this section, the type I interferon (IFN-I) response is introduced, as a foundation for the research presented in Chapter III-2. This type of innate immune response is an important part of the host antiviral defense system, but has a rather complex role during HIV-1 pathogenesis. Both general biology of IFN-I and its association with HIV-1 infection are discussed.

2.1 General biology of type I interferon responses

IFN-I was first described in 1957, as a substance produced by influenza virus exposed cells and able to "interfere" with the replication of other viruses in non-exposed cells [191, 192]. It is now known that these "substances" represent a wide range of cytokines that are usually secreted upon detection of certain "foreign" molecular structures in the cell, so-called pathogen-associated-molecular patterns (PAMPs), by pattern-recognition receptors (PRRs). These cytokines exert their antiviral and other effects by inducing the expression of hundreds of IFN-stimulated genes (ISGs) in both the infected and surrounding cells [193-195] (Figure 2.1). In humans, the most well-defined members of the IFN-I family are IFN-α (represented by 13 different subtypes) and IFN-β. Additional IFN-I cytokines have been described (IFN-ε, IFN-κ and IFN-ω), but their expression is often restricted to specific tissues or cell types and their regulation and antiviral effects are less well characterized [196]. Nearly all human cells are capable of producing IFN-α/β. Humans and certain mammals furthermore possess professional IFN-I producing cells, known as plasmacytoid dendritic cells (pDCs). These cells have the potential to secrete large amounts of IFN-α in response to viral infections [197].

2.1.1 Induction of type I interferon

Production of IFN-α/β by a cell is primarily regulated at the transcriptional level. The sensing of certain PAMPs by specific PRRs leads to activation of transcription factors that induce the expression of IFN-α and IFN-β (Figure 2.1). Members of the IFN-regulatory factor (IRF) family, in particular IRF3 and IRF7, are the key regulators of IFN-I gene expression. The *IFNB* gene is activated by the binding of IRF3/IRF7 homo- or heterodimers to the promoter region and also requires coordinated binding of NF-κB and AP-1 (activator protein 1). Most *IFNA* genes on the other hand, require binding of IRF7 homodimers for efficient transcription. IRF3 is constitutively expressed in most cells and resides in the cytosol in inactive form, until its nuclear translocation is induced by PRR activation. In contrast, IRF7 is present at low amounts in most cell types (except in pDCs), but highly induced as an ISG by IFN-I. Therefore, IFN-I production will occur in different phases in most cell types. Small

amounts of mostly IFN-β are produced in direct response to PRR activation and these will further amplify the production of IFN-β and many IFN-α proteins in a later phase [198, 199].
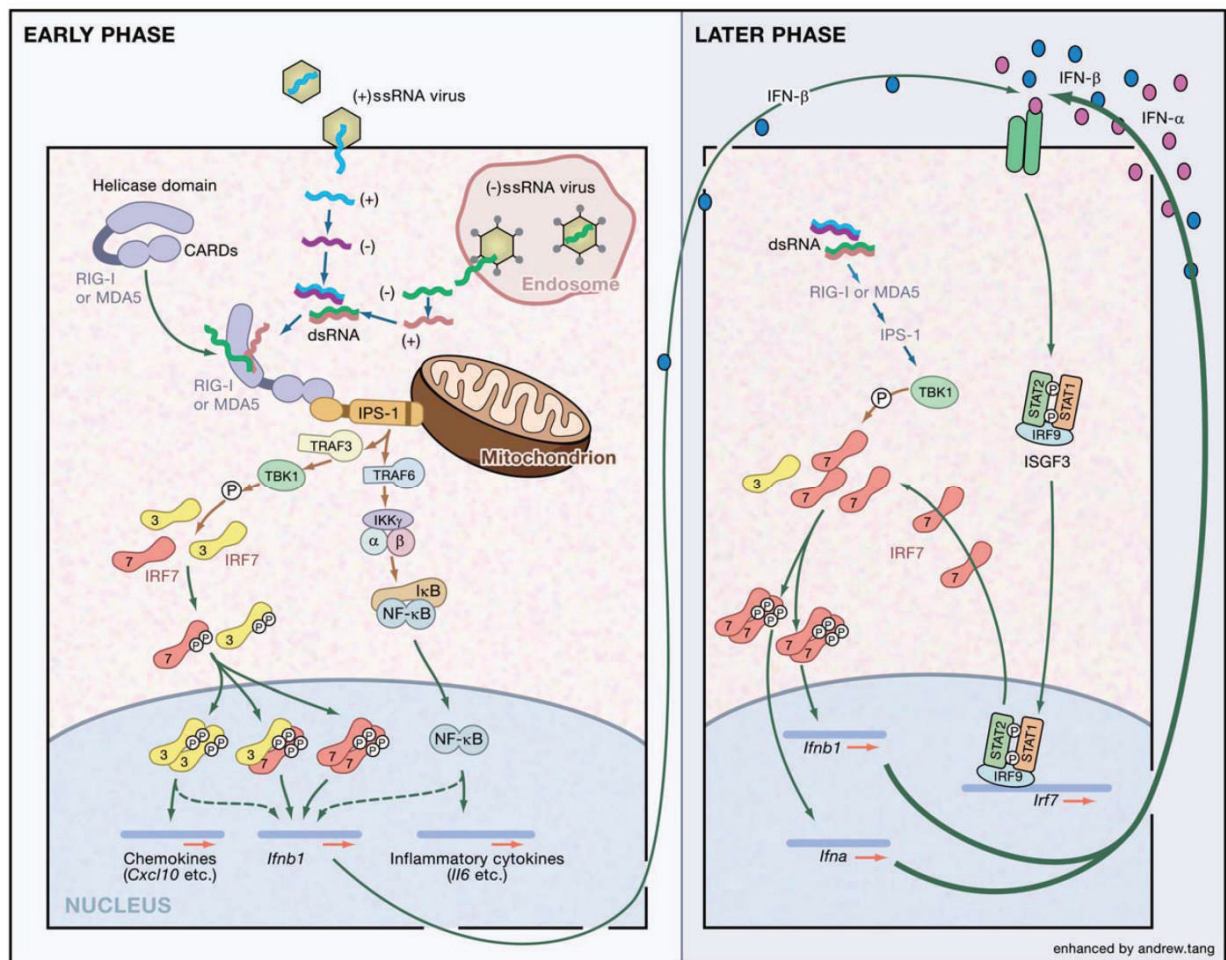

Figure 2.1 General overview of type I IFN response signaling pathways. Recognition of viral replication products by host pattern recognition receptors activates cellular signaling pathways and results in transcription of IFN-I. Secreted IFN-I can bind to the IFN-α/β receptor on the infected cell or surrounding cells and induce hundreds of interferon-stimulated genes with direct or indirect antiviral effects. *Reprint from Honda et al. 2006 [198], with permission from Elsevier, copyright © 2006.*

Various PRRs can activate IRF3 (and IRF7) upon viral infection. Since viruses do not have many features that are suitable for detection, activation often occurs in response to sensing of viral DNA and RNA replication products (although other types of viral PAMPs have also been described [200]). The PRRs that recognize nucleic acids and in turn induce IFN-I in cells can be divided into two groups based on their cellular localization (Figure 2.2).

The first group includes several members of the Toll-like receptor (TLR) family and are found on the endosomal membranes. As such, they monitor the lumen of endosomes and lysosomes for different types of pathogen-derived nucleic acids. TLR3 recognizes double-stranded RNA viruses or replication intermediates, while TLR7 and TLR8 mainly sense

guanosine- and uridine-rich ssRNA. TLR9 is a DNA sensor, that recognizes non-methylated cytidine-phosphate-guanosine (CpG) DNA motifs which are present in some viruses and bacteria [201-203]. The expression patterns of these TLRs are quite different and highly cell type specific. TLR7 and TLR9 are predominantly found in pDCs and to some extent in other immune cells, such as B cells and monocytes/macrophages. On the other hand, TLR8 is primarily expressed in monocytes/macrophages and myeloid dendritic cells. TLR3 has a broader expression range and is found in both hematopoietic cells and others cell types, such as epithelial cells and fibroblasts. The engagement of different TLRs with nucleic acids also triggers distinct signaling pathways. TLR3 activates TRIF (TIR-domain-containing adapter-inducing interferon-β), whereas TLR7/8/9 activate MyD88 (myeloid differentiation primary response gene 88). Both TRIF and MyD88 in turn activate AP-1 and NF-κB. These proteins will mainly induce expression of pro-inflammatory cytokines and chemokines, although they also contribute to IFN-β induction if activated IRFs are present in the cell. IRF3 and IRF7 are also activated by the TRIF signaling cascade. This occurs through activation of TBK1 (TRAF family member-associated NF-κB activator (TANK)-binding kinase 1), which will in turn phosphorylate IRF3/7. MyD88 signaling on the other hand only activates IRF7 and will therefore only contribute to relevant IFN-I production in cells with constitutively high IRF7 levels, such as pDCs [202, 203].
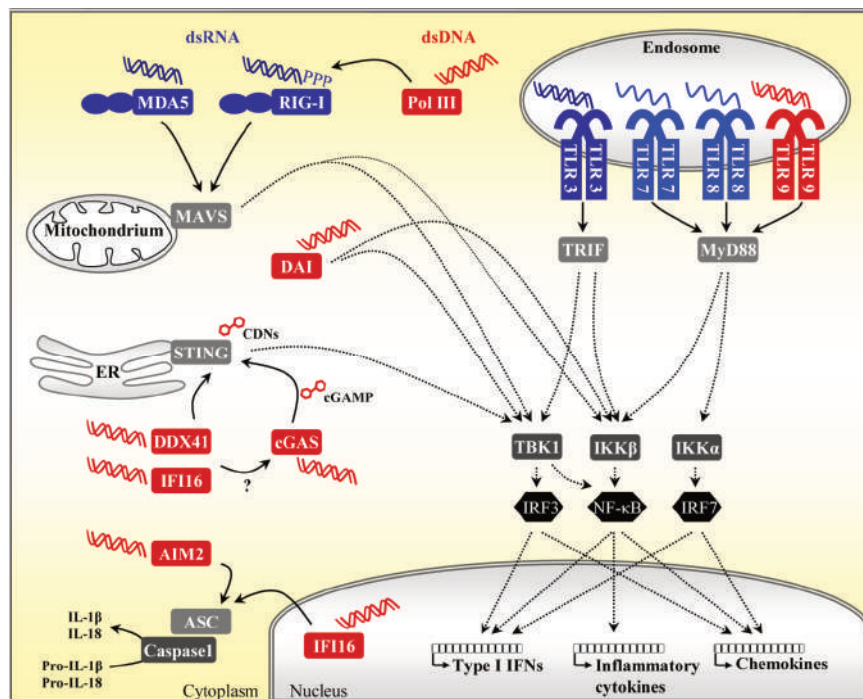

Figure 2.2. Sensing of nucleic acids by different pattern-recognition receptors (PRRs) Overview of different known PRRs that sense DNA (red), dsRNA (dark blue) or ssRNA (light blue) and the signaling pathways used to induce type I IFN and inflammatory proteins. *Reprint from Gürtler et al. 2013 [204] with permission from Elsevier, copyright © 2013.*

The second group of nucleic acid binding PRRs is formed by the cytosolic DNA and RNA sensors. In contrast to most TLRs, they are expressed in both immune and non-immune cells. Sensing of cytosolic RNA, derived from an incoming viral genome or created during viral replication, is mainly performed by members of the RIG-I (retinoic acid inducible gene-I)-like receptor (RLR) family. RIG-I preferentially binds to short dsRNA molecules and ssRNA molecules with an exposed 5'-triphosphate group. The latter are produced by many viruses and allows discrimination from self-ssRNA entities with eg. a capped (mRNA) or cleaved (tRNA) 5' end. MDA5 (melanoma differentiation–associated gene 5) is another member of the RLR family and is thought to mainly recognize long dsRNA. RNA-binding of both RIG-I and MDA5 leads to interaction with the mitochondrium-associated protein MAVS (mitochondrial antiviral signaling protein), which results in aggregation of MAVS proteins and eventually induces activation of IRF3/7 and NF-κB, through respectively TBK1 and IKKβ (IκB kinase β). A third member of the RLR family, LGP2 (laboratory of genetics and physiology 2), can bind to RNA but lacks the N-terminal domains required for MAVS interaction and would mainly serve as a modulator of RIG-I and MDA5 sensing [203, 205].

Cytosolic DNA on the other hand, has been long known to trigger innate immune responses, but the PRRs involved are only recently being identified. An important breakthrough in the field was the discovery of the STING (stimulator of IFN genes) protein as a crucial signaling molecule in the innate response to both transfected DNA and DNA pathogens. STING is a transmembrane protein predominantly found in the ER and induces TBK1-mediated activation of IRF3 as well as NF-κB activation upon stimulation of cells with DNA [206-211]. How cytosolic DNA can trigger the STING-TBK1-IRF3 pathway is still a topic of intense research and many different sensing mechanisms have been proposed [204, 205, 212]. STING can directly bind to cyclic dinucleotides, which are often produced during bacterial replication cycles. However, sensing of actual DNA fragments is thought to occur via PRRs upstream of STING. The cGAS (cyclic GAMP synthase), IFI16 (IFN-Y-inducible protein 16) and DDX41 (DEAD box polypeptide 41) proteins were all shown to bind to cytosolic DNA and to be required for innate immune responses to transfected DNA and infection with DNA viruses in a STING dependent manner [213-215]. For cGAS, it's known that its activation by DNA leads to production of cyclic GMP-AMP (cGAMP) from ATP and GTP. cGAMP in turn functions as a second messenger to activate STING [213, 216]. IFI16 and DDX41 on the other hand co-localize with STING upon DNA stimulation [214, 215], but how this leads to activation of STING is still unclear. Other DNA sensors with a less obvious STING-dependence have also been described. The DAI (DNA-dependent activator of IRFs) protein mediates IFN-I responses to DNA in some specific cell types. RNA polymerase III was shown to transcribe transfected poly(dA-dT) dsDNA into dsRNA, which would in turn serve as a ligand for RIG-I mediated sensing [212, 217, 218]. Finally, two members of the DNA-damage response pathways, the DNA-PKcs/Ku70/Ku80 complex and Mre11 (meiotic recombination 11 homolog A), were also suggested as potential DNA PRRs [219, 220]. Given the recent

discovery of most cytosolic DNA sensors, it remains largely unclear if these proteins have a different preferential affinity for specific DNA ligands or if they operate in different cell types or different cellular compartments. It is also possible that the different DNA receptors cooperate during viral infections to initiate an optimal IFN-I response [212, 221, 222].

2.1.2 Biological effect of type I interferon

Once produced and secreted, all type I interferons exert their effects by binding to a transmembrane IFN-α/β receptor (IFNAR), which consists of an IFNAR1 and IFNAR2 subunit (figure 2.1). In the canonical IFN-I signaling pathway, engagement of the receptor results in activation of the receptor-associated tyrosine kinases, JAK1 (Janus kinase 1) and TYK2 (Tyrosine kinase 2), and subsequent phosphorylation of the receptor. This leads to recruitment and phosphorylation of STAT1 (signal transducer and activator of transcription 1) and STAT2. These molecules will in turn dimerize and translocate to the nucleus where they subsequently recruit IRF9 and form a heterotrimeric complex called ISGF3 (IFN-stimulated gene factor 3). The ISGF3 transcription factor eventually activates transcription of ISGs by binding to IFN-stimulated response elements (ISRE) in the promoter region of these genes [223]. However, signaling of IFN-I extends beyond this primary pathway and ultimately affects a complex network of signaling cascades and regulatory mechanisms. In addition to activation of ISGF3, IFNAR stimulation also induces formation of STAT1 homo-dimers, which activate expression of ISGs that contain gamma activating sequence (GAS) promoter elements and often have a more pro-inflammatory function. Other types of STATs and other signaling pathways, such as MAPK pathways, can also be activated by IFNAR engagement depending on the cell type. Furthermore, different types of IFN-I induce different downstream responses. This probably depends on a different affinity and interaction pattern with the IFNAR subunits. Finally, IFN-I responses are strictly regulated by both autologous ISG-mediated feedback mechanisms as well as heterologous signals generated by other cytokines or microbial factors. These mechanisms altogether regulate the nature and the number of ISGs that are expressed and thereby determine the biological outcome of the IFN-I response [224, 225].

By inducing different ISGs, type I IFNs can exert three broad types of effects on the cells: direct antiviral, anti-proliferative and immune modulatory. The antiviral effects were identified first [191] and are currently best characterized. Several ISGs are known to directly target specific stages of the viral life cycle [195]. Although these different ISGs have different viral specificities and antiviral potentcies, the collective action of multiple ISGs renders the cell in a state that is incompatible with viral replication, the so-called "antiviral state". The importance of the IFN-I antiviral activity is illustrated by the increased susceptibility of IFNAR deficient mice to infection with a broad range of viruses [226]. Among the best studied ISGs are those with a potent and broad antiviral activity, such as PKR (protein

kinase R), OAS (2'–5' oligoadenylate synthetase) proteins and Mx1 (myxovirus resistance protein 1). PKR and OAS proteins both require additional activation by binding of dsRNA. PKR inhibits translation of (viral) RNA by phosphorylating EIF2α (eukaryotic translational initiation factor 2 EIF2α), while OAS proteins synthezise 2'- to 5'-linked oligoadenylates which in turn activate RNAseL to induce cleavage of (viral) RNA. Mx1 is a dynamin-like GTPase and binds to the nucleocapsid of viral particles to trap them in the cell, thereby restricting viral replication [227]. Recently, the Mx1 paralog, Mx2, was also shown to possess potent antiviral activity against primate immunodeficiency viruses, including HIV-1 [228-230]. Mx2 is thought to target the viral capsid proteins and prevent viral integration by a still poorly understood mechanism that affects uncoating [231], nuclear import [228, 229, 232] and/or additional post-nuclear entry steps [230, 232]. In addition, most of the well-characterized host HIV-1 restriction factors, such as APOBEC3G, tetherin, SAMHD1, are also induced by IFN-I [233]. Since IFN-I treatment can enhance the expression of up to 1000 different genes in a cell, many more ISGs with specific antiviral effects probably remain to be identified [234].

The anti-proliferative activity of IFN-I formed the basis for its first application in the clinic, as an anti-tumor agent. These effects result from regulation of multiple pro-apoptotic genes and genes involved in cell cycle control by IFN-I. Several mechanisms for IFN-I-induced cell cycle arrest have been proposed, including the induction of cyclin-dependent kinase inhibitors (CKI), downregulation of the proto-oncogene c-myc and suppression of the growth-promoting transcription factor EF2. Induction of apoptosis or increasing the sensitivity to apoptotic stimuli of cells is mediated through upregulation of pro-apoptotic proteins by IFN-I such as Fas, TRAIL, caspases and Bcl-2 family members [235]. By inducing death of infected cells or prohibiting their proliferation, this IFN-I activity further contributes to limiting the viral spread. In this regard it has to be noted that the effect on immune cells is more complex and IFN-I can both counteract or stimulate their survival and proliferation, depending on the context of IFN-I signaling [236] (see below).

The immune modulatory effects of IFN-I are mainly aimed at further activating innate and adaptive immune responses. IFN-I highly enhances the function and proliferation of NK cells, the differentiation and function of DCs and the function of macrophages. The stimulation of professional antigen-presenting cells (APCs), especially DCs, will in turn enhance activation of adaptive immune cells. In addition, IFN-I regulates cytokine production of APCs and induces secretion of different chemokines, which will further affect the recruitment, activation and function of other immune cells [237]. IFN-I can also target adaptive immune cells directly. Antibody responses by B cells, including plasma cell formation and isotype switching, are stimulated by IFNAR signaling on these cells [238-240]. IFN-I was also shown to promote the development of type 1 immune responses, eg.

differentiation of naïve CD4^{+} T cells into Th1 cells [241, 242] and activation of CD8+ T cells [243, 244].

However, in conditions of sustained IFN-I expression such as chronic viral infections or certain auto-immune diseases, pro-longed IFN-I signaling may lead to hyper-immune activation and exhaustion of immune cells. Recent studies also suggest that IFN-I can adopt immune suppressive functions during chronic infections, possibly to limit host toxicity, and thereby impede antiviral T cell functions. As such, IFN-I could enhance both persistence and pathogenesis of chronic viral infections [245-247].

2.2 Tools to evaluate *in vitro* type I IFN responses

2.2.1 Detection and quantification of IFN-I responses

Similar to most cytokines, type I IFN proteins are extremely potent molecules with biological activity at femtomolar concentration [248]. As such, the sensitivity of an assay is an important factor to consider when selecting methods to detect and quantify IFN-I production. Since IFN-I proteins are secreted by the cell, levels of production can be readily assessed in the supernatant of the cultured cells. This is commonly done by direct protein measurement using sensitive enzyme-linked immunosorbent assays (ELISA) for IFN-β or specific or multiple IFN-α subtypes. Prior to development of such immunoassays, IFN-I activity in biological samples was typically assessed by evaluating the level of interference with replication of a virus (eg. Vesicular Stomatitis Virus (VSV)) in an IFN-I sensitive cell line, so-called antiviral assays [249, 250]. Although these assays are quite tedious, they offer the advantage of specifically measuring biologically active IFN-I. Less labour-intensive IFN-I "bioassays" have more recently become available, by introduction of reporter genes under control of an ISG promoter, into highly IFN-I sensitive cell lines (Figure 2.3). These assays are usually much cheaper compared to ELISA and sometimes even offer higher detection sensitivities [250-252]. Alternatively, very sensitive detection of IFN-I induction can be achieved by measuring IFN-I mRNA transcript abundance in a cell population by real-time quantitative PCR (qPCR). For IFN-α, primers are available targeting regions either specific to or conserved among different subtypes [253-255].

In addition to quantifying IFN-I levels, biological IFN-I activity can be assessed directly in the experimental system by measuring induction of ISGs at protein or mRNA level. Microarray studies indicate simultaneous induction of hundrerds of different genes by IFN-I, usually varying among different cell types and IFN-I (sub)types [193, 234, 256]. Several of these studies have recently been collected in an online database (http://interferome.its.monash.edu.au/interferome/) that allows ISG search by questioning for experimental parameters such as cell type, IFN-I concentration and subtype, treatment

time.... [194, 257]. As such, it can present a helpfull tool to select appropriate ISGs for IFN-I response evaluation in your system.

Figure 2.3. HL116 reporter cell line to measure biological IFN-I activity. HL116 cells were generated by stable introduction of a construct carrying the luciferase gene under control of the IFN-I inducible 6-16 promoter into HT1080 cells [252]. IFN-I activity can be quantified by addition of the IFN-I containing samples to cultured HL116 cells. Binding of IFN-I to IFNAR will subsequently activate a JAK-STAT signaling pathway, resulting in activation of the 6-16 promoter and luciferase expression. The latter can be easily quantified by measuring light emission upon addition of luciferin. *Adapted from Sadler and Williams 2008* [227] *with permission from Macmillan Publishers Ltd: Nature Reviews Immunology, copyright © 2008*
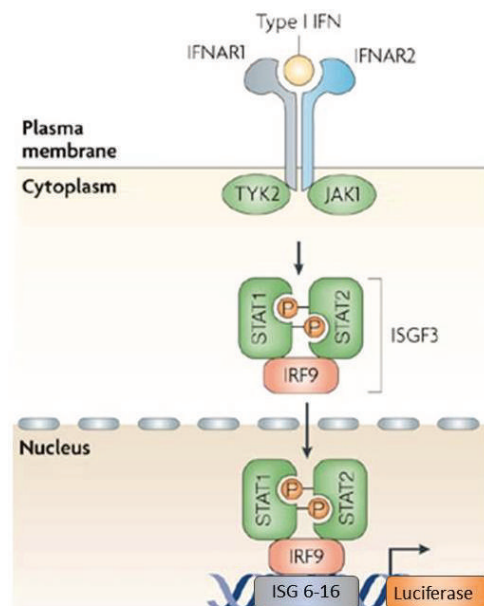
2.2.2 Investigating pathways of IFN-I induction

As described above, many different PRRs are equipped to initiate signaling cascades that lead to IFN-I transcription. Identification of the PRRs and downstream pathways triggered by specific pathogens or PAMPs, is a topic of great research interest in the field of infectious and autoimmune diseases. Similar to most pathways, signal transduction from PRRs is mediated by a series of sequential protein phosphorylation events [258, 259]. As such, analysis of protein phosphorylation status upon PAMP stimulation, eg. by using phosphorylation-state specific antibodies , can provide a first indication on the nature of the activated pathway. However, it has to be noted that most signaling molecules are commonly used by multiple PRRs (Figure 2.2) and therefore this type of analysis will usually not allow to pinpoint a specific PRR. For certain signaling molecules and PRRs, inhibitory molecules have become available, in the form of either small molecule inhbitors [260-262], inhibitory peptides [263] or oligonucleotide-based antagonists [264]. Especially among the TLR family of PRRs, several IFN-I inducing receptors can be efficiently blocked with these molecules [260, 262, 264]. However, for most RLRs and the more recently discovered DNA-sensing PRRs, specific inhibitors are still lacking.

As such, researchers often resort to manipulating the expression of PRRs, in order to evaluate specific PRR involvement in their experimental system. "RNA interference (RNAi)" is the most commonly used technique in this regard, mainly due to its established use and the

wide availability of validated reagents to target almost any one of the known human genes. RNAi allows for knock-down of gene expression by taking advantage of conserved cellular machinery that can process small non-coding RNAs and use them to target complementary RNA for degradation or translational repression (Figure 2.4). Such small RNAs are naturally generated from exogenous long dsRNA molecules or from endogeneously expressed precursor RNAs (eg. microRNAs (miRNA), endogenous small interfering RNAs and piwi-interacting RNAs) and serve important functions in anti-viral defense and regulation of many cellular processes [265-267]. Artificial knock-down of a gene of interest can be achieved by introducing short double-stranded RNA molecules, directed against the target mRNA, into cells. Two main approaches are commonly used in this regard: 1) direct delivery of synthetic dsRNA molecules of 20-25 nucleotides (small interfering RNA (siRNA)) by eg. transfection or 2) delivery of a construct expressing short hairpin RNA (shRNA). The latter mimics a precursor (pre)-miRNA structure and will be processed upon expression by the cellular protein Dicer to yield a mature siRNA duplex. siRNA is subsequently loaded into an RNA-induced silencing complex (RISC) in which one strand of siRNA is ejected (the "passenger strand"), while the other strand will serve as a "guide" to target the RISC complex to complementary mRNA. Hydrolysis of the target mRNA is catalysed by Ago2, the "slicer" component of RISC [268, 269]. A major advantage of shRNA is the ability to use integrating lentiviral vectors for cellular delivery, which does not only allow their introduction into cell types that are difficult to transfect (eg. primary cells) but also ensures stable long-term expression of the RNAi-mediating molecule. Lentiviral shRNA libraries covering the majority of the human genome, such as the Broad Institute TRC Library [270], are now commercially available and allow fast implementation of RNAi to research almost any gene of interest.
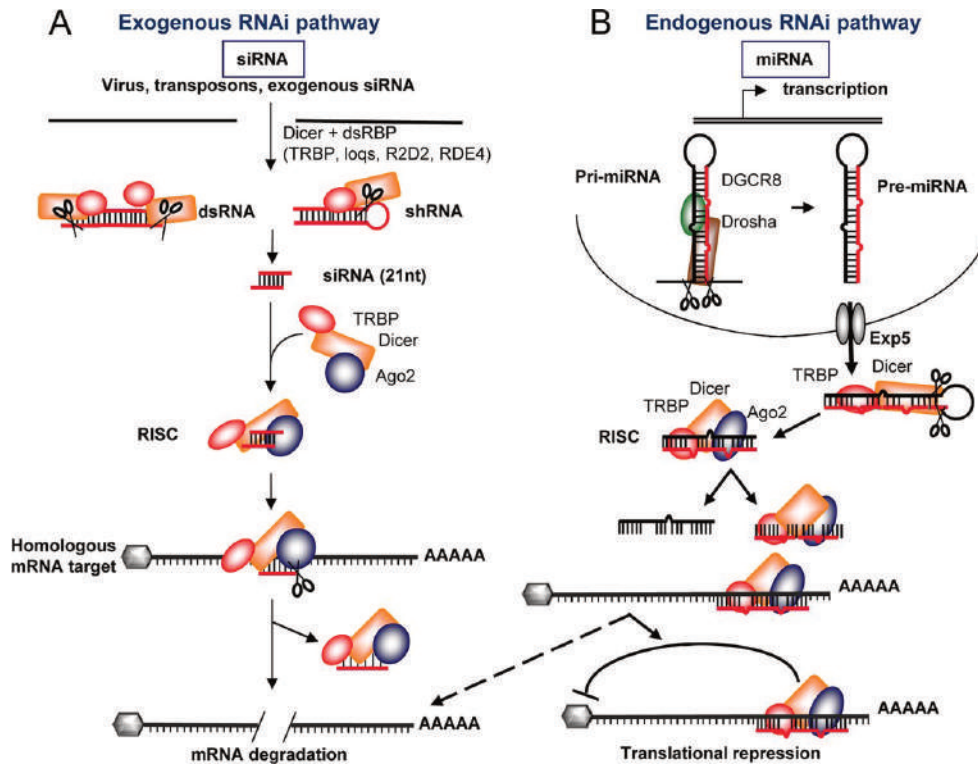
Figure 2.4. RNA interference pathways. (A) Exogenous RNAi pathway mediated by siRNA and shRNA. dsRNA molecules are bound by Dicer and processed to form 21- to 23-nucleotide siRNAs. Alternatively, synthetic siRNA duplexes can be artificially introduced in the cell or generated from shRNA through the pre-miRNA processing pathway (see B). The Dicer complex then recruits Ago2 and forms the RISC. After strand separation, the complex hybridizes to the complementary sequence in an mRNA and mediates its cleavage. (B) Endogenous pathway mediated by miRNAs. Primary mRNAs (pri-miRNAs) are synthesized in the nucleus and processed by Drosha to form the precursor miRNA (pre-miRNA). Pre-miRNAs are exported to the cytoplasm via exportin5 (Exp5), where they are bound and processed by Dicer to form mature miRNAs. Similar to siRNA, miRNAs are loaded into RISC, but they target an imperfectly matched mRNA. This usually leads to repression of translation of this mRNA, although in some cases, cleavage of the target mRNA may also occur (dotted arrow). *Reprint from Daniels and Gatignol 2012 [271] with permission from the American Society for Microbiology, copyright © 2012.*

2.3 Type I IFN responses during HIV-1 infection

Elevated levels of IFN-I were already described in AIDS patients before the viral etiology of the disease was demonstrated [272]. They can be detected at various stages of the infection course. During acute infection, a rapid but transient increase in IFN-α coincides with the appearance of HIV-1 RNA in the plasma [273]. Levels of IFN-α again accumulate in the plasma during progression of the disease and are particularly elevated in patients with advanced disease [274, 275]. However, signatures of IFN-I activity, e.g. increase in ISG expression, are clearly detectable at earlier stages in peripheral blood of patients [276-278], suggesting that release of IFN-I primarily occurs in lymphoid tissue [279, 280]. Several lines of evidence indicate that pDCs are important producers of IFN-I during HIV infection. They

secrete high levels of IFN-I upon exposure to HIV-1 *in vitro* [281] and depletion of pDCs in a humanized mouse model of HIV-1 infection highly decreased IFN-I levels [282]. In rhesus macaques, recruitment of pDCs to the mucosal site of SIV inoculation was observed within one day after exposure [283]. However, the frequency of circulating pDCs declines during the chronic phase of HIV-1 infection [78, 284-286]. Furthermore, pDCs seem to have a decreased ability to produce IFN-I during chronic infection [78, 287, 288]. A recent study suggested that pDCs may only contribute to a transient IFN-α response in the acute phase of SIV infection [289]. Furthermore, IFN-α did not co-localize with pDCs in the spleens of chronically HIV-1 infected patients [285]. Therefore, although pDCs are likely the main source of IFN-I during acute HIV-1 infection, other cell types may contribute to IFN-I levels in the chronic phase of infection [290].

The role of IFN-I responses during HIV and SIV infection is highly ambiguous (Figure 2.3). Early responses may prevent establishment of infection or limit viral spread through upregulation of viral restriction factors (ISGs), apoptosis of infected cells or stimulation of anti-viral innate and adaptive immune responses [69]. The protective potential of early IFN-I is for example illustrated by the protective effect of high APOBEC3G levels in HIV-exposed seronegative individuals [291] and by the higher resistance of HIV-1 founder viruses to *in vitro* IFN-I [292]. During acute SIV infection, high IFN-α levels are also associated with lower viral loads [293]. Furthermore, inhibition of IFN-I signaling in rhesus macaques prior to SIV infection resulted in higher viral loads and accelerated disease progression [294].

However, these same IFN-I responses might in the long-term contribute to chronic hyper-immune activation, depletion or dysfunction of lymphocytes and thereby eventually enhance disease progression [69]. In contrast to acute infection, higher IFN-α levels indeed correlate with higher viral loads during chronic infection in both SIV-infected rhesus macaques and HIV-1 infected humans [279, 293]. Additional evidence for a link between IFN-I and viral pathogenesis comes from studies of pathogenic versus non-pathogenic models of SIV infection, in which a marked difference in IFN-I activity is observed: IFN-I responses are completely down-regulated after acute infection in non-pathogenic models, while they remain elevated throughout the pathogenic infection course [295-297]. Furthermore, a recent study showed that prolonged administration of IFN-α resulted in an increase of cell-associated viral load and CD4+ T cell loss in SIV-infected rhesus macaques [294]. Sustained IFN-I responses might enhance HIV pathogenesis in different ways. Several studies indicate a link between IFN-α activity and T cell activation, eg. IFN-α treatment enhances expression of the activation marker CD38+ on T cells of HCV (hepatitis C virus)- or HIV-infected patients and CD38 expression correlates with IFN-I activity in HIV-1 patients [278, 279, 298]. IFN-I signaling is also associated with increased expression of pro-apoptotic TRAIL and Bak during HIV-1 infection and could thereby contribute to CD4+ T cell depletion [280, 293, 299-301]. Chronic IFN-I signaling during HIV/SIV infection may also cause desensitization to

IFN-I and T cell dysfunction [278, 294, 302], which would further impede the anti-viral responses. Therefore, IFN-I seems to act as a double-edged sword during HIV-1 infection, of which the protective effects during the early stages of infection are eventually overshadowed by disease-aggravating effects during the chronic phase of infection.
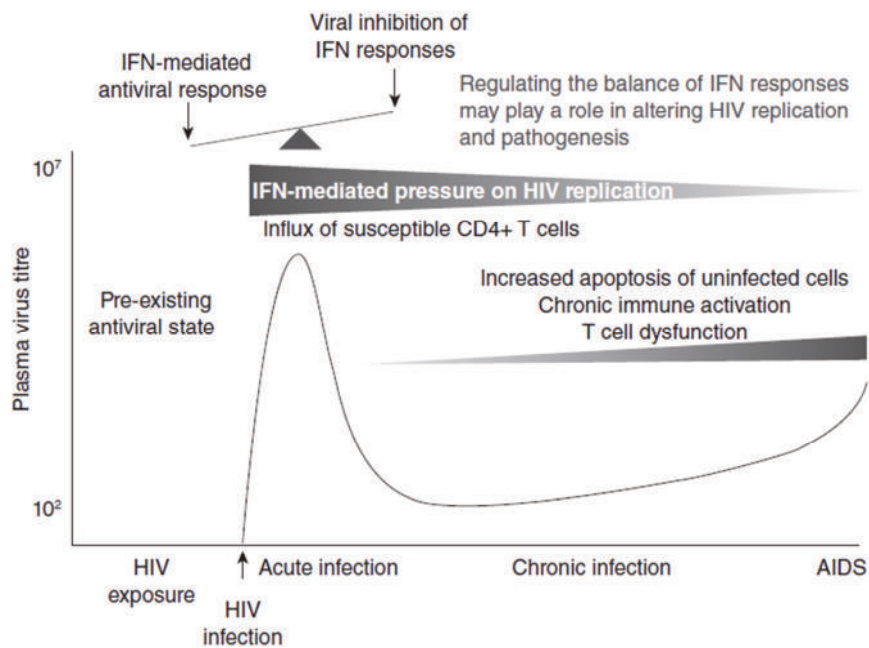

Figure 2.5 Role of type I IFN responses during HIV-1 infection. IFN-I responses have a dual role during HIV-1 infection. In the early stages of infection protective effects probably dominate through induction of antiviral restriction factors and stimulation of adaptive immune responses. Once infection is established, protective and damaging effects are often seen in parallel. Prolonged exposure to IFN-I during chronic infection is likely to fuel pathogenesis by enhacing chronic immune activation and this eventualy overshadows the IFN-I protective effects. *Adapted from Sivro et al. 2014 [69] with permission from Permanyer Publications, copyright © 2014.*

2.4 Innate immune recognition of HIV-1: to sense or not to sense?

The elevated production of IFN-I in HIV-1 patients implies that HIV-1 is recognized or "sensed" by PRRs during infection *in vivo*. In the course of an HIV-1 replication cycle, several products that could serve as a potential PAMP are created. "Foreign" structures such as ssDNA and RNA:DNA hybrids are produced during HIV reverse transcription and dsRNA structures are present in the entering or newly produced HIV ssRNA genome. Molecular structures shared by host and HIV could also be recognized as "foreign" because of to their aberrant location (eg. HIV ssRNA in the endosomes or dsDNA in the cytosol). Since different HIV target cells express a different range of PRRs and since HIV has a varying potential to complete its lifecycle in these cells, sensing of HIV replication products is a cell type specific

phenomenon. Analysis of IFN-I induction by HIV-1 *in vitro* has revealed multiple possible sensing mechanisms. Some of them are successfully triggered by HIV-1 in certain cell types, while other mechanisms seem to be avoided due to viral and host evasion mechanisms.

2.4.1 Sensing of HIV RNA

pDCs produce high levels of IFN-I upon *in vitro* exposure to HIV-1 [281]. They most likely sense HIV through recognition of endosomal HIV ssRNA by TLR7 [303, 304]. Although pDCs are only minimally productively infected by HIV-1 [305], binding of HIV-1 Env to the CD4 receptor is thought to induce endocytosis of the virus and exposure of the HIV genome to endosomal TLRs after endosome acidification [303, 306]. IFN-I is induced by both infectious and non-infectious free viral particles and to a larger extent by exposure of pDCs to HIV-infected cells [304, 306, 307]. It was suggested that the latter involves sensing by both TLR7 as well as a yet unidentified cytosolic PRR [304]. In monocytes, HIV-1 endocytosis might similarly result in sensing of ssRNA by endosomal TLR8, which leads to production of pro-inflammatory cytokines but not IFN-I [308, 309], due to lack of constitutive IRF7 expression in these cells.

Transfection of genomic HIV-1 RNA can also induce an IFN-I response in both peripheral blood mononuclear cells (PBMCs) and cell lines, indicating that cytosolic HIV RNA can be sensed. The RIG-I receptor was identified as responsible PRR [310, 311]. However, this type of sensing is not present during regular HIV-1 infection and one study suggested that sensing is counteracted through degradation of RIG-I by the HIV-1 protease [310].

2.4.2 Sensing of HIV DNA

Similarly to HIV RNA, transfection of HIV-1 ssDNA and dsDNA could trigger IFN-I production in primary macrophages and the cytolosolic DNA sensors IFI16, cGAS and DDX41 were shown to contribute to the response [222]. Furthermore, HIV-1 replication induces IFN-I responses in the human monocytic cell line THP1 prior to viral integration and this was prevented by knock-down of cGAS or IFI16 [222, 312]. Therefore, different DNA sensors have the potential to recognize HIV reverse transcription (RT) products. However, during infection of primary HIV target cells, the occurrence of HIV DNA sensing is more controversial and both successful sensing as well as specific mechanisms of immune evasion have been described in different target cells. This is discussed in more detail below.

In primary macrophages, evasion of DNA sensing was suggested through recruitment of cellular host factors to the entering viral capsid, such as cyclophilin A (CypA) and Cleavage and Polyadenylation Specificity Factor subunit 6 (CPSFE6). These factors are thought to "shield" the replicating virus from the innate sensors, since IFN-I could be artificially induced by depleting these proteins or using HIV-1 interaction mutants. In these conditions,

HIV DNA was sensed prior to integration by cytosolic DNA sensors, including cGAS [313]. In contrast, another group showed that knock-down of IFI16 enhances HIV-1 replication in these macrophages, which suggests that triggering of IFI16-mediated antiviral activity can occur during regular HIV-1 infection, although direct induction of IFN-I by HIV-1 was not demonstrated [222]. HIV may also exploit the host factors to degrade excess HIV DNA and prevent sensing. Yan et al. showed that knock-down of TREX1 (three prime repair exonuclease 1) in primary macrophages and CD4+ T cells resulted in accumulation of cytosolic HIV DNA and IFN-I induction [314]. Later studies suggested that both cGAS and IFI16 mediate DNA sensing in TREX1 deficient cells [222, 312]. However, it remains unclear if the different HIV RT products are all efficiently degraded by TREX1, since HIV DNA is able to induce IFN-I despite the presence of TREX1 in both macrophages (see above) and quiescent CD4+ T cells (see below) [313, 315].

Successful sensing of HIV DNA has been described in lymphoid tissue derived quiescent CD4+ T cells. HIV-1 infection of these cells was shown to induce IFN-β as well as cell death through inflammasome activation and pyroptosis [316, 317]. Entry of HIV-1 in these resting cells does not result in productive infection, but leads to cytosolic accumulation of incomplete reverse transcription products derived from multiple abortive infection events [316]. The same group recently demonstrated that these products are recognized by the DNA sensor IFI16, which triggers both IFN-I production and caspase-1 activation through different pathways [315]. Inhibition of productive infection in quiescent CD4+ T cells is at least in part due to restriction of reverse transcription by SAMHD1. The latter is counteracted by viral Vpx, which is encoded by HIV-2 and certain SIVs, but not HIV-1 [45, 174, 175]. Interestingly, although the lack of Vpx seems to enhance innate immune responses in quiescent CD4+ T cells, it protects HIV-1 from sensing in myeloid DCs. Indeed, when resistance to productive HIV-1 infection in MDDCs is artificially circumvented by co-introduction of Vpx, sensing of HIV-1 DNA and IFN-I induction occurs through cGAS [181, 312]. Surprisingly, IFN-I induction was only observed after integration of the virus, because it required interaction of newly expressed HIV-1 capsid with the host factor cyclophilin A (CypA) [181, 182]. This interaction was suggested to relieve the "shielding" and unmask remaining cytosolic HIV DNA for sensing [181, 318, 319]. A similar requirement of both Vpx and viral integration was observed when MDDCs were infected through viral cell-to-cell transmission from HIV-1 infected T cells. Interestingly, coculture with infected T cells resulted in higher levels of IFN-I production compared to infection of MDDCs with free viral particles [183]. This is in line with the higher multiplicity of infection that is reached during intercellular transmission of virus, a mode of infection that likely predominates during viral spread *in vivo* [320, 321].

2.5 Regulation by HIV-1 accessory proteins

VPU

Vpu was shown to counteract IFN-I induction by HIV-1 in different cell types [135, 322], however the mechanism behind this is not clear. Doehle et al. reported that Vpu induces degradation of IRF3 and thereby prevents transcription of IFN-I after sensing [138, 322]. However, such degradation was not observed by others [135, 323]. A recent study suggested that Vpu rather induces cleavage of IRF3 and that resulting IRF3 fragments act in a dominant-negative fashion [324]. Alternatively, Vpu might suppress IFN-I induction by preventing NF-κB activation [135, 323]. Neil et al. showed that the restriction factor tetherin can initiate NF-κB signaling when tetherin molecules are clustered by restricted viral particles. Counteraction of tetherin by Vpu would prevent such signaling [135]. Other studies indicate that Vpu also inhibits tetherin-independent activation of NF-κB [136, 137, 323, 325, 326] by interfering more downstream in the NF-κB signaling pathway [137, 325].

VIF

Similar to Vpu, the Vif protein was also suggested to induce degradation of IRF3 and was able to counteract IFN-I responses induced by other viruses [327]. However, this was not confirmed by others [328] and a direct effect of Vif on HIV-1 induced IFN-I production has not been demonstrated yet.

VPR

The role of Vpr in regulating innate immune responses to HIV-1 seems more complex and both inhibiting and stimulatory effects have been described. *Vpr*-deleted HIV-1 viruses were shown to induce higher levels of IFN-I in either primary MDDCs [329], primary macrophages [330] or HeLa cells [168], suggesting an inhibiting effect. In HeLa cells, counteraction of IFN-I induction was attributed to activation of the structure-specific endonuclease (SSE) regulator SLX4 complex by Vpr. Similar to TREX1, this complex may process excess HIV DNA and prevent its sensing [168]. Vpr was also suggested to induce degradation or cleavage of IRF3 [324, 328]. In contrast, another study showed that Vpr can stimulate expression of ISGs in primary macrophages [331]. Furthermore, different groups have demonstrated highly variable effects of Vpr on NF-κB signaling, resulting in either suppression [332, 333] or stimulation of NF-κB activation [334, 335], which might in turn affect IFN-I induction.

2.6 Interplay between IFN-I responses and HIV-1 replication

Despite several proposed evasion mechanisms, innate sensing occurs during HIV-1 infection *in vivo* and the virus has to replicate in face of elevated IFN-I levels. The anti-HIV activity of IFN-I is in part counteracted by targeting of IFN-I induced HIV restriction factors by the virus, eg. counteraction of tetherin by Vpu and APOBEC3G by Vif . Furthermore, the virus

may have evolved to use the innate immune responses to its own advantage. For example, HIV-1 Tat was shown to inhibit activation of the antiviral IFN-I induced kinase PKR. This interaction however leads to phosphorylation of Tat, which enhances its capacity to transactivate the HIV-1 LTR [336, 337]. The interferon regulatory factor 1 (IRF1), also induced by IFN-I, can bind to the HIV-1 LTR and stimulate transcription of viral genes both in the absence of Tat or in cooperation with Tat [338]. Similarly, activated NF-κB is an essential inducer of LTR activity [339]. Finally, innate immune responses might indirectly enhance HIV-1 replication by promoting recruitment and activation of HIV-1 target cells or in the long-term by causing immune exhaustion and dysfunction during chronic responses [68, 340].

2.7 Regulating IFN-I responses in HIV-1 patients: treatment potential?

Soon after the start of the AIDS epidemic, IFN-α was one of the first drugs tested for the treatment of HIV patients with AIDS-related Kaposi's sarcoma, based on its well-known antiviral and anti-proliferative properties. Although reduction in circulating HIV-1 p24 levels was observed in several studies [341], the interest in IFN-I for treatment of HIV-1 infection highly decreased when more tolerable and directly acting antiretroviral drugs, like AZT (azidothymidine), became available. Following these early studies, the therapeutic potential of IFN-I has mostly been investigated in combination with AZT or cART or in HIV/HCV co-infected patients. Treatment with pegylated IFN-α during HIV/HCV co-infection is known to decrease HIV viral loads in addition to HCV loads [342-345]. However, the added value of IFN-α administration to cART is less clear : while some studies report beneficial effects on viral load and anti-HIV immune responses [346-349], others have observed only short-term effects [350] or no effect [351, 352]. Interestingly, a recent study by Azzoni *et al.* showed that a subset of patients receiving pegylated IFN-α after cART interruption were able to control viral replication up to 24 weeks and showed decreased levels of integrated HIV-1 DNA. This suggests that IFN-α treatment may have potential to reduce the viral reservoir [353]. However, given the limited sample size and treatment period in this study, larger long-term trials are required to further investigate this observation.

The rationale behind IFN-I therapy is to take advantage of the antiviral effects of IFN-I. However, since IFN-I has a dual role in HIV-1 infection and likely contributes to the hyper-immune activation and disease progression, strategies to counteract IFN-I activity have also been evaluated. Vaccination against IFN-α has been tested in two related studies and was associated with lower rates of disease progression in HIV-1 patients that responded to the vaccine [354, 355]. Another approach was the use of chloroquine, which inhibits endosomal acidification and thereby IFN-α production by pDCs in response to HIV-1. In two separate studies, chloroquine administration significantly reduced T-cell activation as well as other markers of chronic immune activation in HIV patients [356, 357]. However, a third study, in

patients at earlier stages of infection, observed no effect on T-cell activation and even a greater decline in CD4$^+$ T cell count and increase in viral replication [358]. These opposing effects might reflect the different role of IFN-I at different stages of the infection: antiviral responses are important to limit viral spread at early stages, while immune activating effects of IFN-I outweigh the antiviral effects at later stages.

The mixed results obtained by either stimulating or inhibiting IFN-I responses in HIV-1 treatment indicate that patients may benefit from different approaches at different stages of disease [68, 69]. It furthermore highlights that a better understanding of the cellular sources and IFN-I action mechanisms at different stages of disease is required.

Chapter I-3. Quantification of retroviruses and retroviral vectors

In Chapter III-1, an optimized assay for rapid quantification of retroviruses is presented and evaluated. As introduction to the research data, the next section will give an overview of commonly applied methods for retroviral quantification and their application.

3.1 Use of retroviral quantification for clinical and research purposes

Detection and quantification of retroviruses is performed in a variety of settings and can serve different purposes. First, in a clinical setting, direct measurement of retroviral nucleic acids or proteins in plasma or peripheral blood mononuclear cells (PBMCs) is used for early detection of HIV infection (i.e. before seroconversion) or for diagnosis in seropositive infants [359-361]. Furthermore, levels of virus in the plasma need to be monitored after diagnosis in HIV-infected patients, in order to evaluate the efficacy of antiretroviral therapy and disease progression [360]. Similar to HIV, human T-cell lymphotropic virus (HTLV) levels can be measured for diagnostic and monitoring purposes in symptomatic HTLV-infected patients [362, 363]. Second, biological products intended for medical use often need to be screened for the presence of contaminating retroviruses. Material from human origin, such as blood and blood-derived products, can be specifically tested for human retroviruses (HIV, HTLV) [364]. Many other biologicals (eg. vaccines, monoclonal antibodies,...) are produced in living cells or supplemented with reagents derived from living systems. These are known sources of retroviral contamination and screening for a wide range of retroviruses is therefore recommended [365-367]. Third, quantification of retroviruses is also required when using these viruses for research or clinical purposes. This includes the field of retroviral research itself, but also applies to the widespread use of retroviral vectors in research and clinical trials for gene therapy. Retroviral vectors are genetically engineered retroviruses which are exploited to stably insert nucleic acid sequences in the cellular genome and thereby allow for long-term manipulation of gene expression. These vectors can be used to deliver new genes of interest into the target cells or alternatively, they may encode sequences that are able to disrupt the expression of specific genes via different methods (eg. shRNA (small hairpin RNA), ZFN (zinc-finger nucleases), CRISPR (clustered regularly interspaced short palindromic repeats,...)). Conventional retroviral vectors are derived from oncoretroviruses (eg. mouse leukemia virus (MLV) and can therefore only integrate in dividing cells. However, vectors based on lentiviruses, such as HIV and FIV, can efficiently transduce non-dividing cells and are therefore more suitable for use in primary cells and *in vivo* [368-372]. Quantification of retroviruses and retroviral vectors in these settings is performed for several reasons, eg. to perform quality control of retroviral productions, to standardize experiments when using different batches of virus or when comparing different types of viruses, to evaluate retroviral replication in an experimental setting or to obtain a fixed range of

retroviral copies per cell (multiplicity of infection or MOI) in both research and clinical settings.

Several different methods are available to quantify retroviruses. It is important to note that depending on the purpose of quantification, the preferred performance of the method may differ. While quantification for diagnostic or contamination screening purposes requires the highest possible level of sensitivity, methods to measure retroviral production *in vitro* may benefit more from a higher accuracy or broader linear range. Furthermore, quantification can be subtype-specific or alternatively require detection of a broad group of retroviruses. In the next paragraph, several commonly used methods are discussed with a focus on methods to quantify retroviruses/retroviral vectors *in vitro*, either for research or gene therapy purposes.

3.2 Methods for retroviral quantification

In general, methods for retroviral quantification can be divided into techniques that only measure functional viral particles (i.e. particles that are able to transduce or infect a specific cell type) and techniques that measure the total amount of "physical" viral particles, both functional and non-functional [373-375].

3.2.1 Methods for total quantification of retroviral particles

Assays to estimate total amounts of virus usually measure specific components associated with the retroviral particles, such as structural proteins, RNA or reverse transcriptase (RT) activity [374, 375]. HIV-1 and HIV-1 based lentiviral vectors are routinely quantified by using an ELISA directed against the p24 capsid protein. Although this method is straightforward and easily standardized, it suffers from several drawbacks. Inherent to the use of ELISA are the limited dynamic range of detection and the sometimes lower sensitivity compared to PCR based methods. Furthermore, it does not only detect virion-associated p24, but also free p24 derived from disintegrated viral particles or released by the virus-producing cells [369, 376-379]. Another frequently applied method involves quantification of viral RNA genomes. Here, viral RNA is isolated from the retrovirus-containing sample, transformed into cDNA and quantified by PCR [374]. Such assays can be optimized to reach very high sensitivities and are the gold standard for monitoring plasma viral load in HIV-infected patients [380]. A disadvantage is that they are dependent on specific primers targeting the viral/vector genome and accurate quantification of *in vitro* retroviral productions requires removal of plasmid DNA from sample [374, 375, 381]. These methods may also detect free, non-incorporated viral RNA, although the stability of free RNA is expected to be smaller than the one of viral proteins (eg. p24). A third type of methods is based on measuring RT activity in retroviral samples. Usually this is done by adding an RNA template and quantifying the amount of DNA that is synthesized by the endogenous

retrovirus associated RT [374]. The sensitivity of such assays has highly increased by performing PCR-based amplification of the newly synthesized DNA prior to detection, which are known as product-enhanced RT (PERT) assays [382-384]. DNA is subsequently quantified by using DNA gel electrophoresis [382, 385, 386], southern blot [383, 384, 387, 388] or qPCR [389-393]. The latter allows an improved accuracy of quantification and is less labor-intensive [388-390]. A major advantage of RT assays is that they detect most, if not all, types of retroviruses. As such, PERT assays are the first method of choice to demonstrate the absence of retroviral contamination in biologicals [365, 386, 390, 394, 395]. However, their use for determination of HIV viral loads [379, 396, 397] and for quantification of *in vitro* produced retroviruses [393] has also been suggested. Similar to p24 assays, RT-based assays also detect viral particles that do not contain viral RNA [398] and might detect free RT proteins. However, a better correlation with infectious particles has been suggested, possibly because these assays only quantify functional RT enzymes which may have a short half-life in culture [399, 400].
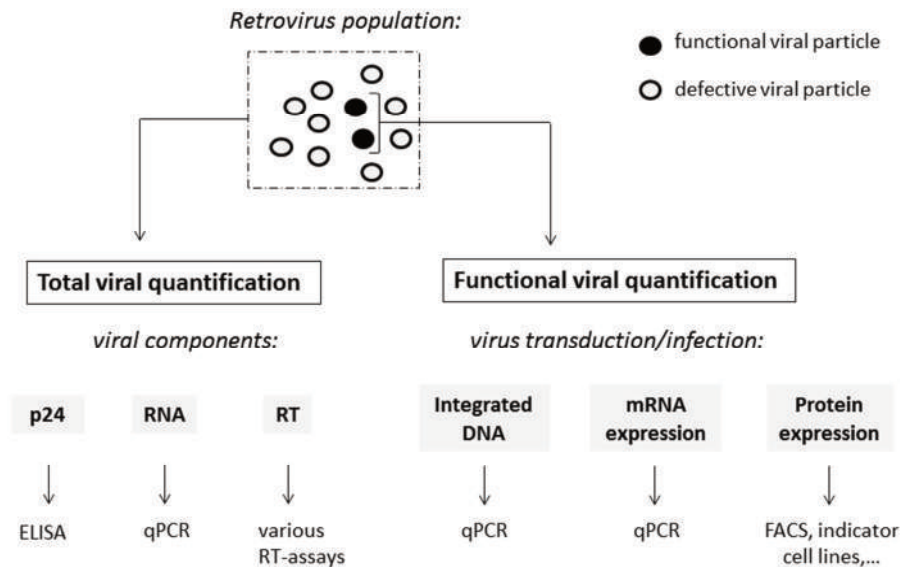

Figure 3.1. Overview of methods for retroviral quantification. Total quantification methods measure both functional and non-functional viral particles by quantifying viral components. Functional quantification methods require infection/transduction of target cells and measure post-integration events to quantify functional viral particles. RT: reverse transcriptase.

3.2.1 Methods for quantification of functional retroviral particles

Retroviral particles generated *in vitro* or *in vivo* often have intrinsic defects that render them incapable of infecting/transducing a target cells [401-403]. Depending on the methods used for viral production and quantification, functional to physical particle ratios of $1/10^2 - 1/10^7$ have been reported for *in vitro* produced retroviruses [373-375, 402, 404]. Therefore, if a

fixed amount of transduced/infected target cells is required for experimental purposes or an estimate of functional particles has to be made for other reasons, the use of functional quantification methods is recommended. These methods are more labor-intensive because they require transduction/infection of target cells and subsequent quantification of successful events [374, 375]. The latter can be done in several ways. Relatively fast assessment is possible if the virus/vector in question contains a reporter gene. Fluorescent proteins such as GFP (green fluorescent protein) or antibiotic resistance markers are commonly used in this regard [405]. For several retroviruses, indicator cell assays are also available, in which easy detectable proteins are produced upon successful infection or replication [406-410]. If these options are not applicable and other viral/vector proteins are difficult to quantify, measurement of transgene expression at mRNA level can be used to estimate functional infection/transduction levels [375, 411]. Alternatively, the number of integrated proviral copies per cell can be determined by qPCR. An inherent drawback of the latter is that not all integrated proviral genomes may lead to gene expression, due to integration in DNA regions with reduced transcriptional activity. In this regard, several groups have demonstrated that proviral titers largely overestimate titers based on protein expression [373, 411, 412]. A general critical point with functional quantification methods is that both efficiency of infection/transduction and of gene expression are highly dependent on the cell type used for titer determination. It is therefore important to keep in mind that the number of particles that are "functional" in the titration cell line will be different in the cell type used for experimental purposes and that titers estimated on different cell-types are difficult to compare.

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Chapter II :
Research objectives

Scope and research objectives

Type 1 interferons (IFN-I) have extremely potent antiviral effects and are a key part of the immune response against multiple viral pathogens. However, during chronic viral infections, such as HIV-1, the role of IFN-I is dual. While early IFN-I responses can counteract establishment of infection and early viral spread, prolonged exposure to IFN-1 is associated with hyper-immune activation and dysfunction, both hallmarks of pathogenic HIV-1 infection. Accordingly, targeted modulation of IFN-I responses during HIV-1 infection may have therapeutic potential. This first requires an in depth understanding of the cellular sources and mechanisms that could contribute to elevated IFN-I levels, as detected in HIV-1 patients. So far, very few cell-types have been shown to produce IFN-I during HIV-1 infection *in vitro*. As such, the origins of *in vivo* IFN-I, especially during the chronic phase of infection, remain incompletely understood.

Therefore, a <u>primary goal </u>of this thesis was to **evaluate if the main HIV target cells, activated CD4$^+$ T cells, are able to produce IFN-I during HIV-1 infection**. To this end, several assays were implemented to evaluate induction of both IFN-I and ISGs during *in vitro* infection of primary cells. This included an assessment of induction kinetics during viral replication and evaluation of both laboratory adapted and primary HIV-1 or HIV-2 strains. The potential antiviral effect of HIV-induced IFN-I on viral replication was also investigated.

It is known that several isolated HIV replication products have affinity for pattern-recognition receptors (PRRs). However only some of these sensing mechanisms have been shown to be activated during actual HIV-1 infection and this often occurs in a cell-type specific manner. Furthermore, strategies adopted by the virus to evade IFN-I induction have been suggested. A <u>second goal</u> was therefore to **investigate host and viral mechanisms that regulate HIV-induced IFN-I production in primary CD4$^+$ T cells**. In order to identify potential host innate immune sensors and signaling pathways that are triggered by HIV-1 in these cells, we performed shRNA-mediated knock-down of several host genes and evaluated the effect on IFN-I induction. We also assessed at which stage of the HIV-1 replication cycle IFN-I induction occurred, to obtain further information on the HIV-1 replication products that are required to trigger the IFN-I response. Finally, we evaluated the potential role of HIV-1 accessory proteins in this regard. These viral proteins are known to modify immune responses in order to optimize the viral replication environment. Two of them, Vpr and Vpu, have been previously implicated in regulation of IFN-I responses. We therefore assessed their involvement in IFN-I induction in primary CD4+ T cells. Several HIV-1 variants containing targeted mutations in these genes or encoding alternative HIV/SIV alleles were also evaluated, in order to obtain a preliminary insight in the underlying mechanism of regulation.

The mechanistic evaluation of IFN-I induction by HIV-1 required the use of a large set of modified HIV variants and the use of lentiviral vectors to perform shRNA knock-down of host proteins. This introduced the need for a method to evaluate lentiviral productions, to normalize infection experiments and quantify HIV replication. The current standard methods for *in vitro* retroviral quantification suffer from several drawbacks, including a limited dynamic range of detection, a narrow virus specificity, a high cost and labor-intensiveness. As such, they are not compatible with high-throughput production of different lentiviruses or lentiviral vectors. A <u>third goal</u> that emerged during this project was therefore **to optimize and evaluate a general method for retroviral quantification that is both fast, cheap as well as accurate**. Although qPCR-based PERT assays aimed at quantifying viral RT activity have not yet found their way to basic research environments, they can be easily modified to obtain these very qualities. We optimized such an assays for use with commercially available reagents, allowing an easier implementation and option for better standardization. The performance of this assay was evaluated for both HIV-1 and retroviral vectors and in comparison to the gold standard method, p24 ELISA. We also used the assay to assess the informative value of RT activity by comparing it to methods for functional lentiviral quantification.

Chapter III-1 :

Quantification of reverse transcriptase activity by real-time PCR as a fast and accurate method for titration of HIV, lenti- and retroviral vectors.

Quantification of reverse transcriptase activity by a real-time PCR-based assay as a fast and accurate method for titration of HIV and retroviral vectors

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Quantification of Reverse Transcriptase Activity by Real-Time PCR as a Fast and Accurate Method for Titration of HIV, Lenti- and Retroviral Vectors

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Abstract

Quantification of retroviruses in cell culture supernatants and other biological preparations is required in a diverse spectrum of laboratories and applications. Methods based on antigen detection, such as p24 for HIV, or on genome detection are virus specific and sometimes suffer from a limited dynamic range of detection. In contrast, measurement of reverse transcriptase (RT) activity is a generic method which can be adapted for higher sensitivity using real-time PCR quantification (qPCR-based product-enhanced RT (PERT) assay). We present an evaluation of a modified SYBR Green I-based PERT assay (SG-PERT), using commercially available reagents such as MS2 RNA and ready-to-use qPCR mixes. This assay has a dynamic range of 7 logs, a sensitivity of 10 nU HIV-1 RT and outperforms p24 ELISA for HIV titer determination by lower inter-run variation, lower cost and higher linear range. The SG-PERT values correlate with transducing and infectious units in HIV-based viral vector and replication-competent HIV-1 preparations respectively. This assay can furthermore quantify Moloney Murine Leukemia Virus-derived vectors and can be performed on different instruments, such as Roche Lightcycler® 480 and Applied Biosystems ABI 7300. We consider this test to be an accurate, fast and relatively cheap method for retroviral quantification that is easily implemented for use in routine and research laboratories.

Introduction

Retroviral vectors have become an indispensable tool in any modern molecular biology laboratory. They allow stable expression of a gene of interest in dividing cells, as well as stable gene knock-down by expression of short hairpin RNA (shRNA). A subset of vectors, derived from lentiviruses such as human immunodeficiency virus (HIV-) 1 and 2 and feline immunodeficiency virus (FIV), can be used for efficient transduction of non-dividing cells and have therefore received increased attention for both basic research and clinical applications [1,2,3,4]. Although methods for accurate quantification of retroviral vector titers will be indispensable in a clinical setting, also the basic research environment can benefit from a fast and inexpensive method to evaluate the quality of retroviral vector productions. Furthermore, research laboratories investigating the replication of retroviruses, such as HIV, require routine assays to determine retroviral titers after production and during viral infection. Multiple methods for retroviral titer quantification are currently available (see [5,6] for an overview of lentiviral titration methods), but they often have some inherent drawbacks. Determination of the proportion of

transduced/infected cells, by evaluating viral integration or transgene expression, provides a good estimate of the number of functional viral particles, but is time-consuming as it requires transduction/infection of the cells and several days of incubation. Other more rapid methods measure both functional and non-functional viral particles in the supernatant, by quantifying the levels of retroviral Gag protein (such as the HIV p24 protein) and the levels of viral genomic RNA. The former is often done by enzyme-linked immunosorbent assay (ELISA) and consequently has a limited linear range and high cost. The latter relies on quantitative real-time PCR (qPCR)-based amplification of cDNA of virion-associated RNA and requires target-specific primers. Furthermore, both methods are still quite labor-intensive.

An alternative retroviral titration method involves quantification of the reverse transcriptase (RT) activity, which is associated with all retroviral particles. In these assays, an exogenous RNA template is added to the viral supernatant and RT activity is estimated by determining the amount of RNA that is converted to cDNA by the retroviral RT. In the first generation RT assays, cDNA production was monitored by measurement of labeled nucleotide incorporation [7,8,9]. Sensitivity was highly increased

when a PCR amplification step of the synthesized cDNA was introduced prior to product detection. These types of assays are commonly known as product-enhanced RT (PERT) assays. However, quantification of PCR products still required labor-intensive techniques such as DNA gel electrophoresis, Southern Blot or ELISA [10,11,12,13,14,15]. The newest PERT generation therefore uses integrated qPCR techniques for fast cDNA quantification, further increasing both the accuracy and linear range of the assays. Most qPCR based PERT assays use cDNA-specific fluorogenic labeled probes (Taqman® chemistry) for signal generation (F-PERT) [16,17,18,19,20], although a one-step PERT assay using the more accessible and cost-efficient SYBR Green-I chemistry (SG-PERT) was also recently developed [21]. PERT assays are now routinely used for detection of retroviral contaminants in biological products intended for human use [22,23,24,25,26,27,28,29,30,31]. However, in basic research environment, the implementation of real-time PCR based PERT assays is still limited, despite their low cost and fast procedure.

In this paper, we present an adapted version of the SG-PERT assay described before by Pizzato et al. [21]. The assay was adapted for use with different commercial ready-to-use SYBR Green I qPCR reaction mixes, to allow an easy implementation of the assay in any research lab with qPCR experience and to avoid possible compositional variation inherent to in house prepared qPCR mixes. In addition, RNA from bacteriophage MS2, which also lacks a DNA phase in its life-cycle, was used instead of RNA from the Brome Mosaic Virus (BMV) that was used as a template in the original assay, but is no longer commercially available. Sensitivity and specificity of the assay were determined, as well as the variation on repeated RT activity measurement within and between runs. We used the assay to evaluate the informative value of the RT activity for lentiviral titer determination, by comparing it to more commonly used titration methods. We observed excellent correlation with the p24 antigen concentration in both replication-competent HIV-1 virus supernatant and replication-incompetent HIV-based lentiviral vector preparations, as well as with the levels of transducing units or infectious units. This particular assay outperformed p24 ELISA by its lower inter-run variation, lower cost and higher linear range. Furthermore, it was far less time-consuming than both p24 ELISA and determination of transducing or infectious units. We therefore believe that this assay forms an attractive alternative to routine retroviral and lentiviral titer determination in routine and research laboratories.

Materials and Methods

Cell Culture

Human lymphoblastoid Jurkat E6.1 (ATCC Cell Biology Collection, Manassas, VA, USA), Jurkat CD4 CCR5 (Programme EVA Centre for AIDS Reagents, NIBSC, UK), human embryonal kidney 293T (DZSM, Braunschweig, Germany), 293TN (System Biosciences, Mountain View, CA, USA), Phoenix-Amphotropic packaging (Phoenix A) cells (Dr P. Achacoso and Dr G.P. Nolan, Stanford University School of Medicine, Stanford, CA, USA) [32] and P4.R5 MAGI cells (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Germantown, MD, USA) [33] were cultured at 37°C in a 7% CO2 humidified atmosphere, in IMDM complete medium: Iscove's modified Dulbecco's medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Thermo-fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Life Technologies) and 100 µg/mL streptomycin (Life Technologies).

Production of Replication-competent HIV-1 Virus

Replication-competent HIV-1 virus was produced by transfection of 293T cells with one of the following pNL4-3 proviral constructs: the NLENG1-IRES vector (kindly provided by Dr. D.N. Levy, New York University college of Dentistry, New York, NY) [34], the NL4-3-IRES-HSA vector (kindly provided by Dr. M.J. Tremblay, Faculté de Médecine, Université Laval, Québec, Canada) [35] or the HIV-1 NL4-3-IRES-eGFP vector (kindly provided by Dr. F. Kirchhoff, Institute of Virology, University of Ulm, Ulm, Germany) [36]. Transfection was performed with Calcium Phosphate Transfection Kit (Life Technologies) or JetPei® (Polyplus, Sélestat, France), according to manufacturer's instructions. Viral supernatant was harvested 48 hours or 72 hours after transfection and centrifuged at 900 g for 10 min, to clarify the supernatant from remaining cells. High-titer viral supernatant, that was used to produce a standard curve for the SG-PERT assay, was obtained by infection of Jurkat CD4 CCR5 cells with HIV-1 (140 ng p24 equivalent per mL) and subsequent collection of the culture medium 12 days after infection. During infection, culture medium was refreshed every two or three days.

Production of Replication-incompetent Lentiviral and Retroviral Vectors

All replication-incompetent lentiviral vectors used in this study were produced using the pLKO.1-puro Non-Mammalian shRNA control plasmid from the Sigma MISSION® product line (Sigma-Aldrich, Bornem, Belgium), in which the puromycin resistance gene was replaced by an eGFP encoding sequence. 293T or 293TN cells were transfected with this plasmid using Calcium Phosphate Transfection Kit, Lipofectamine® 2000 (Life Technologies) or Fugene® 6 (Promega, Leiden, The Netherlands). HIV packaging genes and the VSV-G heterologous viral envelope gene were provided in the cells by simultaneous cotransfection with either the MISSION® Lentiviral Packaging Mix (Sigma-Aldrich) or the p8.91/pMD.G plasmids [32] and virus was harvested two days after transfection. The Moloney Murine Leukemia Virus (MoMLV)-based retroviral vector was produced by transfection of Phoenix Amphotropic packaging cells with the LZRS-IRES-EGFP vector using the Calcium Phosphate Transfection kit, as described before [37,38].

Reagents Required for the SG-PERT Assay

MS2 RNA was purchased from Roche Diagnostics (Vilvoorde, Belgium; Catalog #10165948001). Primers to amplify MS2 cDNA in the SG-PERT reaction were obtained from Eurogentec (Seraing, Belgium) and had the following sequence: FWD (5′-TCCTGCTCAACTTCCTGTCGAG-3′) and REV (5′-CA-CAGGTCAAACCTCCTAGGAATG-3′), as published [19]. Ribolock™ RNase inhibitor was from Fermentas (St. Leon-Rot, Germany; Catalog # EO0381). For SG-PERT on the Light-Cycler® 480 (Roche Diagnostics), the LightCycler® 480 SYBR Green I Master mix from Roche was used as reaction mix (Catalog #04707516001). For SG-PERT on the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA), a reaction mix was made using the ROX-containing qPCR Core kit for SYBR Green I from Eurogentec (Catalog #RT-QP73-05). Recombinant HIV Reverse Transcriptase was purchased from Ambion (Life Technologies, Catalog # AM2045). 2× concentrated lysis buffer was composed of 0.25% Triton X-100, 50 mM KCL, 100 mM TrisHCL pH 7.4, 40% glycerol and prepared as described previously [21]. 2 µL of RNAse inhibitor was added per 100 µL of the 2× lysis buffer immediately prior to use.

SG-PERT Assay

Cell-free viral supernatant was generally used without prior dilution as input for the assay. 10-fold dilution series of viral supernatant or HIV recombinant RT were generated in IMDM complete. 5 µL of the viral supernatant or recombinant RT solution was added to a well of a 96-well U-bottom plate (Beckton Dickinson, Erembodegem, Belgium) and mixed with 5 µL of 2× concentrated lysis buffer, already containing RNAse inhibitor. Samples were incubated for 10 minutes at room temperature and subsequently diluted by addition of 90 µL nuclease-free water (Life Technologies). After brief centrifugation, the lysates were resuspended and used as input for the assay. For SG-PERT assays on the LightCycler® 480 instrument, 9.6 µL of the lysate was transferred to a 384-well plate (LightCycler® 480 Multiwell Plates 384, white, Roche Diagnostics), that already contained 10.4 µL of a reaction mix consisting of 10 µL 2× Roche SYBR Green I Master mix, 0.1 µL 10× diluted RNAse inhibitor, 0.1 µL MS2 RNA and 0.1 µl of both the MS2 FWD and REV primer (100 µM, to obtain final concentration of 500 nM in 20 µL reaction volume). After brief centrifugation of the plate, the reaction was carried out according to the following program: 20 minutes (min) at 42°C for RT reaction, 5 min at 95°C for activation of FastStart Taq DNA polymerase and 40 or 50 cycles of amplification: 5 seconds (sec) at 95°C for denaturation, 5 sec at 60°C for annealing and acquisition, 15 sec at 72°C for elongation. Fluorescence acquisition was done at the end of annealing phase in our experiments, but can alternatively be done at the end of elongation phase. For SG-PERT assays on the ABI 7300 qPCR system, 9 µL of the lysate was added to a 96-well plate (MicroAmp Optical 96-well reaction plate, Applied Bisosystems) together with 11 µL of a reaction mix consisting of 10.6 µL mastermix from the Eurogentec qPCR core kit for SYBR Green I (2 µL 10× reaction buffer, 1.4 µL of 50 mM MgCl2, 0.8 µL of 5 mM dNTP mix, 0.1 µL of HotGoldSTar Taq polymerase, 0.6 µL SYBR Green I and 5.7 µL of nuclease-free water.), 0.1 µL 10× diluted RNAse inhibitor, 0.1 µL MS2 RNA and 0.1 µl of both the MS2 FWD and REV primer (100 µM). Following reaction conditions were used on the ABI 7300 instrument: 20 min RT reaction at 42°C, 2 min activation of the HotGoldStar Taq enzyme at 95°C and 40 cycles of amplification: 5 sec denaturation at 95°C, 30 sec annealing and acquisition at 60°C, 15 sec elongation at 72°C.

All reagents were kept on ice or on a cooling block during preparation of the assay. For each sample lysate, an SG-PERT reaction was always performed in duplo. Cycles of quantification (Cq) values were generated by the software of the qPCR instruments, after manual threshold determination for the ABI 7300 instrument and according to the second-derivative maximum method for the LightCycler® 480. Melting peaks were calculated automatically by the software of both instruments.

To perform absolute quantification of RT activity values, a standard curve of replication-competent HIV-1 containing supernatant with known RT activity levels was run in parallel in each assay and values were extrapolated from the obtained Cq values. The standard curve was produced by serial dilution of a large batch of high-titer supernatant. Dilutions were aliquoted for use in different SG-PERT assays, to avoid loss of RT activity by repeated freeze-thaw cycles. RT activity values of the standard curve were determined by running a dilution series of commercial recombinant RT in parallel in at least four independent experiments.

Calculations and Statistics

Standard deviation (STDEV), coefficient of determination (R^2) and regression equations were calculated with Excel 2007 (Microsoft). Coefficient of variation (CV) was calculated as follows: (STDEV/AVERAGE) x 100%. Column scatter plots were created with GraphPad Prism version 5.04. Statistical significance of difference between inter-run coefficient of variation of p24 ELISA test and SG-PERT assay was analyzed with the one-tailed non-parametric Mann-Whitney U test (GraphPad Prism).

Determination of p24 Concentration, Transducing and Infectious Units in Viral Supernatant

Concentration of the p24 antigen was measured in HIV-1 or HIV-based lentiviral vector containing supernatant with the INNOTEST® HIV Antigen mAb ELISA kit (Innogenetics, Zwijnaarde, Belgium), according to manufacturer's instructions. Multiple dilutions of each sample were tested, to ensure that concentration was within the linear range of the assay. For correlation analysis of p24 antigen and RT activity values, all samples were measured within the same ELISA assay, to avoid the introduction of inter-run variation. For lentiviral vectors, the number of transducing units per volume of supernatant (TU/mL) was determined by transduction of Jurkat E6.1 cells with a limiting dilution series of each sample, using polybrene (8 µg/mL; Sigma-Aldrich) and spinoculation (30 min, 950 g, 32°C). The percentage of transduced cells was determined 72 hours after transduction by FACS analysis (MACSquant® Analyzer, Miltenyi Biotec, Leiden, The Netherlands) of eGFP expression. Vector titers (TU/mL) were calculated in cultures with 0.5% to 4% of eGFP expressing cells according to following formula: { (% eGFP expressing cells/ 100) x number of cells at moment of transduction x dilution factor }/volume of viral supernatant used for transduction (mL).

For replication competent HIV-1 virus, relative levels of infectious units (IU) were determined by single-cycle infection of P4.R5 MAGI indicator cells. Briefly, 10,000 cells were plated per well of a 96-well flat bottom plate (Beckton Dickinson). Twenty-four hours after plating, HIV-1 NL4-3 viral supernatant was added to the cells in the presence of 1 µM of the HIV protease inhibitor ritonavir (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Germantown, MD, USA), to avoid multiple rounds of infection. Cells were subsequently spinoculated at 950 g for 90 min at 32°C. Forty-eight hours after infection β-galactosidase activity was assessed using a colorimetric assay (Mammalian β-galactosidase Assay kit; Thermofisher Scientific) according to manufacturer's instructions. Optical density at 405 nm was quantified using a Versa Max Plate Reader (Molecular Devices, Sunnyvale, CA, USA) and obtained values were corrected for background signal by subtraction of the optical density value obtained with non-infected cells. For correlation analysis of RT activity and relative levels of infectious units, we used cell-free HIV NL4-3 viral supernatant harvested 48 hours or 72 hours after transfection, with the Calcium Phosphate Transfection Kit, of 293T with either the NLENG1-IRES vector or the NL4-3-IRES-HSA vector. To ensure that β-galactosidase activity levels were within the linear range of the assay, the viral concentration in the culture was limited to maximum 360 mU RT activity/mL. To determine absolute levels of infectious units/mL, P4.R5 MAGI cells were infected with serial dilutions of HSA encoding HIV-1 virus in presence of ritonavir. Forty-eight hours after single-cycle infection, cells were stained with APC (allophycocyanin)-labeled anti-mouse-CD24 antibody (HSA, heat stable antigen; clone M1/69, BioLegend, San Diego, CA, USA) and the percentage of infected cells was determined by FACS analysis (FACSCalibur flow cytometer; Becton Dickinson)of HSA expression. Viral titers (IU/mL) were calculated according to the following formula: {(% HSA expressing cells/100)×number of

plated cells × dilution factor}/volume of viral supernatant used for infection (mL).

Results

Sensitivity and Specificity of the SG-PERT Assay

A one step SYBR Green I-based real-time PERT assay (SG-PERT) was developed, that uses MS2 RNA as a substrate for reverse transcription and commercially available ready-to-use reaction mixes for MS2 cDNA quantification by SYBR Green I-based qPCR (Figure 1). We first performed experiments using the Roche Master Mix for qPCR on the LightCycler® 480. Inherent to such a SYBR Green I-based detection system, is the possible contribution of non-specific PCR fragments to the measured signal. To evaluate the presence of any non-specific products, we performed a melting curve analysis on the PCR products. Only a single PCR fragment with a melting peak of 80.6°C was detected in the final PCR product of both highly and weakly positive samples (Figure 2A).

In order to determine the sensitivity and linear range of the assay, 10-fold serial dilutions of a recombinant HIV-1 reverse transcriptase (RT) enzyme were used as input for SG-PERT. Amplification of the MS2 substrate correlated with the input amount of recombinant RT (Figure 2B). When plotting the RT input against the obtained Cq, the correlation was linear over 7 orders of magnitude, ranging from 10^{11} pU to 10^4 pU recombinant HIV-1 RT per reaction (Figure 2C). Lower amounts of recombinant RT (10^3 and 10^2 pU) were still detected, but Cq values were considered to be outside the linear range of the assay. Furthermore, when using nuclease-free water (NFW) as input for the assay, a weak signal was occasionally obtained after 37 or more cycles (Figure 2D). This signal might indicate a weak background reverse transcriptase activity of the Taq DNA polymerase in the Roche mix or might be caused by carry-over contamination between SG-PERT experiments. Since Cq values obtained in reactions with 10^3 and 10^2 pU input HIV-1 RT enzyme were not consistently above these occasional background values, the detection limit of this assay is considered to be 10^4 pU recombinant HIV-1 RT.

Subsequently, the ability of the assay to measure the RT activity associated with complete viral particles was tested. When using cell-free supernatant of replication competent HIV-1 infected Jurkat CD4 CCR5 cells as input for the assay, a linear correlation between the input virus dilution and Cq values was again obtained over six 10-fold serial dilutions of the original supernatant. Similar to the results with recombinant HIV-1 RT, higher dilutions of viral supernatant were still detectable (Cq ≥35), but outside the linear range of the assay (Figure 2D and 2E). The undiluted sample used here, had a p24 antigen concentration of 3,100 ng/mL according to ELISA measurement. Therefore, this SG-PERT assay can detect and quantify RT activity in HIV-1 supernatant with a p24 equivalent as low as 0.0031 ng/mL. Since only 0.48 μL of supernatant is used per reaction, this corresponds to a detection limit of 1.5 fg p24 or ±20 viral particles (assuming that an HIV core is composed of 2,000 p24 capsid molecules [6]). Alternatively, when the lowest detectable number of virions is calculated based on virion associated RT activity (400- 200 pU/virion [9,18]), a similar detection limit of 25–50 virions is obtained.

Since RT activity is associated with all retroviruses, a more prominent application of this assay can be the quantification of recombinant lenti- and retroviral vectors, for instance to check the quality of retroviral production. We used two types of commonly used replication-defective viral vectors, HIV-based and MoMLV-based, to evaluate the correlation between vector concentration and RT activity measured by SG-PERT. Both the RT activity associated with HIV-based MISSION® lentiviral particles as well as the activity of the MoMLV RT enzyme could be detected in supernatant of lentivirus producing 293T cells and retrovirus-producing Phoenix A packaging cells respectively. Activity of both enzymes showed a linear correlation with the input vector dilution over four to five 10-fold dilutions (Figure 2E). When plotting the input concentration versus the obtained Cq values, a curve with similar slope was obtained for recombinant RT, replication competent HIV-1, HIV-based lentiviral vectors and MoMLV-based retroviral vectors (Figure 2C and Figure 2D; varying from −3.6 to −3.8). Therefore, PCR efficiencies must be in the same range and the linear range for retro- and lentiviral vector quantification might be similar to the one of recombinant RT quantification. In order to express the sensitivity of viral vector quantification by the SG-PERT assay, the functional titer of both the lenti- and retroviral vector supernatant used here was determined by limiting dilution titration on Jurkat E6-1 cells and was found to be resp. 1.12×10^7 transducing units (TU)/mL and 4.9×10^5 TU/mL. Since the assay could quantify a 10^5 dilution of the HIV-based lentiviral supernatant, a sensitivity of 1.12×10^2 TU/mL or 0.056 TU/reaction can be assumed. Similarly, for MoMLV-based retroviral vector supernatant,
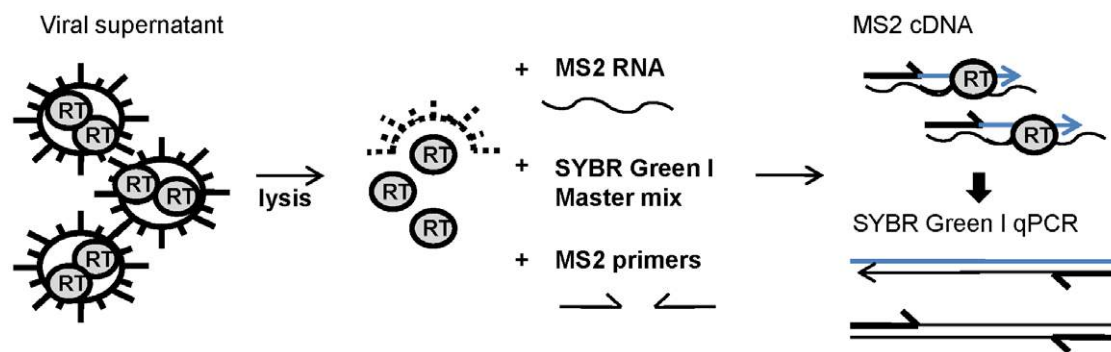

Figure 1. Principle of the SG-PERT assay. Cell-free retrovirus containing supernatant is lysed and added to a reaction mix containing the MS2 RNA template, MS2 complementary primers and a SYBR Green I qPCR mastermix. During a one-step reaction, the reverse transcriptase (RT) enzymes derived from the retroviral particles will convert the MS2 RNA into cDNA and cDNA is subsequently quantified by qPCR amplification of the MS2 cDNA. The amount of synthesized cDNA represents the level of RT activity in the viral supernatant and is thereby a measure of the amount of retroviral particles.
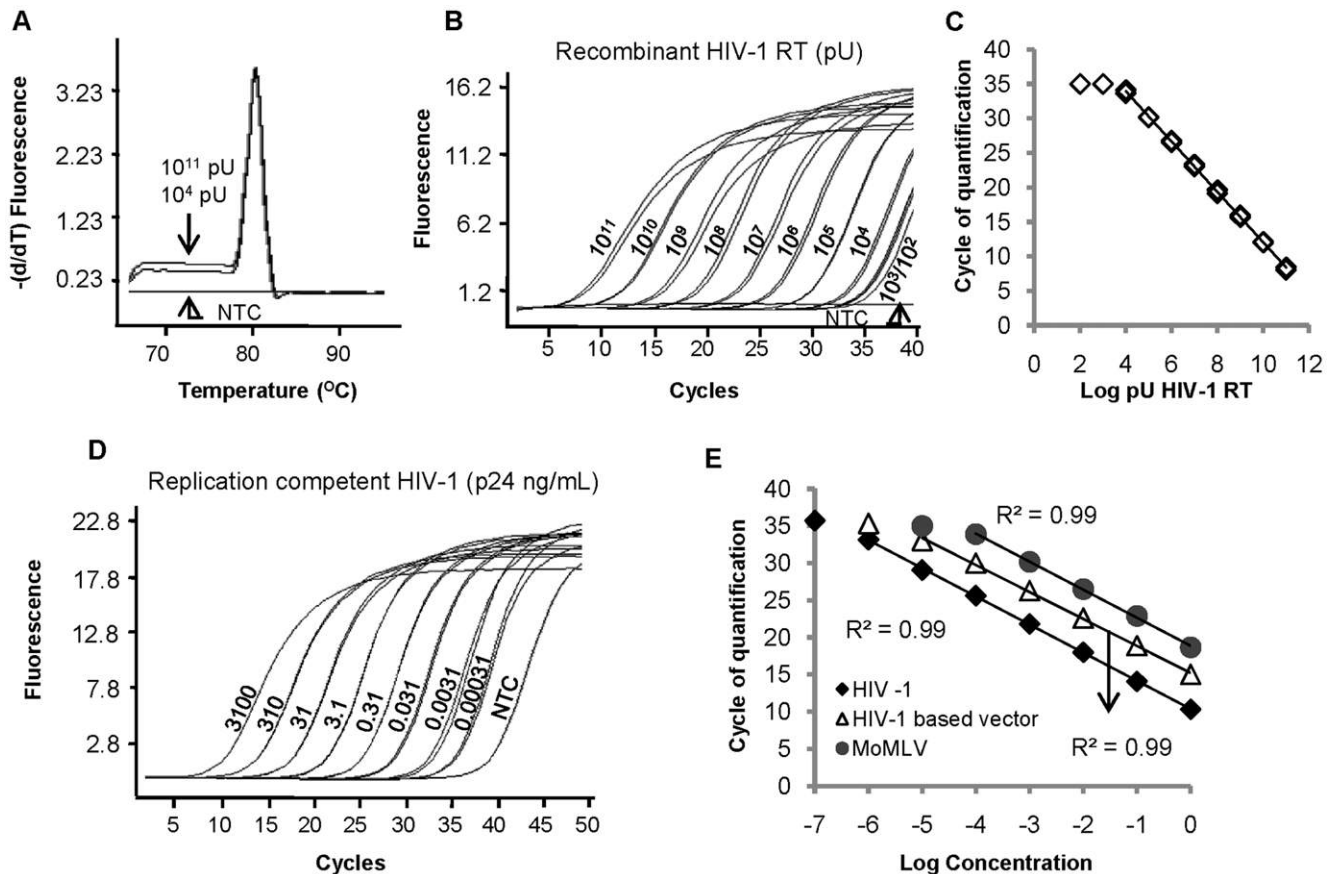
74

Figure 2. Sensitivity and specificity of the SG-PERT assay. (A) Melting curves of PCR products obtained by SG-PERT assay on the LightCycler® 480 when using 10^{11} or 10^4 pU recombinant HIV-1 RT or nuclease-free water (non-template control = NTC) as input for the assay, as indicated. (B, D) Amplification curves of indicated amount of (B) recombinant HIV-1 RT (pU), (D) replication competent HIV-1 (NL4-3 strain) (ng p24/mL) or nuclease-free water (NTC) obtained by SG-PERT on the LightCycler® 480. (C,E) Relation between input of (C) recombinant HIV-1 RT, (E) replication competent HIV-1 (HIV-1), HIV-1 based lentiviral vectors (HIV-1 based vector) or Moloney Murine Leukemia-based retroviral vectors (MoMLV) and obtained cycle of quantification (Cq) values by SG-PERT on the LightCycler® 480. Viral titers in the undiluted samples in (E) (value of ''0'' on x-axis) were 3,100 ng p24/mL for the replication competent HIV-1 virus, 1.12×10^7 transducing units/mL (TU/mL) for the HIV-1 based viral vector and 4.9×10^5 TU/mL for the MoMLV-based vector. Only input levels within linear range of the assay were included for correlation analysis.
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quantification of up to 49 TU/mL or 0.024 TU/reaction is possible.

Inter- and Intra-run Variation of the SG-PERT Assay

When determining RT activity in lenti- or retroviral supernatant with the SG-PERT assay, absolute quantification can be done by running a standard curve of recombinant reverse transcriptase in the same assay as the sample of interest. For frequent use of the assay, a cheaper alternative is the use of a retroviral standard curve. In this case, the RT activity of high titer retroviral supernatant is determined once, using a standard curve of recombinant reverse transcriptase. For subsequent assays, a dilution series of this retroviral supernatant can be used as standard curve and absolute quantification can be done by using the known RT activity of the retroviral standard curve.

To obtain such a standard curve, supernatant of HIV-1 infected Jurkat CD4 CCR5 cells with an RT activity of 47,000 mU/mL was serially diluted and aliquots of each dilution were stored for use in different assays. Cq values of this standard curve, obtained in 12 independent SG-PERT experiments on the LightCycler® 480, are shown in Figure 3A. They were found to be highly reproducible, with a standard deviation of maximum 1 Cq value

over the different assays for each dilution point, corresponding to a variation of two-fold over the measurement average. If a precise determination of RT activity is not required, it is therefore possible to only occasionally include a standard curve in the SG-PERT experiment and directly derive the RT activity from the obtained Cq value instead (using a fixed standard curve for calculations).

Subsequently, the reproducibility of RT activity quantification using the SG-PERT assay and the HIV-1 standard curve, was evaluated on the LightCycler. To determine intra-run reproducibility, 8 aliquots from each of 6 different HIV-containing samples were separately lysed and RT activity in the different lysates was determined in the same run. The coefficient of variation (CV) ranged between 8.6% to 15% of the RT activity (Figure 3B), with an average CV of 12%. Inter-run variation was evaluated on 11 different HIV-1 containing samples by determining their RT activity in at least 3 independent SG-PERT experiments. We found an average CV of 19.9%. Except for the sample with the lowest titer, CV was lower than 30%. The sample with very low RT activity (0.2 mU/mL; sample 1 in Figure 3C) showed a CV of 43%, which might indicate a decreased reproducibility of the SG-PERT measurement in the region close to the detection limit of the assay (Figure 3C).
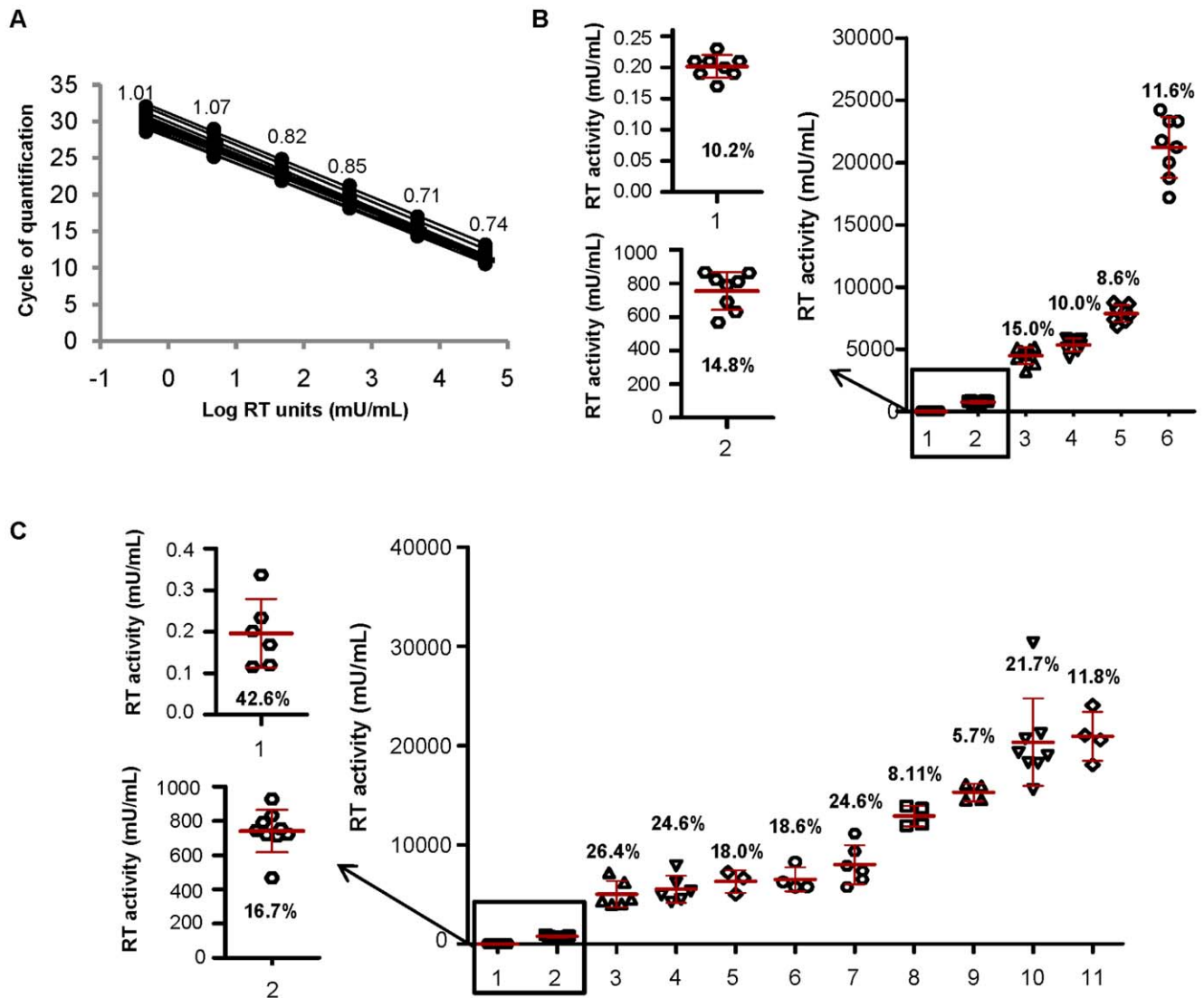
Figure 3. Intra- and inter-run variation of the SG-PERT assay. (A) Standard curve composed of a pre-made six 10-fold serial dilution series of replication-competent HIV-1 containing supernatant measured in 12 independent SG-PERT experiments. For each experiment obtained Cq values are plotted versus the RT activity in each sample. RT activity values were determined by running a dilution series of recombinant HIV-1 RT in parallel. Standard deviation on the obtained crossing point values is indicated for each dilution. (B) RT activity values obtained for 8 repeated measurements of different HIV-1 samples (sample number 1 to 6) within the same run. The average RT activity value for each sample is indicated by a red line, error bars represent standard deviation on the obtained RT activity values. Numbers indicate intra-run variation for each sample, expressed as percentage of the average RT activity values (coefficient of variation). (C) RT activity values obtained for different HIV-1 samples (sample number 1 to 11) in at least 3 independent SG-PERT experiments. The average RT activity value for each sample is indicated by a red line, error bars represent standard deviation on the obtained RT activity values. Numbers indicate inter-run variation for each sample, expressed as percentage of the average RT activity values (coefficient of variation). Experiments were performed on the LightCycler® 480.
doi:10.1371/journal.pone.0050859.g003

Comparison of the SG-PERT Assay with Other Lentiviral Titration Methods

Currently, the most frequently used methods for lentiviral titer determination measure the concentration of the p24 antigen in the supernatant or determine the number of transducing units (TU) or infectious units (IU) per volume of the supernatant. The latter can be done by assessing the level of transduced or infected cells after limiting dilution of replication incompetent or replication competent lentiviruses respectively. Alternatively, for HIV viruses, relative levels of infectious units are often determined by single-cycle infection of indicator cell lines. To evaluate the informative value of the RT activity determined by SG-PERT, we investigated its correlation with the p24 antigen concentration levels and the levels of TU or IU in the same samples.

Supernatants with HIV-1 virus (NL4-3 strain) or replication incompetent HIV-based MISSION® lentiviral particles were used for both p24 antigen concentration and RT activity determination by ELISA and SG-PERT respectively. We found a very strong correlation between the obtained p24 and RT values, both for combined and separate analysis of the lentiviral vectors and HIV-1 viruses. The two values correlated with each other according to a power function, with a power number very close to 1, indicating an almost linear relation. The ratio between the RT and p24 values was calculated, that seemed higher for the lentiviral vectors compared to the replication competent HIV-1 viruses (Figure 4A

Table 1. Evaluation of different lentiviral titration methods.

	RT activity (mU/mL)	p24 (ng/mL)	ratio RT/ p24	TU/mL	p24 based # VP/mL	RT based # VP/mL	ratio p24 based/ RT based # VP	ratio TU/p24 based # VP	ratio TU/RT based # VP
HIV-1 sup 1	1.96E-01	2.78E-02	7.0		3.33E+05	6.52E+05	1.95		
HIV-1 sup 2	7.41E+02	1.30E+02	5.7		1.56E+09	2.47E+09	1.58		
HIV-1 sup 3	5.03E+03	7.69E+02	6.5		9.23E+09	1.68E+10	1.82		
HIV-1 sup 4	5.44E+03	7.57E+02	7.2		9.08E+09	1.81E+10	2.00		
HIV-1 sup 5	5.52E+03	9.26E+02	6.0		1.11E+10	1.84E+10	1.66		
HIV-1 sup 6	6.34E+03	1.14E+03	5.6		1.37E+10	2.11E+10	1.55		
HIV-1 sup 7	8.00E+03	1.43E+03	5.6		1.72E+10	2.67E+10	1.55		
HIV-1 sup 8	1.26E+04	2.00E+03	6.3		2.40E+10	4.19E+10	1.75		
HIV-1 sup 9	1.39E+04	2.01E+03	6.9		2.41E+10	4.64E+10	1.92		
HIV-1 sup 10	2.00E+04	1.46E+03	13.7		1.75E+10	6.65E+10	3.81		
HIV-1 sup 11	2.06E+04	3.76E+03	5.5		4.52E+10	6.88E+10	1.52		
LV sup 1	7.32E+00	9.24E-01	7.9	2.82E+04	1.11E+07	2.44E+07	2.20	393	865
LV sup 2	4.91E+01	2.85E+00	17.2	1.03E+05	3.42E+07	1.64E+08	4.78	330	1581
LV sup 3	3.84E+02	5.54E+01	6.9	9.40E+05	6.65E+08	1.28E+09	1.92	708	1362
LV sup 4	8.14E+02	1.12E+02	7.2	1.72E+06	1.35E+09	2.71E+09	2.01	786	1580
LV sup 5	1.13E+03	1.21E+02	9.3	2.68E+06	1.45E+09	3.76E+09	2.59	541	1401
LV sup 6	1.66E+03	1.31E+02	12.7	2.39E+06	1.58E+09	5.54E+09	3.52	659	2319
LV sup 7	3.53E+03	3.78E+02	9.3	1.13E+07	4.54E+09	1.18E+10	2.60	402	1045

Table shows SG-PERT RT activity measured on the LightCycler® 480 and p24 antigen concentration in different productions of replication-competent HIV-1 virus supernatant (HIV-1, sup 1–11) and replication-incompetent HIV-1-based MISSION® lentiviral vectors (LV, sup 1–7). TU/mL: transducing units/mL (only determined for lentiviral vectors). The number of viral particles (#VP) was calculated from p24 values by assuming 12 viral particles per fg p24. For viral particle calculation from RT activity values, an activity of 300 pU per viral particle was assumed.
doi:10.1371/journal.pone.0050859.t001

and Table 1). Of note, the excellent correlation between the p24-RT values of HIV-1 viral supernatants was not caused by the sample with very low titer, since the coefficient of determination (R^2) was still 0.92 if this sample was removed from the analysis (data not shown).

The MISSION® lentiviral particles used in this experiment express eGFP as a marker gene and therefore allow easy determination of TU/mL, by FACS measurement of the proportion of transduced cells. The number of TU/mL obtained after limiting dilution of the different lentiviral supernatants on Jurkat E6-1 cells strongly correlated with the RT activity levels in these samples (Figure 4B). When calculating the number of viral particles in the supernatant, assuming an RT activity of 300 pU per virion [9,18], we found a ratio of 865 to 2,319 RT-containing viral particles per functional transducing unit. Viral particle numbers in the same range were obtained when calculations were based on p24 content, although the estimated particle concentration was slightly higher when based on RT activity ("RT based/p24 based" ratio of 1.5–3.8 for HIV-1 containing supernatant and 1.9–4.8 for lentiviral vector containing supernatant) (Table 1). This shows that as for p24, RT activity is representing both transducing and non-transducing viral particles.

For replication competent HIV-1 virus, relative levels of infectious units in the supernatants were assessed by infection of P4.R5 MAGI indicator cells. These CD4, CXCR4 and CCR5 expressing HeLa cells express bacterial β-galactosidase under control of the Tat-responsive HIV long terminal repeat and therefore express β-galactosidase upon productive HIV infection [33]. The levels of β-galactosidase activity induced by HIV-1 supernatant in these types of assays have been shown to correlate with infectious titers determined by end-point dilution (tissue culture infectious dose 50% or TCID50) [39]. When infecting the cells with a serial dilution of HIV-1 supernatant, the input viral concentration linearly correlated with the β-galactosidase activity measured in the cell culture 48 h after single-cycle infection. For high levels of viral input a saturation of β -galactosidase activity occurred at a level corresponding to an optical density value around 2 (Fig. 4C). To compare levels of infectious units present in different productions of HIV-1 NL4-3 viral supernatant, an appropriate dilution of each supernatant was used for single cycle infection. We found a high correlation between the levels of β-galactosidase activity and the RT activity, indicating a correlation between the number of infectious units and RT activity in the supernatant (Fig. 4D).

Furthermore, we evaluated the inter-run variation of p24 concentration measurement by ELISA and compared this to the SG-PERT inter-run variation determined above. For 7 different HIV-1 containing samples, from the same set that was used to determine SG-PERT reproducibility, p24 concentration was determined in at least 3 independent ELISA experiments. Due to the restricted linear range of p24 ELISA, careful consideration was given to appropriate dilution of the supernatants in each experiment. Coefficient of variation ranged from 16% to 51.4% and was on average higher than the inter-run variation of the SG-PERT assay (p-value = 0.0426 with Mann-Withney U test) (Figure 4E–F and compare to Figure 3C). Quantification of the sample with low viral titer and high inter-run variation of RT activity determination (sample 1 in Figure 4E), was only slightly more reproducible with p24 ELISA compared to SG-PERT (Figure 4E, compare to Figure 3C).
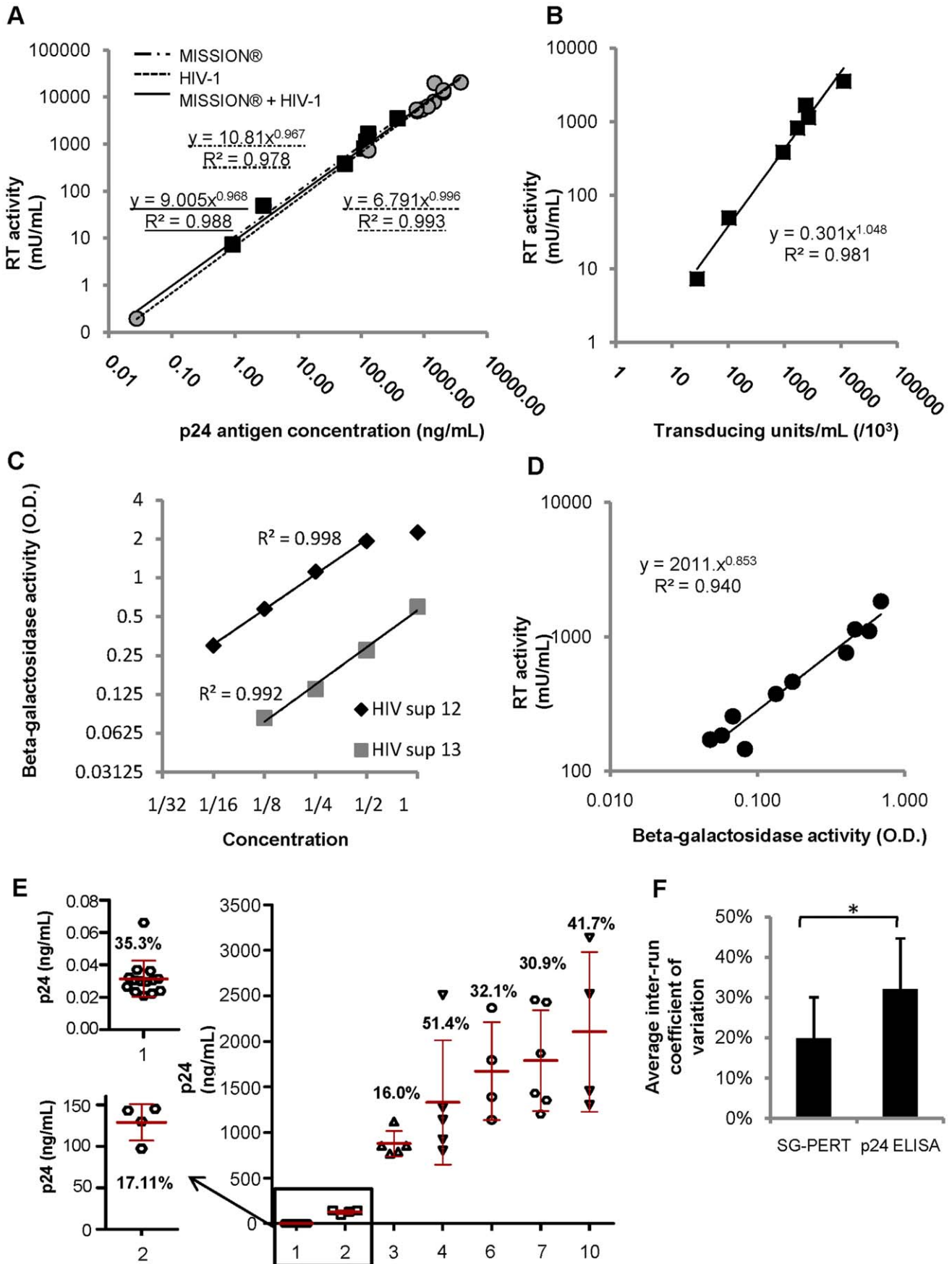
Figure 4. Comparison of SG-PERT assay with other lentiviral titration methods. (A) Correlation between RT activity levels measured with SG-PERT and p24 antigen concentration levels measured with ELISA in supernatant containing replication-competent HIV-1 virus or HIV-1 based MISSION lentiviral vectors. Coefficient of determination and correlation functions are shown for HIV-1 virus (- - -) and lentiviral vectors (-·-·) separately or combined (-). To avoid introduction of interrun variation, p24 ELISA measurements for all samples were performed in the same experiment. (B) Correlation between RT activity levels measured by SG-PERT and levels of transducing units determined by limiting dilution on Jurkat E6.1 cells in HIV-1 based MISSION® lentiviral vector preparations. (C) Relation between the level of β-galactosidase activity, as a marker of HIV-1 Tat production and the relative amount (serial dilution) of replication competent HIV-1 NL4-3 virus used to infect the cells, in the culture of P4.R5 MAGI indicator cells 48 h after single-cycle infection. Viral input in the undiluted samples (value of "1" on x-axis) was 1.76×10^3 mU RT/mL for HIV sup 12 and 2.33×10^2 mU RT/mL for HIV sup 13. For HIV sup 12, the undiluted sample was outside the linear range of the assay and was therefore not included for calculation of the determination coefficient (R^2) shown in panel C. (D) Correlation between the level of β-galactosidase activity in the culture of P4.R5 MAGI indicator cells 48 h after single-cycle infection with different productions of HIV-1 NL4-3 supernatant and the RT activity measured in the supernatant by SG-PERT. (E) p24 antigen concentration values obtained for different HIV-1 containing samples in at least 3 independent ELISA experiments. Sample numbers in x-axis correspond to those in Figure 3C. The average p24 concentration value for each sample is indicated by a red line, error bars represent standard deviation on the obtained p24 concentration values. Numbers indicate inter-run variation for each sample, expressed as percentage of the average p24 concentration (coefficient of variation = CV). (F) Average inter-run coefficient of variation for SG-PERT and p24 ELISA assays, calculated from the CV's obtained in Figure 3C and Figure 4E respectively. Error bars indicate standard deviations. The asterix (*) indicates a statistical significant difference between the two values according to a one-tailed Mann-Withney U test (p-value = 0.0426).
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SG-PERT Assay for Applied Biosystems qPCR Instruments

For the characterization of the SG-PERT assay above we used the Roche SYBR Green I Master Mix for qPCR quantification on the LightCycler. However, other qPCR platforms, such as most of the Applied Biosystems qPCR instruments, require the presence of a passive reference dye in the master mix to normalize for non-PCR related fluorescence signal variations. To compare, we performed the assay on an ABI 7300 Real-Time PCR System using the ROX containing qPCR core kit for SYBR Green I from Eurogentec as reaction mix (see material and methods). Sensitivity and linear range of the assay were determined by using the same 10-fold serial dilution of HIV-1 supernatant as was used before on the LightCycler for this purpose. Similar to the results on the LightCycler, the SG-PERT assay could quantify RT activity in a sample with p24 antigen concentration as low as 0.0031 ng/mL and Ct values correlated linearly with the input virus dilution over six orders of magnitude (Figure 5A and 5B). When using samples with even higher RT activity, by using recombinant RT, we did notice a loss of linearity, probably by saturation of the assay (data not shown). However, such RT activity levels are far above the ones commonly obtained in supernatant of viral productions or medium of HIV infected cells. Nuclease-free water did not generate a detectable signal on the ABI 7300 instrument when used as input for SG-PERT (Figure 5A). Furthermore, melting curve analysis of the PCR products confirmed the absence of non-specific amplification in the assay (data not shown). Therefore, retroviral quantification by SG-PERT can be performed on ABI qPCR instruments with equal efficiency as on the LightCycler.

Discussion

Assessment of retroviral titers is a requisite in quality assurance, virology and molecular biology research laboratories for quality control of cell derived preparations; quality control of viral productions; for standardization of experiments using different batches of virus preparations and for normalization of viral particles numbers when comparing different types of viruses. In this paper, we present the first one-step SYBR Green I qPCR-based PERT assay using commercial ready-to-use reaction mixes and show that quantification of RT activity in viral supernatant by this assay, can provide a robust and accurate alternative for other frequently applied methods of retroviral titer determination.

The SG-PERT assay presented here, uses MS2 RNA as a substrate for reverse transcription by retrovirus-associated RT molecules and subsequently quantifies MS2 cDNA by SYBR Green I-based qPCR, using commercially available reagents and ready-to-use reaction mixes. Since the introduction of a qPCR cDNA quantification step to conventional PERT, a number of assays applying this modification have been published [16,17,18,19,20,21]. Most of these assays follow a two-step protocol, separating the reverse transcription and qPCR step [17,18,19,20]. While such a separation offers the possibility to remove non reverse transcribed RNA templates after the RT step, thereby avoiding possible background signals of the Taq polymerase [20], it renders the assay more labor-intensive and time-consuming. For quantification of retroviral titers in research laboratories, easiness and speed of the assay is often more important than the elimination of minute background levels, that are often far below the signal generated by the retroviral supernatant. Based on assay durations reported in literature, the SG-PERT assay described here seems considerably faster compared to other published two-step and one-step qPCR-based PERT assays [16,18,20]. Furthermore, most available qPCR based PERT assay use cDNA-specific fluorogenic labeled probes (Taqman® chemistry) for signal generation [16,17,18,19,20], while SYBR Green I chemistry is more accessible and is not sensitive to possible DNAse activity present in the test samples, as has been reported for fluorogenic labeled probes in one-step qPCR based PERT assays [17]. Subsequently, the ability to perform the SG-PERT assay with commercial ready-to-use SYBR Green I qPCR reaction mixes largely facilitates its implementation in laboratories equipped for qPCR and eliminates possible compositional variation inherent to in house prepared qPCR mixes. Of note, we have confirmed that another commercially available SYBR Green PCR ready mix (the widely used Qiagen QuantiTect SYBR Green PCR Kit), is fully compatible with the linear quantification of HIV-1 RT activity and therefore most likely is an alternative to the Roche and Eurogentec mixes evaluated in this report.

During evaluation of the one-step SG-PERT assay, we found that obtained RT activity values correlated with input levels over six to seven orders of magnitude. Because of this extraordinary linear range, prior extensive serial dilution of the viral supernatant is not required for titer determination, thus potential introduction of variation is avoided. The sensitivity of the assay corresponded to a p24 equivalent of 3 pg/mL for HIV-1 supernatant and to ±50 or ±110 TU/mL for MoMLV-based and HIV-based retroviral vectors respectively. The original SG-PERT assay described by Pizzato et al. was reported to have a sensitivity of 10^2–10^3 pU recombinant HIV-1 RT per reaction [21]. Although similar input levels were still detected in the current modified assay on the LightCycler® 480, the obtained values were outside the linear range of the assay. The higher sensitivity observed by Pizzato et al. could be due to the use of a different qPCR instrument, the BMV RNA template-primer combination or the performance of home-
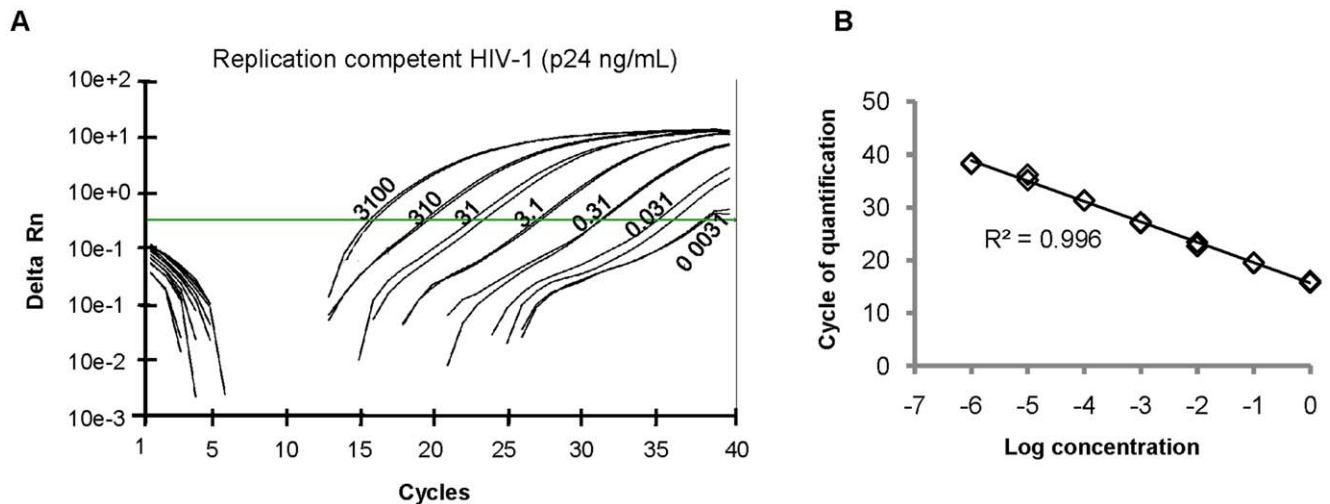
Figure 5. SG-PERT assay on ABI 7300 Real-Time PCR System. (A) Amplification curves of indicated amount of replication competent HIV-1 (NL4-3 strain) (ng p24/mL) obtained by SG-PERT on the ABI 7300 instrument. The horizontal line represents the threshold line used to calculate Cq values. (B) Correlation between input levels of replication-competent HIV-1 virus and Cq values obtained by SG-PERT on the ABI 7300 instrument. p24 antigen concentration in the undiluted samples (value "0" on x-axis) was 3,100 ng/mL.
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made PCR reaction mix. Nevertheless, the sensitivity of the current assay is sufficient for most applications in virology research laboratories and similar to the one of most commercial p24 ELISA kits (eg. Innogenetics INNOTEST®: 10 pg/mL; Perkin-Elmer ALLIANCE® test: 12.5 pg/mL). The assay was furthermore able to accurately quantify RT activity in samples containing retroviruses of different origins (HIV-1 and MoMLV). This offers the advantage that viral (vector) preparations of different origins can all be evaluated within a single assay. In this regard, Ma et al. recently demonstrated that a standard curve of recombinant HIV-1 RT can be used to perform absolute quantification of other types of retroviruses by qPCR-based PERT [18], although the efficiency of different viral reverse transcriptase enzymes should be evaluated in our SG-PERT assay. However, for routine evaluation of a specific type of retrovirus, we recommend to determine absolute RT activity levels of a high-titer preparation of this virus by running a standard curve of the appropriate recombinant RT in parallel. For subsequent assays, a dilution series of this high-titer preparation can then be used as a standard curve.

Due to the accurate quantification capacity of qPCR, a low intra- and inter-run variation has been reported for different PERT assays [17,18,21]. However, variation is usually determined on the obtained Cq values and consequently underestimates the variation on the actual RT activity values. Therefore, we expressed variation of the SG-PERT assay as percentage of the actual RT values, which are calculated from the obtained Cq values by running a standard curve with known RT activity in parallel. We show an acceptable intra- and inter-run reproducibility of the assay, although, as expected, an increase in variation is observed in regions close to the detection limit of the assay. Inter-run variation of viral titer determination was on average lower when using the SG-PERT assay compared to p24 ELISA. A high inter-run variation for p24 antigen quantification by ELISA has been reported by others [5,40], and might be due to extensive sample dilution or the multiple handling steps inherent to the assay, which are both sources of variance introduction.

Since the use of qPCR-based PERT assays as a retroviral titration method is still limited, one goal of this paper is to evaluate the informative value of retroviral titers based on RT activity levels. We show an excellent correlation of RT activity with both p24 antigen concentration and levels of transducing and infectious units.

For replication-incompetent lentiviral vectors, we calculated the number of viral particles from the obtained RT activity and found an average 1/1,450 ratio between transducing units and RT-containing particles. A functional/physical particle ratio in the same range has been reported for lentiviral vectors, when the latter was determined by quantification of viral RNA copies [5,41,42,43] or p24 antigen concentration [44,45]. However, these ratios are highly dependent on different characteristics of the both the vector and transduction process, such as the vector backbone and envelope protein, the transduction method and the cell line used to determine functional titers [5,43]. Therefore, it is important to note that the correlation between RT activity and levels of transducing units was evaluated on lentiviral vector preparations produced with the same transfer plasmid, although the packaging plasmids and transfection methods used were different. We also calculated the ratio between the absolute number of infectious units and RT-containing viral particles for two productions of replication competent HIV-1 virus. The former was determined by analysis of the HSA marker gene expression, encoded by HIV-1 replication competent reporter viruses, in P4.R5 cells infected with a serial dilution of supernatant, the latter based on an RT activity of 300 pU per virion [9,18]. We found ratios of 1/63,500 and 1/111,800 between infectious units and total viral particles (data not shown). In line with replication-incompetent lentiviral vectors, highly variable ratios of infectious/noninfectious units have been reported in literature for *in vitro* produced HIV-1 supernatant (ranging from $1/10^2$ to $1/10^7$) [46,47,48,49]. These ratios are influenced by several factors, such as the type of virion producing cell, type and density of the target cell, the infection protocol and the HIV-1 strain [40,48,49,50,51]. If a precise estimation of the transducing or infectious capacity is necessary, it is therefore recommended to establish titers of the particular vector or HIV virus with a functional titration method immediately on the cell line of interest [5,52]. However, if fixed transduction or infection levels are not necessary, a more rapid estimation of physical particle content, with for instance the SG-

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PERT assay, is often sufficient to assess quality control of the viral production or to normalize the number of viral particles before transduction or infection. It should be noted that the actual number of RT-containing viral particles might be different from the ones estimated in Table 1, since the RT activity per virion might be dependent on both the origin of the HIV RT packed in the viral particles as well as the source of the recombinant RT used for quantification in the SG-PERT assay. In addition, we cannot exclude that some of the RT activity is not bound to a viral particle. Nevertheless, these results indicate that, similar to the p24 antigen concentration, the virion associated RT activity most likely provides an estimate of the physical particle content in lentiviral supernatant, which strongly correlates with the functional particle content for both replication-incompetent lentiviral vectors and replication-competent HIV-1 virus.

Correlation between RT activity and infectious units of replication competent HIV-1 virus has been investigated by others in the past [39,40,53,54]. Although one study found RT activity to be a poor predictor of virion infectivity [54], most of them observed a correlation between infectivity and the RT activity measured in the supernatant by conventional non-PERT RT assays [39,40,53]. It has to be noted that these studies were done on primary HIV isolates and the presence of correlation appeared to be dependent on whether or not viruses were grouped according to coreceptor usage or subtype [39,40]. Since our study evaluated different productions of the same HIV-1 strain (NL4-3), a correlation between RT activity and relative levels of infectious units is in agreement with these studies and indicates that the SG-PERT assay is a fast and worthy alternative for non-PERT RT assays in these types of studies. In contrast to RT activity, p24 antigen levels show a poor correlation with levels of infectious HIV units in most studies [39,40,53,54]. In this regard, it has been reported that RT activity might present an intermediate of physical and functional particle concentration. This was based on the observation that the levels of RT activity and infectious units in HIV-1 infected cell cultures over time show a rapid decrease after reaching peak levels, while p24 antigen levels further accumulate and subsequently reach a plateau phase. These authors therefore assume a short half life of RT activity, while p24 proteins are still detected upon decay of infectious particles [39,40]. In the present study, p24-RT activity correlation was evaluated in supernatant collected from transfected 293T or 293TN cells at 2 or 3 days post transfection. Since the levels of transducing units in these preparations also correlated with p24 levels (data not shown), viral particle decay is probably still limited at this time-point [5]. However, when monitoring lentiviral replication levels over prolonged periods of time, assessment of RT activity levels might provide a more accurate result compared to p24 antigen levels.

Implementation of the SG-PERT assay in our laboratory was found to be easy. A lab equipped for qPCR assays only needs to acquire the MS2 RNA template, MS2 specific primers, and a small amount of the recombinant RT of interest. The Triton X-100 based lysis buffer can be prepared from routine chemical ingredients and a standard curve can be established from any high-titer retroviral preparation of choice. We calculated that the current reagents cost of the SG-PERT assay using the Light-Cycler® 480 and associated reaction mix is about 10 times lower per retroviral quantification compared to determination of p24 antigen levels with a commercial ELISA kit. Moreover, since the limited linear range of quantification of the ELISA assay often requires the measurement of 3 different dilutions per sample, cost and labor increase even more. Furthermore, while titer quantification of 30 samples by ELISA can take up to six hours of hands-on labor time, the SG-PERT assay requires less than 2 hours hands-on time and is amenable to automation and further reduction in reaction volumes.

In summary, this paper shows that the SG-PERT assay with commercially available MS2 RNA and qPCR reaction mixes is a robust and accurate method for retroviral quantification. Titer determination by this assay correlates well with those of other frequently applied methods. Combined with its low cost, fast procedure and easy implementation, it is an attractive alternative for use in virology and molecular biology research.

Acknowledgments

Author Contributions

Conceived and designed the experiments: JV MP BV. Performed the experiments: JV EN HV. Analyzed the data: JV BV. Contributed reagents/materials/analysis tools: AL VI AVN TT. Wrote the paper: JV BV.

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Chapter III-2 :

HIV triggers a Vpu- and Vpr-regulated, cGAS-dependent type I interferon response in CD4$^+$ T cells.

HIV triggers a Vpu- and Vpr-regulated, cGAS-dependent type I interferon response in CD4^{+} T cells.

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Summary:

Viral replication products are recognized by different cellular pattern recognition receptors. During HIV infection, viral sensing and subsequent type I interferon (IFN-I) production indeed occurs in specific cell types and infection conditions, however it was considered to be virtually absent in the main HIV target cells. Here, we show that activated CD4$^+$ T cells sense HIV through the cytosolic DNA sensor cGAS and this induces a bioactive IFN-I response. Surprisingly, efficient induction of IFN-I by HIV-1 requires proviral integration of the virus and is subject to regulation by newly expressed viral proteins: Vpr potentiates, while Vpu represses IFN-I induction. Notably, Vpr also amplified innate sensing of HIV-1 in Vpx treated dendritic cells. These results identify cGAS as an immune sensor of HIV-1 in CD4$^+$ T cells and demonstrate that the IFN-I response is modulated by the viral accessory proteins Vpr and Vpu.

Introduction

Type I interferons (IFN-I) are key players in the innate immune response against viral pathogens. They comprise a group of heterogeneous cytokines, including IFN-β and different subtypes of IFN-α. IFN-I production is induced through recognition of specific viral replication products by a multitude of pattern recognition receptors. IFN-I proteins mediate their powerful antiviral effects by binding to an interferon receptor and activating transcription of a large number of immunomodulatory and antiviral interferon-stimulated genes (ISGs) (reviewed in Goubau et al., 2013). Over the last years, different ISGs with profound activity against HIV, such as APOBEC3G, TRIM5α, BST2/tetherin and SAMHD1, have been identified (reviewed in Harris et al., 2012).

The induction of IFN-I in response to HIV infection itself has become a subject of great interest. In HIV-infected patients, serum levels of IFN-I are strongly elevated during acute infection (Stacey et al., 2009) and higher levels of IFN-I protein and activity are detected in chronic infections (Hyrcza et al., 2007; Hardy et al., 2013). Although IFN-I responses initially limit viral spread, prolonged exposure to IFN-I in the chronic phase of HIV/SIV infection is associated with desensitization and detrimental hyper-immune activation and can therefore paradoxically contribute to disease progression (Sivro et al., 2014). During SIVmac infection this was clearly illustrated, since both the *in vivo* blockage of IFN-I receptors as well as prolonged treatment with IFN-I accelerated the progression to AIDS in rhesus macaques, characterized by reduced sensitivity to IFN-I and enhanced cell-associated viral load and

CD4^{+} T-cell depletion (Sandler et al., 2014). Furthermore, sustained IFN-1 activity in the chronic phase of infection is only detected in pathogenic models of SIV infection, while a complete down-regulation is observed after acute infection in non-pathogenic models (Bosinger et al., 2009; Harris et al., 2010). Accordingly, modulation of IFN-I responses might have therapeutic potential.

Plasmacytoid dendritic cells (pDCs) are known to produce high levels of IFN-I in response to HIV (Fonteneau et al., 2004; Lepelley et al., 2011). During acute infection, they are most likely the main source of IFN-I (Kader et al., 2013; Li et al., 2014). However, levels of circulating pDCs as well as their capacity to produce IFN-I decreases during progressive infection, suggesting that other cell types contribute to IFN-I levels, particularly during the chronic phase of HIV infection (Soumelis et al., 2001; Tilton et al., 2008; Nascimbeni et al., 2009). Yet in the main HIV target cells, IFN-I induction during HIV infection is generally thought to be modest (Rasaiyaah et al., 2013; Yan et al., 2010), suggesting a lack of sensing or evasion by the virus. Therefore, we investigated whether sensing does occur in HIV-infected activated CD4^{+} T cells, which sensor is involved and how sensing is regulated by the virus.

We show that HIV infection induces a bioactive IFN-I response in its main target, activated CD4^{+} T cells. Interestingly, IFN-I induction was dependent on integration of the virus, indicating that productive infection is required for the sensing to occur. We identified the cytosolic receptor cGAS as an HIV sensor inducing IFN-I in infected CD4^{+} T cells. Furthermore, we found that the HIV-1 Vpr protein potentiates the IFN-I response, in both CD4^{+} T cells and dendritic cells. Conversely, the HIV-1 Vpu protein suppresses IFN-I induction, independently of its ability to downregulate the innate sensor tetherin. These findings indicate that cGAS sensing of HIV reverse transcription products is enabled through the assistance of newly expressed viral replication products with a pivotal role for the Vpr protein and that HIV evolved counteraction of innate sensing by Vpu.

Results

HIV induces an IFN-I response in activated primary CD4^{+} T cells

In order to determine if HIV replication induces IFN-I responses in CD4^{+} T cells, PHA/IL-2 activated primary cells were infected with the HIV-1 NL4-3 strain and expression of IFN-I and different ISGs was evaluated (Figure S1A). A clear induction of IFN-α and IFN-β mRNA (Figure 1A) as well as secretion of bioactive IFN-I protein (Figure 1B) was observed in the
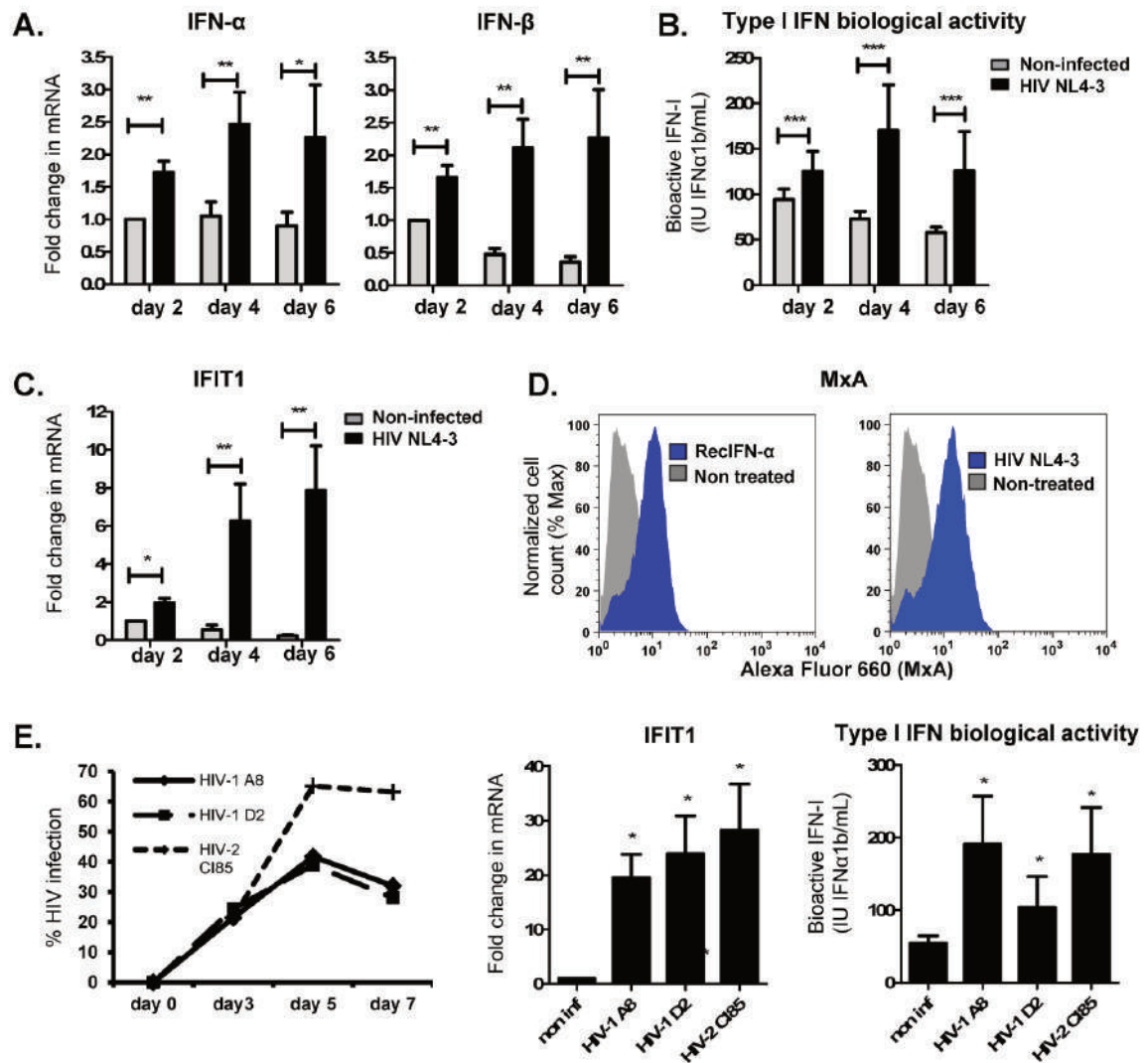
Figure 1. **HIV induces an IFN-I response in primary CD4+ T cells**.
(A). Fold change in IFN-α and IFN-β mRNA levels in HIV NL4-3-GFP-I infected (black bars) primary CD4+ T cells, at 2, 4 or 6 days after infection, relative to non-infected (grey bars) cells at day 2 (n=8).
(B) IFN-I protein activity in supernatant of cells in described in **(A)**.
(C) Fold change in *IFIT1* mRNA levels in cells in described in **(A)**.
(D) Flow cytometric analysis of non-treated, IFN-α treated (1000 IU/mL, IFNα1b) or HIV NL4-3 infected primary CD4+ T cells after intracellular staining for MxA, 3 days after infection or treatment. Histograms show expression of MxA in a representative of 4 experiments.
(E) Infection levels and IFN-I response in primary CD4+ T cells infected with T cell-line grown HIV-1 and HIV-2 primary isolates (n=6). (Left) Percentage of infected cells at different time points, measured by intracellular p24 staining (HIV-1) or by surface CD4 staining (HIV-2); (middle) fold change in *IFIT1* mRNA levels 5 days after infection relative to non-infected cells; (right) IFN-I protein activity measured in supernatant of cells described above.
Graphs in **(A-C), (E)** represent mean (± SEM). * p<0.05; ** p<0.01; *** p<0.001 (Wilcoxon matched pairs test). See also Figure S1.

HIV infected cultures. CD4$^+$ T cells purified by standard methods contain very few remaining plasmacytoid dendritic cells (pDCs) (Figure S1B). In all experiments, additional depletion of pDCs was furthermore performed prior to infection (typically ≤1 pDC per well of 250,000 cells), excluding them as the source of IFN-I. The levels of HIV-induced IFN-I varied in CD4$^+$ T cells derived from different donors, but usually progressed simultaneously with the level of HIV infection in the culture (Figure S1C). Similarly, IFN-I induction correlated with the amount of spreading infection when different initial inocula of HIV were used (Figure S1D). In addition to IFN-I, multiple ISGs were induced in the HIV infected CD4$^+$ T cell cultures, including *IFIT1* mRNA (Figure 1C, Figure S1D) and intracellular MxA (MX1) protein. The induction of the latter by HIV was comparable to the one obtained after treatment of cells with recombinant IFN-α (IFNα1b, 1000 IU/mL) (Figure 1D). Altogether, these results demonstrate that HIV is capable of inducing a bioactive IFN-I response in primary CD4$^+$ T cells. In order to ascertain that IFN-I induction by HIV was not restricted to the HIV-1 NL4-3 strain, CD4$^+$ T cells were infected with three different T-cell line expanded HIV-1 and HIV-2 primary isolates. Both HIV-1 group M as well as the HIV-2 isolate clearly induced *IFIT1* and bioactive IFN-I during infection, indicating that different HIV strains efficiently trigger an IFN-I response in primary CD4$^+$ T cells (Figure 1E).

HIV-induced IFN-I affects HIV-1 replication levels

Type I IFNs are known to block both early and late stages of the HIV life cycle, at least in part by inducing cell-intrinsic antiviral restriction factors (reviewed in Boasso, 2013). To determine if the IFN-I induced by HIV in CD4$^+$ T cells suppresses HIV replication, neutralizing antibodies against IFN-α and/or IFN-β were added to T cell cultures during HIV infection. Addition of both types of antibodies largely decreased induction of the ISG *IFIT1* by HIV (Figure 2A). When added separately, the IFN-α and –β antibodies each enhanced HIV replication to a similar extent. Combining both antibodies had a more potent effect (Figure 2B). This indicates that both IFN-α and –β contribute to the antiviral effect of IFN-I induced by HIV in CD4$^+$ T cells. On average, we observed a 1.6 fold increase in the number of HIV infected cells by IFN-α and –β blockage (Figure 2B). This effect is rather modest, consistent with previous reports describing a partial resistance of HIV-1 to IFN-I in culture (Vendrame et al., 2009, Cordeil et al., 2013).
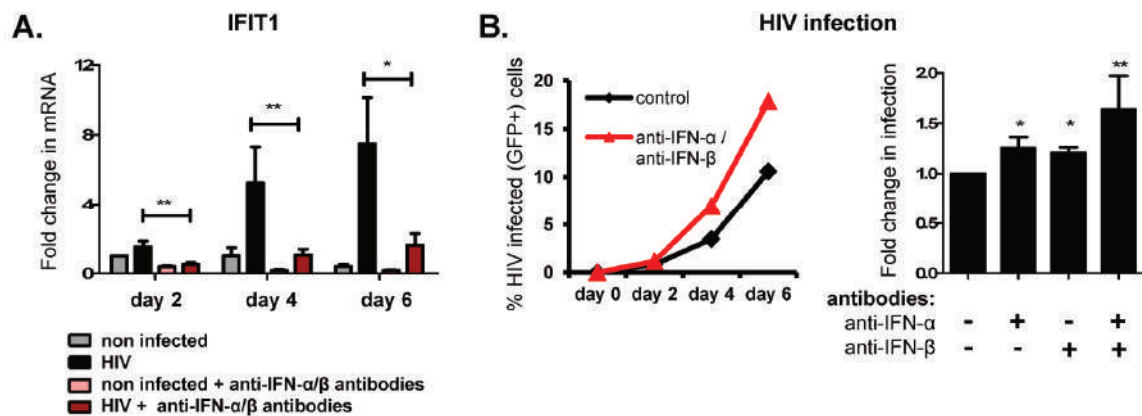
Figure 2. HIV-induced IFN-I suppresses HIV replication.
Primary CD4^{+} T cells were infected with HIV NL4-3-GFP-I in presence or absence of neutralizing antibodies (Ab) against IFN-α and IFN-β as indicated.
(A) Fold change in *IFIT1* mRNA levels at different time points after infection relative to non-infected non-treated cells at day 2 (n= 7 or 8).
(B) (Left) Percentage of infected cells (GFP+) at different time points in a representative experiment; (right) fold change in percentage of infected cells at peak of infection relative to infection in absence of antibodies (n=7).
Graphs in **(A)** and **(B)** (left) represent mean ± SEM. * p<0.05, ** p<0.01 (**(A)** Wilcoxon matched pairs test; **(B)** (left) Kruskal-Wallis ANOVA followed by **Dunn's** multiple comparisons **post hoc test**, treated compared to untreated control)**.**

HIV proviral integration is required for IFN-I induction in primary CD4^{+} T cells.

To determine which steps of the HIV-1 replication cycle are required for IFN-I induction in CD4^{+} T cells, cells were infected with HIV NL4-3 in the presence of reverse transcriptase (nevirapine) or integrase inhibitors (raltegravir). Induction of an IFN-I response was compared to the levels obtained in the presence of a protease inhibitor (ritonavir), in order to limit replication to a single cycle of infection. Inhibition of reverse transcription blocked IFN-I and *IFIT1* mRNA induction by HIV, while a small induction was observed in presence of the integrase inhibitor. However, induction was clearly enhanced when integration was allowed (Figure 3A and Figure S2A). The importance of viral integration for IFN-I induction was further confirmed with an HIV NL4-3 virus containing a point mutation that inactivates integrase (HIV D116N) (Engelman et al., 1995). This virus failed to trigger IFN-I and *IFIT1* mRNA induction (Figure 3B and Figure S2B). Since the number of remaining p24-expressing cells was much lower during infection with HIV D116N compared to infection in the presence of raltegravir (Figure S2B and S2A), remaining levels of integration might account for the small IFN-I induction by HIV observed with raltegravir (Figure 3A). Similar to the integrase-deficient virus, a *tat*-mutated virus did not induce significant levels of IFN-I and *IFIT1* mRNA during single cycle infection of CD4^{+} T cells (Figure 3C and Figure S2C), indicating that Tat-

dependent expression of viral genes, rather than the integration process itself, triggers IFN-I induction. Together, these results indicate that induction of an IFN-I response by HIV-1 in primary CD4^{+} T cells is dependent on integration and subsequent expression of the proviral genome.
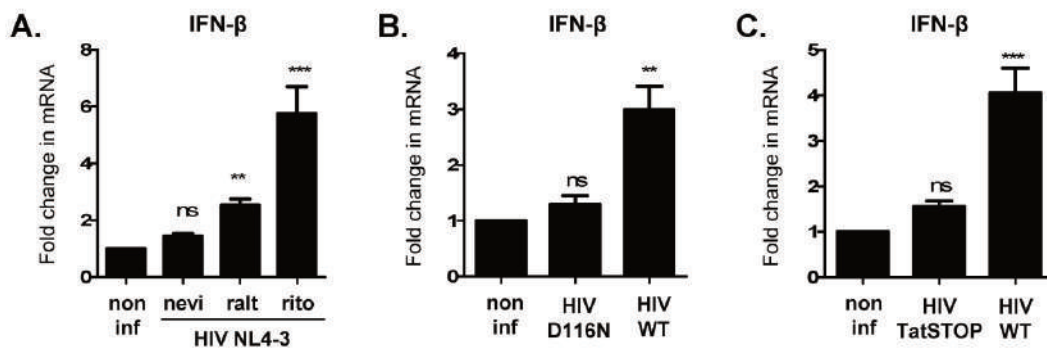

Figure 3. Type I IFN induction in primary CD4^{+} T cells requires HIV integration.
Fold change in IFN-β mRNA levels relative to non-infected cells in primary CD4^{+} T cells infected with:
(A) HIV NL4-3 in presence of a reverse transcription inhibitor (nevirapine, nevi), integration inhibitor (raltegravir, ralt) or protease inhibitor (ritonavir, rito) 48 h after infection (n=8);
(B) HIV NL4-3 wild-type (HIV WT) or D116N integrase mutant (HIV D116N) 24 h after infection (n=8);
(C) HIV NL4-3-HSA-I wild-type (HIV WT) or *tat*-mutated virus (HIV TatStop) in the presence of ritonavir 48h after infection (n=7).
Graphs represent mean ± SEM." ns" not significant, **p<0.01; ***p<0.001 (Friedman test followed by **Dunn's** multiple comparisons **post hoc test, infected compared to non-infected control)**. See also Figure S2.

IFN-I induction by HIV in primary CD4+ T cells is mediated through the cGAS pathway

Next, we aimed to identify host innate sensing pathways that are triggered by HIV-1 in CD4^{+} T cells, using shRNA-mediated knock-down of different IFN pathway genes. Initial screening experiments with shRNA-encoding lentiviral vectors indicated a role for the DNA-sensing cGAS receptor in IFN-I induction by HIV-1 (data not shown). We therefore performed knock-down of the known cGAS pathway members (reviewed in Cai et al., 2014) (*MD21D1*/cGAS, *TMEM173*/STING, *TBK1* and *IRF3*) in primary CD4^{+} T cells and measured the effect on IFN-β expression upon HIV infection. Transduction with shRNA-encoding lentiviral vectors decreased expression of all target genes (Figure S3A). The transduced primary CD4^{+} T cells were subsequently infected by co-culturing them with HIV-1 infected MT4 T cells, which allows for efficient infection of primary cells within 24 hours (Figure 4A). This system was chosen to avoid the use of long-term cultures of HIV-infected lentivirally transduced primary

CD4$^+$ T cells, in which the effect of knock-down of any IFN pathway gene on IFN-I induction was difficult to establish. IFN-β induction was neither detected in HIV infected MT4 cells cultured alone, nor in co-cultures of non-infected MT4 cells with primary CD4$^+$ T cells (Figure S3B). It was however clearly induced in infected co-cultures verifying that HIV-infected primary CD4$^+$ T cells are the source of IFN-I in this system. Knock-down of cGAS in the primary CD4$^+$ T cells highly decreased IFN-β induction (Figure 4B). This was further confirmed with two independent shRNA sequences targeting cGAS (Figure S3C). A similar effect on type-1 IFN response was observed after knock-down of the downstream signaling molecules in the cGAS pathway: STING, TBK1 and IRF3 (Figure 4B). Together, these results indicate that the cGAS pathway is triggered during HIV-1 infection of primary CD4$^+$ T cells, resulting in induction of IFN-I.
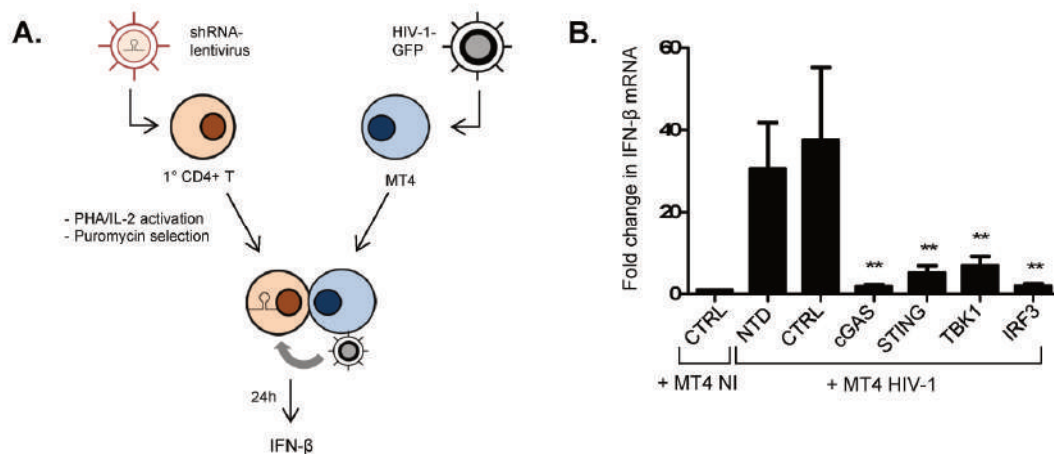

Figure 4. cGAS and downstream signaling molecules are required for IFN-I induction by HIV in primary CD4$^+$ T cells.
(A) Experimental set-up to measure host protein involvement in IFN-I induction by HIV-1: primary CD4$^+$ T cells are transduced with pLKO.1 shRNA-encoding vectors, activated with PHA/IL-2 and efficiently transduced cells are selected with puromycin. MT4 are in parallel infected with HIV NL4-3-GFP-I virus cells. Both cell types are subsequently co-cultured and IFN-β mRNA levels are measured 24 h after the start of co-culture.
(B) Fold change in IFN-β mRNA levels in co-cultures of non-infected or HIV infected MT4 cells with primary CD4$^+$ T cells transduced with vectors encoding shRNA targeting the indicated genes, with the shRNA control vector (CTRL) or non-transduced cells (NTD) (n=6).
Graph represent mean ± SEM. **$p < 0.01$ (Mann-Whitney test).
See also Figure S3.

IFN-I induction by HIV-1 is regulated by Vpu and Vpr

The involvement of the cGAS, a DNA sensor, suggests that recognition of reverse transcribed HIV cDNA is driving the innate immune response to HIV-1 in CD4$^+$ T cells.

Paradoxically, our previous results also indicated that IFN-I induction is dependent on integration and expression of the HIV provirus. We reasoned that newly expressed viral proteins might be required for cGAS-mediated IFN-I induction. In monocyte-derived dendritic cells (MDDCs), such a mechanism was recently demonstrated during HIV-1 infection in the presence of Vpx. Indeed, Manel et al. showed that sensing of HIV DNA by cGAS is prevented before integration by the HIV-1 capsid (CA), which might "shield" the viral DNA by forming complexes with the host factors cyclophilin A (CypA) (Lahaye et al., 2013; Manel et al., 2010) and/or CPSF6 (Rasaiyaah et al., 2013). The interaction of newly expressed HIV-1 capsid (CA) with CypA was required for sensing by cGAS after integration (Lahaye et al., 2013; Manel et al., 2010), and might function by relieving the "shielding". We therefore evaluated the importance of this interaction for IFN-I induction in CD4$^+$ T cells, by using the same panel of HIV-1 CA mutated viruses, complemented with a wild-type capsid protein during production, as described by these authors (Manel et al., 2010). Surprisingly, in T cells, viruses expressing capsid that is able (wild-type, T54A/N57A, Q63A/Q67A) or not (G89V) to interact with CypA induced a similar IFN-I response (Figure S4A). This indicates that IFN-I induction in primary CD4$^+$ T cells does not require CypA interaction with the newly expressed capsid.

We subsequently evaluated the role of two HIV-1 accessory proteins, Vpu and Vpr, in IFN-I induction by HIV in CD4$^+$ T cells. These proteins were previously implicated in IFN-I induction, although both were found to counteract rather than enable the IFN-I response in some (Doehle et al., 2012a; Galao et al., 2012; Laguette et al., 2014) but not all (Zahoor et al., 2014) studies.

We first evaluated the effect of Vpr on IFN-I response, by infecting CD4$^+$ T cells with wild-type (WT) or *vpr*-mutated HIV NL4-3 viruses. Surprisingly, *vpr*-mutated virus induced much lower levels of IFN-I and *IFIT1,* despite comparable infection rates (Figure 5A, Figure S4B). This indicates a potentiating rather than suppressing effect of Vpr on IFN-I induction by HIV in CD4$^+$ T cells. A similar lower IFN-I induction was observed with viruses containing a single Q65R substitution in the *vpr* gene, which is known to disrupt binding of Vpr to DCAF1 (DeHart et al., 2007). Viruses expressing the Vpr R77Q variant, associated with reduced pro-apoptotic activity of Vpr (Lum et al., 2003), were able to induce a potent IFN-I response (Figure 5B, Figure S4C). Infection with *vpr*-mutated viruses that were complemented with a wild-type Vpr protein during production resulted in a similarly reduced IFN-I response (Figure 5C, Figure S4D). This suggests that Vpr protein produced after integration of the virus, and not virion-incorporated Vpr is responsible for enhancing IFN-I induction. The potentiating effect of newly expressed Vpr could therefore explain why the IFN-I response occurs post-integration.
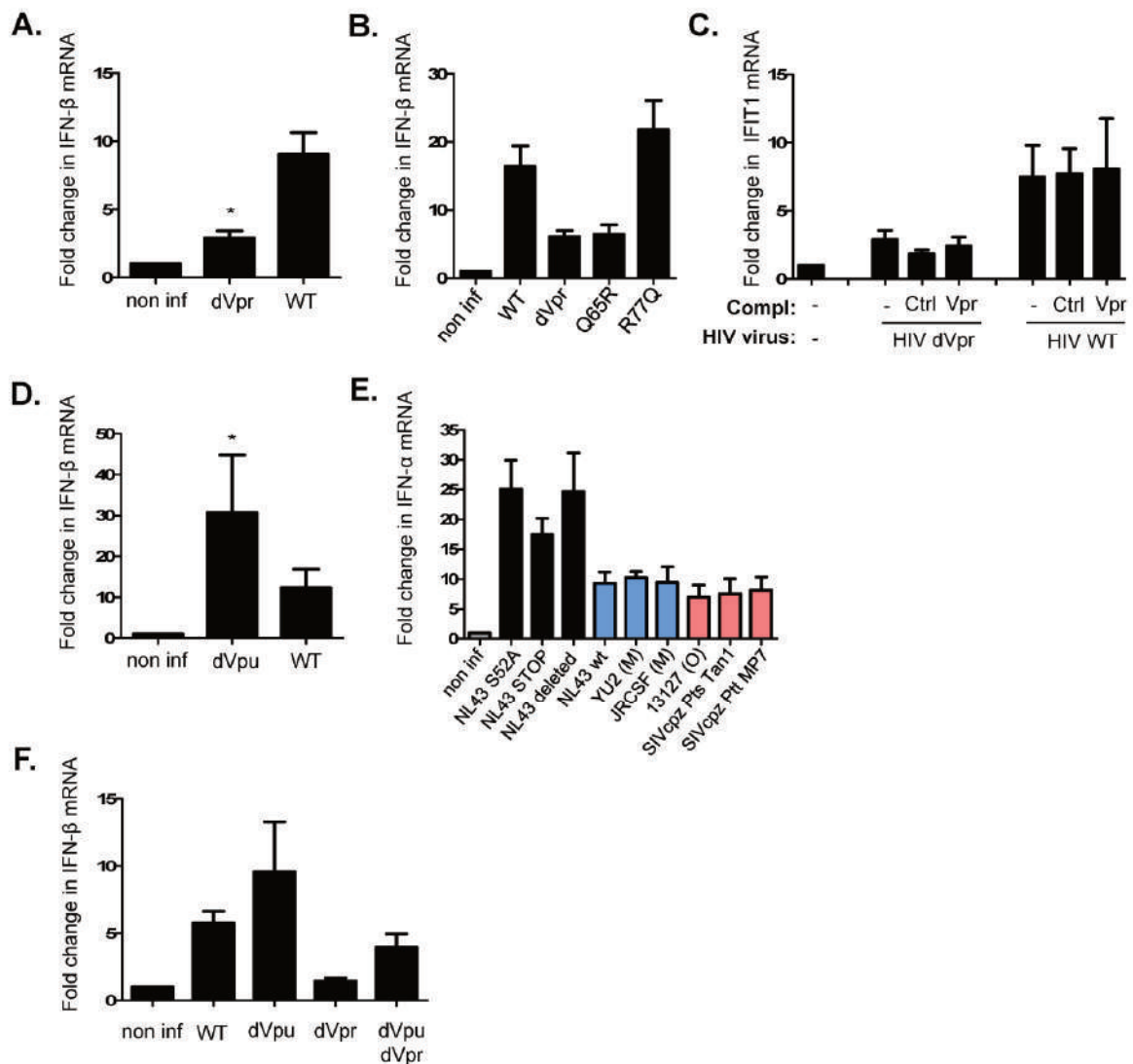
Figure 5. Effect of Vpu and Vpr on IFN-I response in primary CD4+ T cells.
IFN-I response relative to non-infected (non inf) cells in HIV infected primary CD4+ T cells.
(A) IFN-β mRNA levels 48-72 h after infection with HIV NL4-3-HSA-I wild-type (WT) or *vpr-* mutated virus (dVpr) (n=7).
(B) IFN-β mRNA levels 3 days after infection with HIV NL4-3 viruses: wild-type (WT), *vpr-* deleted (dVpr), viruses with Q65R or R77Q mutation in the *vpr* gene (n=4).
(C) *IFIT1* mRNA levels 48 h after infection with HIV NL4-3-HSA-I wild-type (WT) or *vpr-* mutated (dVpr) viruses, not complemented (-), complemented with a control vector (Ctrl) or Vpr (Vpr) during production. Infection was done in the presence of ritonavir (n=5). **(D)** IFN-β mRNA levels 48 h after infection with VSV-pseudotyped HIV NL4-3-I-EGFP wild-type (WT) or *vpu*-deleted (dVpu) virus (n=7).
(E) IFN-α mRNA levels 48 h after infection with VSV-pseudotyped HIV NL4-3-Vpu-IRES-Env viruses in which the original *vpu* allele was replaced by the *vpu* allele of the indicated HIV or SIV strain. Black bars show viruses with mutated NL4-3 *vpu* alleles, blue bars show viruses with *vpu* alleles that have antagonizing activity against human tetherin, red bars show viruses with *vpu* alleles that do not have antagonizing activity against human tetherin (n=4).
(F) IFN-β mRNA levels 48 h after infection with VSV-pseudotyped HIV NL4-3-I-EGFP wild-type (WT), *vpu*-deleted (dVpu), *vpr*-deleted (dVpr) or *vpu*- and *vpr*-deleted (dVpu dVpr) viruses (n=4).
Graphs represent mean ± SEM. *p<0.05 (Wilcoxon matched pairs test).
See also Figure S4.

We confirmed the suppressing effect of the Vpu protein: *vpu*-deleted HIV NL4-3 viruses induced a larger IFN-I response compared to WT viruses in primary CD4$^+$ T cells (Figure 5D, Figure S4E). The mechanism behind Vpu's effect on IFN-I induction is currently unclear, but might be related to its ability to inhibit NF-κB activation (Galao et al., 2012; Hotter et al., 2013; Wang et al., 2010), which in turn might (Galao et al., 2012) or might not (Bour et al., 2001; Pickering et al., 2014) be dependent on counteraction of the restriction factor and innate sensor tetherin by Vpu. To asses if Vpu's ability to downregulate tetherin is required to suppress the HIV-induced IFN-I response in primary CD4$^+$ T cells, we infected cells with HIV NL4-3 viruses containing *vpu* alleles with varying abilities to antagonize tetherin. While Vpu proteins from HIV-1 M viruses were previously shown to efficiently counteract tetherin, Vpu's from HIV-1 group O and SIVcpz viruses have no activity against human tetherin (Sauter et al., 2009). Interestingly, all these viruses induced a comparable IFN-I response that was lower than the one induced by Vpu-defective HIV NL4-3 viruses (Figure 5E, Figure S4F). This indicates that Vpu can dampen the IFN-I response induced by HIV in CD4$^+$ T cells, independent of its ability to antagonize tetherin. On the other hand, an HIV NL4-3 construct containing a mutation in the β-TrCP binding motif of Vpu (S52A) lost the ability to suppress the HIV-induced IFN-I response (Figure 5E, Figure S4F).

To simultaneously evaluate the effect of Vpu and Vpr on IFN-I induction during HIV infection, CD4$^+$ T cells were infected with matched HIV NL4-3 viruses carrying a deletion in *vpr*, *vpu* or both genes. The *vpr-vpu*-deficient virus induced an intermediate IFN-I response, usually larger than the one generated by a *vpr*-mutated virus, but smaller than the one obtained in absence of Vpu alone (Figure 5F, Figure S4G). This indicates that both proteins can exert their effect on IFN-I induction independently from each other.

Given the unexpected potentiating effect of Vpr on IFN-I production in activated CD4$^+$ T cells, we determined if Vpr plays a similar role in other immune cells. Myeloid dendritic cells are generally largely resistant to productive HIV-1 infection due to restriction by SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011). However, the addition of Viral-like Particles (VLP) carrying Vpx allows HIV-1 infection and promotes cGAS dependent IFN-I release (Lahaye et al., 2013; Manel et al., 2010). As we previously reported that HIV-infected T cells are much more potent inducers of IFN-I in MDDCs compared to cell-free virions (Puigdomenech et al., 2013), we measured bioactive IFN-I levels in co-cultures of Vpx-treated MDDCs and MT4 cells, infected with either HIV NL4-3 *vpr*-deficient or WT viruses (Figure 6A). MDDCs became productively infected and similar levels of Gag+ cells were observed with WT or *vpr*-mutated viruses (Figure 6B). HIV-1 infection of MDDCs was associated with IFN-I release in the supernatants. Interestingly, IFN-I production was strongly reduced in the absence of Vpr

(Figure 6C). These results show that Vpr potentiates IFN-1 production both in activated CD4$^+$ T cells and in dendritic cells.
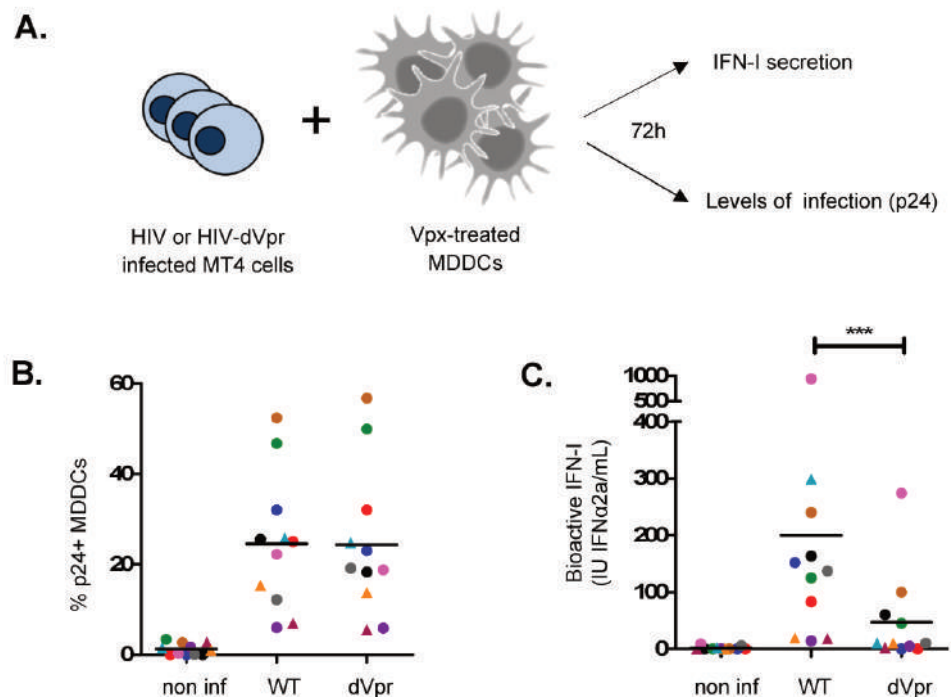

Figure 6. Vpr potentiates HIV-1 induced IFN-I production in dendritic cells.
(A) Experimental outline: MDDCs were pre-treated with Vpx VLPs to allow productive infection. MT4C5 cells were infected with HIV NL4-3 wild-type (WT) or *vpr*-deleted (dVpr) viruses and subsequently co-cultured with MDDCs. After 72 h, supernatant was harvested for quantification of IFN-1 and infection was monitored by intracellular p24 staining.
(B) Percentage of HIV-1 infected MDDCs (p24+).
(C) IFN-I activity in supernatants of co-cultures.
Graphs show data and mean of experiments in cells from 11 independent donors. ***p<0.001 (Wilcoxon matched pairs test).

Discussion

We show that activated CD4$^+$ T cells are able to sense HIV replication products and produce IFN-I through a post-integration mechanism that requires the cGAS receptor and its downstream signaling pathway. Furthermore, we found that this response is subject to both potentiation by Vpr and counteraction by Vpu. These findings are important, since part of the IFN-I released during the chronic phase of HIV infection is thus likely to originate from infected CD4$^+$ T cells and may contribute to the detrimental high levels of immune activation that drive progression to AIDS. One can speculate that the incomplete repression by Vpu or the inherent property of Vpr to enable sensing, is the result of an evolutionary drive on the

virus to orchestrate innate immune responses to its benefit, e.g. by stimulating the recruitment or activation of potential target cells or by impeding antiviral T-cell responses (Boasso et al., 2008; Gonzalez-Navajas et al., 2012; Hardy et al., 2013), thereby ultimately enhancing viral replication and thus transmission rates.

Triggering of an innate immune response during wild-type HIV-1 infection of non-manipulated primary cells has so far been observed in pDCs and aberrantly infected resting CD4^{+} T cells (Fonteneau et al., 2004, Doitsh et al., 2010). Except for two studies showing induction of ISGs (Nasr et al., 2012) or IFN-I protein (Imbeault et al., 2009), IFN-I production was not observed after HIV-1 infection of activated CD4^{+} T cells (Doehle et al., 2012a; Doehle et al., 2009; Goldfeld et al., 1991; Yan et al., 2010). We noticed that detection of IFN-I was highly enhanced when HIV infection levels were higher, e.g. after spreading replication. In addition, IFN-I induction was absent in MT4 cells (Figure 4B) and other T cell lines (data not shown). These are possible reasons why IFN-I induction by HIV in T cells might have been overlooked in the past. It was previously shown that degradation of HIV DNA by the host protein TREX1 prevents sensing in macrophages and CD4^{+} T cells (Yan et al., 2010). However, in line with our data, DNA synthesized by HIV-1 mutants is sensed despite the presence of TREX1 in macrophages (Rasaiyaah et al., 2013) and probably also in aberrantly HIV-1 infected resting CD4^{+} T cells (Monroe et al., 2014). It is possible that certain DNA products are not detected by TREX1 or that DNA is shielded from both TREX1 and cGAS prior to integration.

We show that activated CD4^{+} T cells are able to sense HIV through cGAS and produce IFN-I during productive infection. Since cGAS is considered to be activated by binding of cytosolic DNA (Kranzusch et al., 2013; Sun et al., 2013), the innate immune response is most likely triggered by HIV reverse transcription products. Our results also show that this response is only activated after integration of the virus and expression of new viral proteins, since *integrase*- or *tat*-deficient viruses or viruses cultured with an integrase inhibitor induced insignificant or strongly reduced levels of IFN-I. This indicates that sensing of HIV DNA in the cytoplasm is enabled through the action of newly expressed HIV replication products and implies that the simultaneous presence of both an integrated virus as well as DNA reverse transcription products in the cell is required. The latter could originate from either simultaneous or previous abortive infection events. As such, sensing *in vivo* is most likely to occur at sites where high viral infection rates are reached. The frequency of multiple infections per cell is still a topic of debate, but has been detected especially in splenic tissues of patients (Gratton et al., 2000; Jung et al., 2002; Suspene and Meyerhans, 2012). Interestingly, a study evaluating the nature of IFN-α producing cells in spleens of HIV+ patients, found that most of these cells were B and T lymphocytes, not pDCs (Nascimbeni et

al., 2009). In such niches, cGAS-mediated post-integration sensing could be a driving mechanism for IFN-I production.

Our data indicate that sensing of HIV DNA products is efficiently prevented prior to integration. Therefore, innate triggering in activated CD4$^+$ T cells seems to differ from the one in lymphoid tissue-derived resting CD4$^+$ T cells, in which incomplete HIV RT products accumulate due to abortive infection and HIV DNA is sensed by the IFI16 protein prior to integration (Doitsh et al., 2010; Monroe et al., 2014). In macrophages, it was suggested that HIV DNA is "shielded" by the host proteins CypA and/or CPSF6 (Rasaiyaah et al., 2013) and a similar mechanism might prevent sensing in activated CD4$^+$ T cells prior to integration. However, our results indicate that recognition of HIV DNA products in the cytosol is enabled after integration in activated CD4$^+$ T cells. As mentioned in the results, a comparable phenomenon has been described in MDDCs during Vpx complemented HIV-1 infection, in which interaction of newly expressed capsid with CypA might unmask cytosolic HIV DNA (Manel et al., 2010; Manel and Littman, 2011). However, in activated CD4$^+$ T cells, such an effect of newly expressed capsid was not observed by us, since normal levels of IFN-I were induced by viruses expressing different mutated capsid proteins, including the CypA interaction mutant G89V. Rather, we show the HIV-1 Vpr protein to be required for full post-integration IFN-I induction: *vpr*-deleted viruses induced lower levels of IFN-I and virion complementation experiments indicate that virion packaged Vpr is insufficient for IFN-I induction.

Our evaluation of Vpr-mutated viruses revealed that the interaction of Vpr with DCAF1 is important for potentiating the IFN-I response. DCAF1 is used by Vpr to recruit the DDB1-Cul4 E3 ubiquitin ligase complex. Although this complex is involved in different Vpr functions (Casey et al., 2010), the activation of the DNA damage response by Vpr is particularly interesting in this regard, because of the large overlap and interplay between cellular DNA damage response and innate immune response pathways (reviewed in Chatzinikolaou et al., 2014). Vpr might for instance enhance NF-κB activation or production of IRFs (and thereby IFN-I induction) as a consequence of its effect on DNA damage proteins (Brzostek-Racine et al., 2011; Hinz et al., 2010; Roshal et al., 2003). Activation of NF-κB by Vpr has indeed been demonstrated by several groups (Liu et al., 2014; Roux et al., 2000). Alternatively, Vpr might modify cGAS sensing itself e.g. by its binding to CypA (Zander et al., 2003) or unintegrated HIV DNA (de Rocquigny et al., 2000). Correlation studies of Vpr's ability to potentiate IFN-I induction and other known Vpr functions will be required to obtain further mechanistic insights. In contrast to the *integrase*-mutant, IFN-I induction by the *vpr*-deleted viruses was not completely blocked in the CD4$^+$ T cells. Especially in cells infected with *vpu-vpr-* deleted viruses, IFN-I was readily detectable. Therefore, Vpr is most likely an important, but not the

sole viral factor triggering post-integration IFN-I induction. A similar IFN-I potentiating effect of Vpr was recently described in monocyte-derived macrophages (MDMs) (Zahoor et al., 2014) and confirmed herein for HIV-1 infection of Vpx treated MDDCs. In contrast, other studies have suggested a suppressive role of Vpr in IFN-I production, either in non-Vpx-treated MDDCs (Harman et al., 2011), in MDMs (Mashiba et al., 2014) or in non-immune cell lines, such as HEK293 or HeLa (Doehle et al., 2009; Laguette et al., 2014; Okumura et al., 2008). However, the mechanism of IFN-I induction in these cells by (*vpr*-deleted) HIV is not clear and is unlikely to occur through cGAS-mediated post-integration sensing, as in CD4$^+$ T cells and Vpx-treated MDDCs (Lahaye et al., 2013). Our data strongly indicate that in these cell types, cGAS-mediated IFN-I induction is enhanced rather than suppressed by Vpr. We speculate that potentiation of post-integration IFN-I induction by Vpr results from a Vpr-host interaction with other indispensable beneficial effects during the viral life cycle, e.g. NF-κB activation to enhance proviral expression or as a consequence of G2 arrest. While the innate immune responses resulting from this post-integration Vpr interaction might on one hand enhance recruitment and activation of surrounding HIV target cells, high levels of IFN-I are likely to be avoided by virus. As such the virus might have employed another late HIV-1 protein, Vpu, to partially counteract the Vpr effect and keep IFN-1 levels in check.

In line with previous reports (Doehle et al., 2012a; Galao et al., 2012), we observed a higher IFN-I induction in CD4$^+$ T cells by viruses lacking Vpu. This Vpu effect was previously attributed to either degradation of IRF3 (Doehle et al., 2012b) or tetherin counteraction, thereby preventing tetherin-mediated NF-κB activation (Galao et al., 2012). However, Vpu-mediated IRF3 degradation was not observed by us and others (Galao et al., 2012; Hotter et al., 2013). Furthermore, the loss of IFN-I induction we observed after IRF3 knock-down, indicates that IRF3 is activated by HIV despite the presence of Vpu. In our experiments, IFN-I induction was suppressed to an equal extent by viruses with and without tetherin-antagonizing ability, indicating that targeting of tetherin and suppressing IFN-I are two separate functions of Vpu. However, a virus with a mutation in the β-TcrP binding domain (S52A) of Vpu failed to counteract IFN-I induction in CD4$^+$ T cells. It was previously suggested in cell lines that sequestration of β-TcrP by Vpu could inhibit down-stream NF-κB activation (Besnard-Guerin et al., 2004; Bour et al., 2001). cGAS-mediated activation of STING is known to induce activation of NF-κB (Kato et al., 2013) and silencing of NF-κBp65 was previously shown to partially decrease IFN-β production in response to dsDNA (Abe and Barber, 2014). Therefore, Vpu might dampen IFN-I induction in primary CD4$^+$ T cells by interfering with cGAS-mediated NF-κB activation.

Our work provides insight in the virus-host interplay that regulates HIV-1 sensing in its main target cells. Contribution of HIV infected CD4$^+$ T cells to IFN-I levels in the chronic phase of

infection and as such to the natural disease course, might provide the basis for novel therapeutic strategies.

Experimental Procedures

Viral constructs, experimental outline and applied methods are indicated in figure legends and explained in detail in Extended Experimental Procedures.

Quantification of IFN-I responses: IFN-α, IFN-β and *IFIT1* mRNA levels were measured by qPCR using specific primers as listed in Table S1; IFN-I biological activity was measured in supernatant by HL-116-luciferase reporter cells; MxA protein levels were quantified by intracellular staining and flow cytometry.

HIV viral constructs and infection: HIV NL4-3 derived constructs are listed in Table S2. Viruses were produced in 293T cells using standard methods, complemented if indicated by co-transfection with Gag- or Vpr-expressing plasmids. HIV-1 and HIV-2 isolates were propagated on peripheral blood mononuclear cells (PBMCs) and Jurkat CD4 CCR5 cells. Primary CD4+ T cells, obtained from blood of healthy donors, underwent extra pDC depletion and were infected with HIV by spinoculation. Antiretrovirals or IFN neutralizing antibodies were added prior or immediately after spinoculation respectively and maintained during culture. Monocytes were isolated from PBMCs and differentiated in presence of IL-4 and GM-CSF. MDDCs were treated with VLPs carrying Vpx and infected with HIV-1 through co-culture with MT4C5 cells.

Knock-down of host genes and co-culture with HIV-1 infected cells: origin and properties of pLKO.1-puro shRNA-encoding lentiviral construct are listed in Table S3. Lentiviruses were produced in 293T cells using Mission™ Lentiviral Packaging Mix. Primary CD4+ T cells were transduced using polybrene, stimulated with PHA/IL-2, selected with puromycin and co-cultured with HIV infected MT4 cells.

Statistical analysis: Statistical tests were performed with GraphPad Prism 5.0 as indicated in the figure legends.

Author contributions

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Supplementary figures

Figure S1
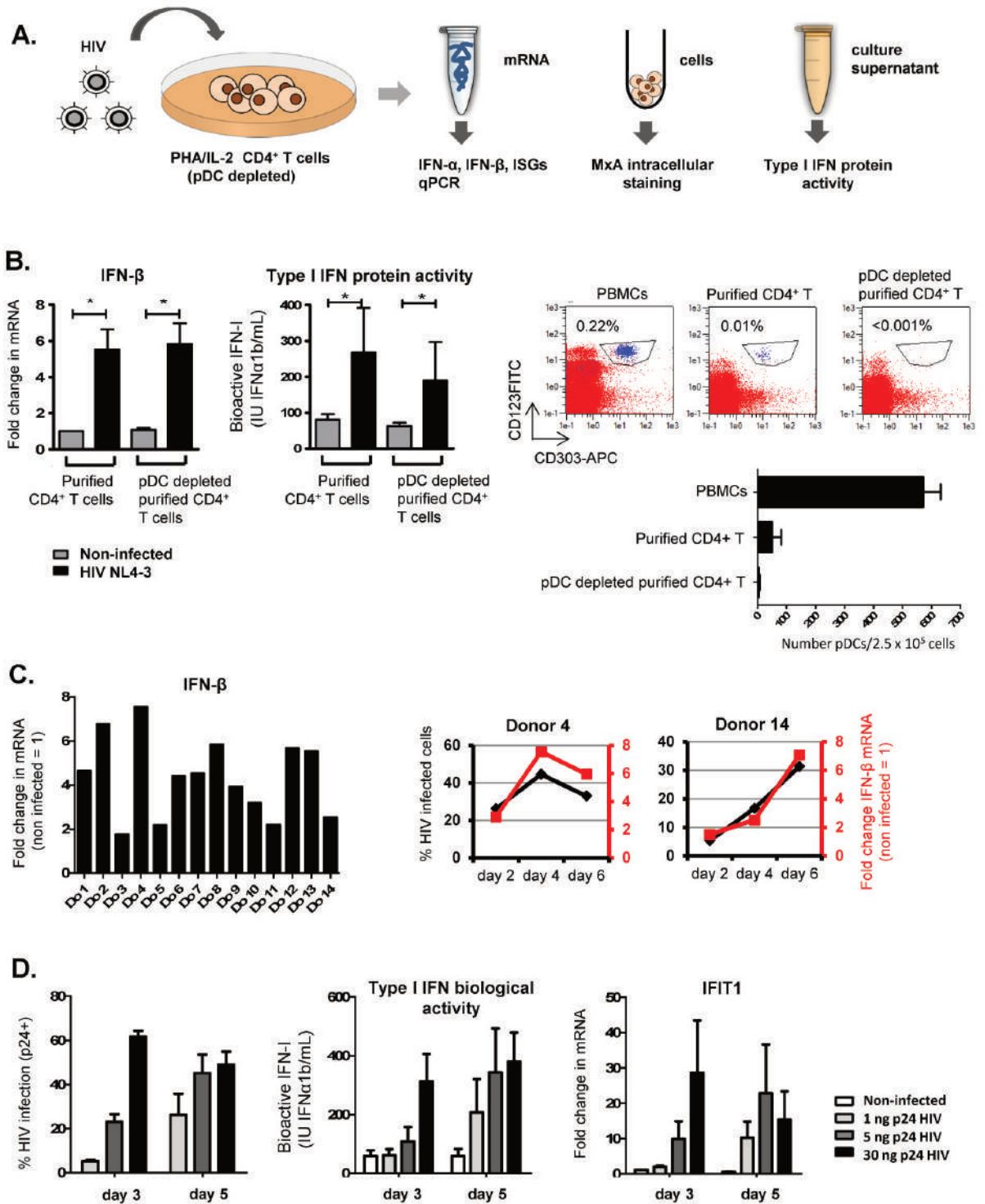
Characterization of IFN-I response during HIV infection primary CD4$^+$ T cells.
Related to Figure 1.
(A) Experimental scheme to measure type 1 IFN response: PHA/IL-2 activated, pDC depleted primary CD4$^+$ T cells are infected with HIV. IFN response is quantified by isolation of mRNA from the cells to measure IFN-α, IFN-β and IFN-stimulated gene (ISGs) expression by qPCR, by intracellular staining of the cells for MxA protein and by measuring IFN-I activity in the supernatant of the cells with the HL-116 assay.
(B) (Right) Fold change in IFN-β mRNA levels relative to non-infected cells and (middle) IFN-I biological activity for purified CD4$^+$ T cells with or without additional pDC depletion at peak levels of HIV NL4-3-GFP-I or HIV NL4-3-HSA-I infection (4 or 6 days after infection) (n=6); (left) dot-plots of flow cytometric data showing expression of pDC markers (CD123-FITC versus CD303-APC) in PBMCs and purified CD4$^+$ T cell populations with or without additional pDC depletion, immediately after isolation of cells in a representative experiment. Red dots represent cells gated on viable (propidium iodide-negative) cells, blue represents cells with additional gating on CD304+CD123+ cells. Numbers indicate the percentage of CD304+CD123+CD303+ cells among viable cells. Graph shows average number of CD304+CD123+CD303+ cells per 250,000 cells detected as described above (n=6). Purified CD4$^+$ T cells populations typically contained around 20 pDC's per culture of 250,000 cells, while CD4$^+$ T cells with additional pDC depletion contained ≤ 1 pDC per culture.
(C) (Left) Fold change in IFN-β mRNA levels relative to non-infected cells, 4 days after HIV NL4-3-GFP-I or HIV NL4-3-HSA-I infection of primary CD4$^+$ T cells derived from 14 different donors; (right) percentage of HIV infected cells (left y-axis) and fold change in IFN-β mRNA levels relative to non-infected cells (right y-axis) at different time points after infection in cells of two of these donors.
(D) Infection levels and IFN-I response in primary CD4$^+$ T cells infected with indicated amounts of HIV NL4-3 virus (n=3). (Left) Percentage of infected cells at different time points after infection, measured by intracellular p24 staining; (middle) IFN-I protein activity in supernatant of cells; (right) fold change in *IFIT1* mRNA levels at different time points after infection, relative to non-infected cells at day 3.
Graphs in **(B)**, **(E)** represent mean ± SEM. *p<0.05 (Wilcoxon matched pairs test)

Figure S2
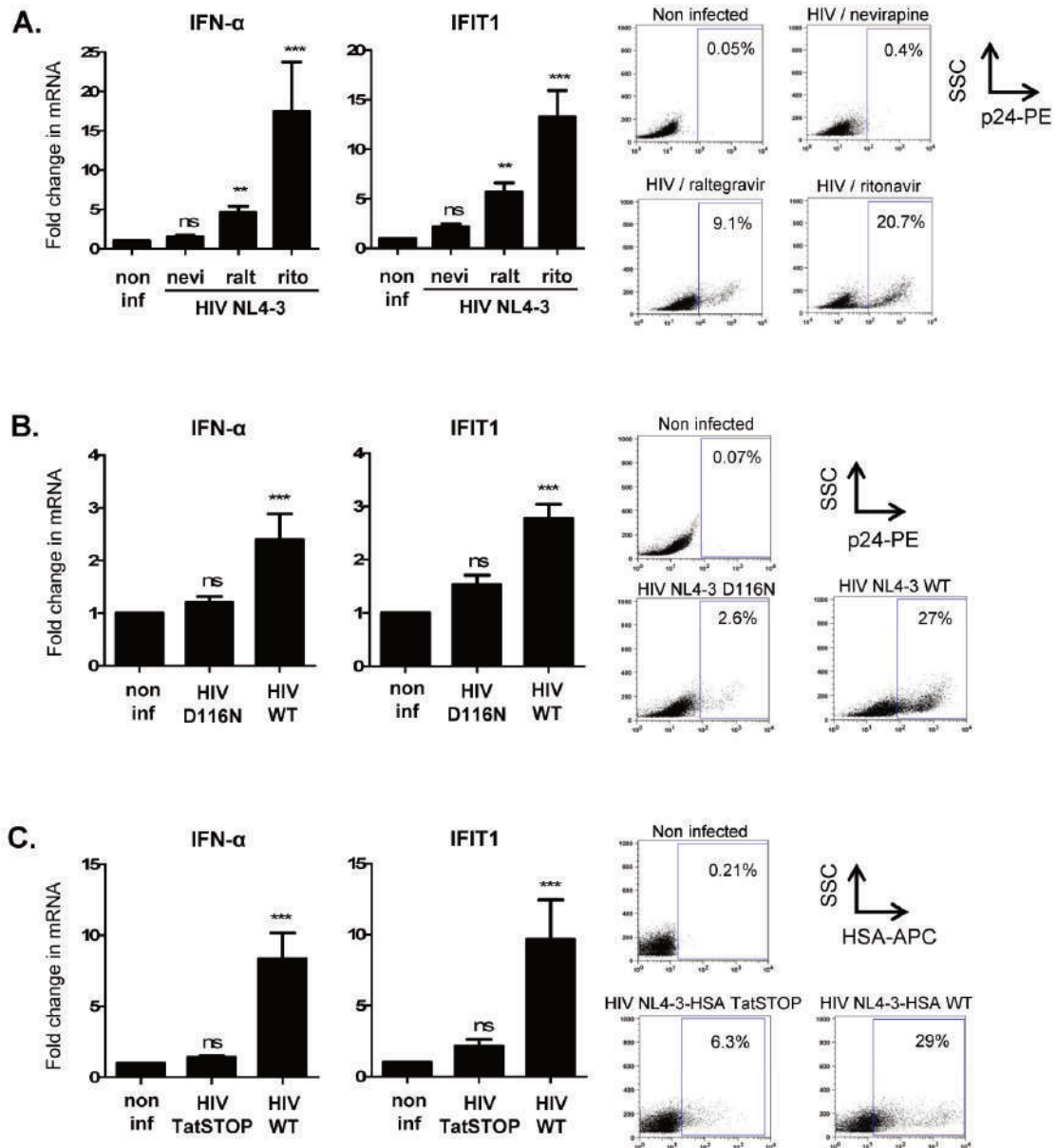
Requirement of HIV-1 integration and Tat-mediated viral gene expression for IFN-I induction in primary CD4$^+$ T cells.

Related to Figure 3.

(Left) Fold change in IFN-α and *IFIT1* mRNA relative to non-infected (non inf) cells; (right) dot-plots of flow cytometric data showing side scatter (SSC) versus p24-PE **(A, B)** or HSA-APC **(C)** fluorescent intensity levels in primary CD4$^+$ T cells infected with:

(A) HIV NL4-3 in presence of a reverse transcription inhibitor (nevirapine, nevi), integration inhibitor (raltegravir, ralt) or protease inhibitor (ritonavir, rito) 48 h after infection (n=8).

(B) HIV NL4-3 wild-type (HIV WT) or D116N integrase mutant (HIV D116N) 24 h after infection (n=8).

(C) HIV NL4-3-HSA-I wild-type (HIV WT) or Tat mutated virus (HIV TatStop) in the presence of ritonavir 48 h after infection (n=7).

Graphs represent mean ± SEM." ns" not significant, **$p<0.01$; ***$p<0.001$ (Friedman test followed by **Dunn's** multiple comparisons **post hoc test**, infected compared to non-infected control).

Figure S3
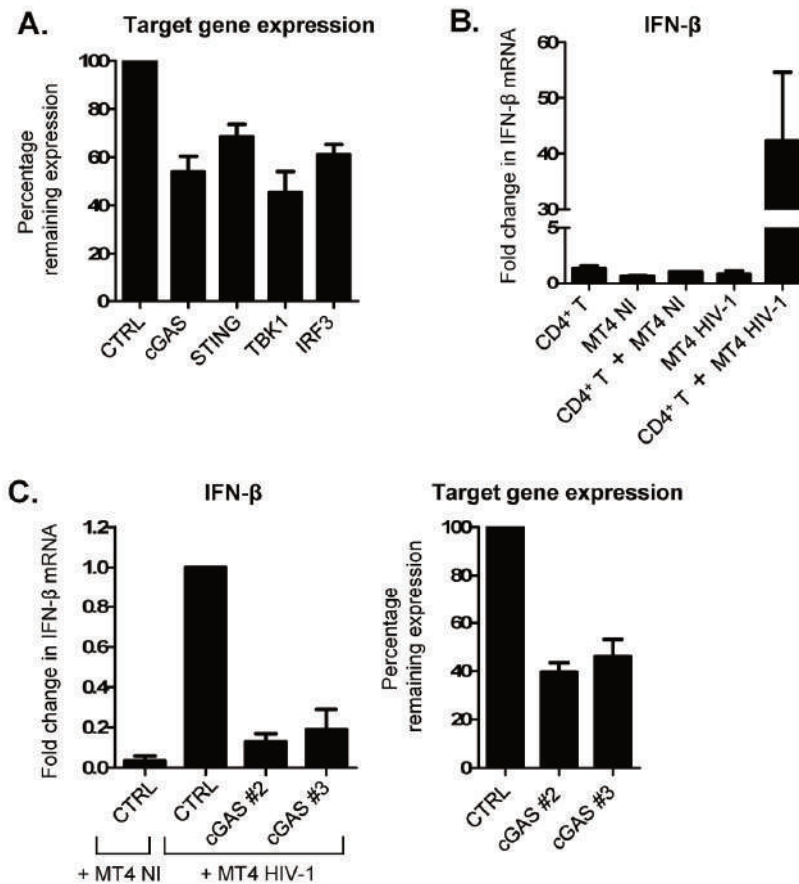
cGAS is required for IFN-I induction by HIV in primary CD4$^+$ T cells.
Related to Figure 4.
(A) Percentage of remaining mRNA of the indicated genes in primary CD4$^+$ T cells transduced with shRNA encoding pLKO.1 vectors targeting the indicated genes, relative to levels in cells transduced with a non-targeting shRNA control vector (100%) immediately prior to co-culture (n=2-4).
(B) Fold change in IFN-β mRNA levels in cultures of non-infected primary CD4$^+$ T cells transduced with the scrambled shRNA control vector (CD4$^+$ T) (n=4), non-infected MT4 cells (MT4 NI), HIV NL4-3-GFP-I infected MT4 cells (MT4 HIV-1) (n=3) or co-cultures of these cells (n=4), relative to levels in co-cultures with non-infected MT4 cells.
(C) (left) Fold change in IFN-β mRNA levels in co-cultures of non-infected (NI) or HIV NL4-3-GFP-I infected MT4 cells with primary CD4$^+$ T cells transduced with shRNA targeting cGAS (#2 and #3 represent use of different shRNA sequences) or the shRNA control vector (CTRL), relative to levels obtained in co-cultures of CTRL cells and HIV-infected MT4 cells (n=2); (right) percentage of remaining cGAS mRNA in primary CD4$^+$ T cells described above immediately prior to co-culture, relative to levels in cells transduced with a non-targeting shRNA control vector (CTRL) (100 %) (n=2).
Graphs in represent mean ± SEM.

Figure S4
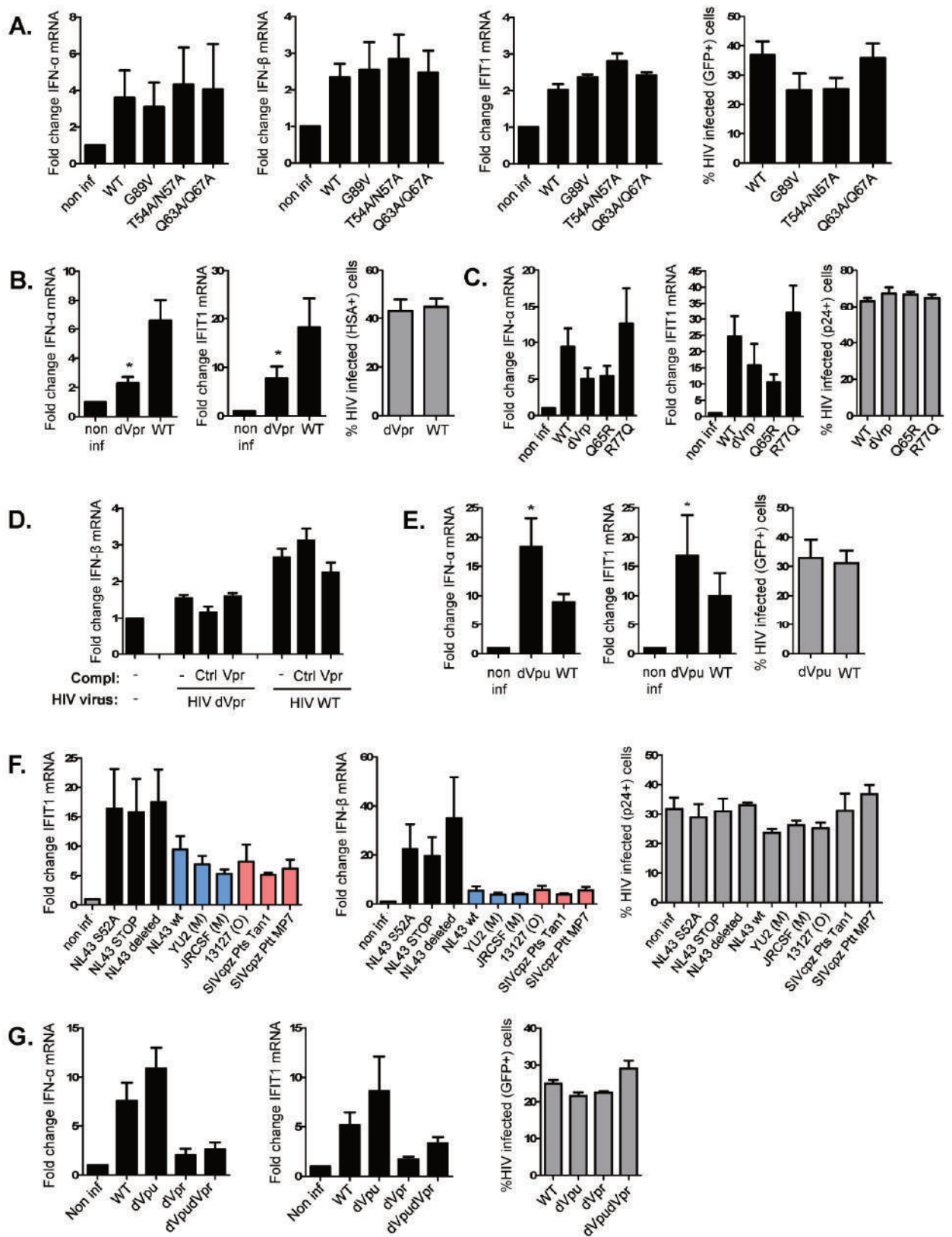
Effect of HIV-1 Capsid-CypA interaction, Vpu and Vpr on HIV-induced IFN-I response in primary CD4$^+$ T cells.

Related to Figure 5.

Infection levels and IFN-1 response in primary CD4$^+$ T cells, relative to non-infected (non inf) cells.

(A) Infection with VSV-pseudotyped HIV-1 LAI dEnv dNef viruses (WT) or with viruses with additional mutations in capsid (G89V, T54A/N57A,Q63A/Q67A), 48 h after infection (n=2). All viruses were complemented with wild-type capsid during production. (Left to right) Fold change in IFN-α, IFN-β or *IFIT1*, mRNA levels; percentage of infected cells (GFP+).

(B) Infection with HIV NL4-3-HSA-I wild-type (WT) or *vpr*-mutated virus (dVpr), 48-72 h after infection (n=7). (Left and middle) fold change in IFN-α and *IFIT1* mRNA levels; (right) percentage of infected (HSA+) cells.

(C) Infection with HIV NL4-3 viruses: wild-type (WT), *vpr*-deleted (dVpr), viruses with Q65R or R77Q mutation in the *vpr* gene, 72 h after infection (n=4). (Left and middle) Fold change in IFN-α and *IFIT1* mRNA; (right) percentage of infected (p24+) cells.

(D) Infection with HIV NL4-3-HSA-I *vpr*-mutated (dVpr) or wild-type (WT) virus, not complemented (-), complemented with a control vector (Ctrl) or with Vpr (Vpr) during production. Infection was done in the presence of ritonavir (n=5). Fold change in IFN-β mRNA levels 48 h after infection.

(E) Infection with VSV-pseudotyped HIV NL4-3-I-EGFP *vpu*-deleted (dVpu) or wild-type (WT) virus, 48 h after infection (n=7). (Left and middle) fold change in IFN-α and *IFIT1* mRNA levels; (right) percentage of infected (GFP+) cells.

(F) Infection with VSV-pseudotyped HIV-NL4-3-Vpu-IRES-Env viruses in which the original *vpu* allele was replaced by the *vpu* allele of the indicated HIV or SIV strain. Black bars show viruses with mutated NL4-3 *vpu* alleles, blue bars show viruses with *vpu* alleles that have antagonizing activity against human tetherin, red bars show viruses with *vpu* alleles that do not have antagonizing activity against human tetherin, 48 h after infection (n=4). (Left and middle) fold change in *IFIT1* and IFN-β mRNA levels; (right) percentage of infected (p24+) cells.

(G) Infection with VSV-pseudotyped HIV NL4-3-I-EGFP wild-type (WT), Vpu deleted (dVpu), Vpr deleted (dVpr) or Vpu and Vpr deleted (dVpu dVpr) viruses, 48 h after infection (n=4). (Left and middle) Fold change in IFN-α and *IFIT1* mRNA levels; (right) percentage of infected (GFP+) cells.

Graphs represent mean ± SEM. *p<0.05 (Wilcoxon matched pairs test)

Extended Experimental Procedures

Isolation and culture of cells

Primary CD4$^+$ T cells were isolated from buffy coats of healthy donors (Red Cross, Ghent, Belgium), donated after informed consent, approved by Ghent University ethical committee. Peripheral blood mononuclear cells (PBMCs) were obtained on Lymphoprep (Axis-Shield PoC, Oslo, Norway) and used for CD4+ T cell isolation by negative selection with a commercial kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Depletion of pDCs was done on PBMCs prior to isolation of CD4$^+$ T cells, by negative selection after staining with CD304-PE antibody (clone AD5-17F6, Miltenyi Biotec) in the presence of human FcR blocking reagent (Miltenyi Biotec) and subsequent staining with anti-PE paramagnetic microbeads (Miltenyi Biotec). Purity of the CD4$^+$ T cell population was measured by flow cytometry (MACSquant® Analyzer using MACSQuantify v2.4 software, Miltenyi Biotec), showing a fraction of at least 95 % CD4+CD3+ double positive cells and less than 0.0004 % CD123+CD304+CD303+ cells (measured on 1.5 x10^6 cells, corresponds to ≤1 pDC per culture of 250,000 cells). Antibodies used for staining were CD3-PE (clone SK7, Becton Dickinson (BD) Biosciences, Erembodegem, Belgium) and CD4-APC (clone MT4 66, Miltenyi Biotech) or CD123-FITC (clone AC145, Miltenyi Biotech), CD303-APC (clone AC144, Miltenyi Biotech) and CD304-PE. Except when used for lentiviral transduction (see below), primary CD4$^+$ T cells cells were cultured for 3 days after isolation at 37 °C in a 5 % (v/v) CO_2 humidified atmosphere in RPMIc: Gibco® RPMI medium 1640 (Life Technologies, Merelbeke, Belgium) supplemented with 2 mM L-glutamin (Life Technologies), 10 % (v/v) heat-inactivated fetal calf serum (Hyclone, Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). RPMIc was supplemented with 20 ng/mL interleukin-2 (IL-2; specific activity 10 U/ng, Peprotech, London, United Kingdom), and with 1 µg/mL phytohemagglutinin (PHA) mitogen (Thermo Fisher Scientific). Subsequently, cells were infected with HIV. 293T cells (DZSM, Braunschweig, Germany), MT4 cells (kind gift from Dr. Katrien Fransen, Institute of Tropical Medicine, Antwerp, Belgium) used for co-culture with primary CD4$^+$ T cells and Jurkat CD4 CCR5 cells (Programme EVA Centre for AIDS Reagents, NIBSC, UK) were cultured at 37°C in a 7 % (v/v) CO_2 humidified atmosphere, in IMDMc: IMDM (Life Technologies) supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamin, 100 U/mL penicillin and 100 µg/mL streptomycin. MT4C5 cells (a derivate MT4 cells expressing CCR5) were used for co-culture with MDDCs and were cultured as described in Lepelley et al. 2011. HL-116 cells (kindly provided by Dr. Uze, University of Montpellier II, France (Uze et al., 1994)) were cultured at 37 °C in a 7 % (v/v) CO_2 humidified atmosphere in DMEM (Life Technologies) supplemented with 10 % fetal bovine serum and 2 mM L-glutamin (DMEMc).

Monocytes were isolated by positive CD14 immunomagnetic selection (Miltenyi Biotec) from PBMCs of healthy donors. MDDCs were generated by culturing monocytes for 5 days in presence of IL-4 (50 ng/mL) and GM-CSF (10 ng/mL) as described in (Puigdomenech et al., 2013).

Viral constructs

Characteristics and sources of the plasmids used for production of HIV-1 in this study are described in Table S2.

The *tat*-mutated and *vpr*-mutated NL4-3-IRES-HSA vectors were created by site directed mutagenesis on a part of the parental backbone, followed by substitution of the mutated fragment (with verified sequence) in the parental backbone. For mutation of *tat*, a TGA stop-codon was introduced after the ATG start codon of the first exon of *tat.* For mutation of *vpr*, the CTAGAGCTTTTAGAGAA sequence at the end of the *vif-vpr* overlapping reading frame, was replaced by a CTAG**TGA**T**TGA**A**TAG**GAA sequence, thereby introducing a stop-codon in *vpr* in each of the reading frames after the end of *vif*.

The HIV NL4-3 constructs encoding viruses with Q65R and R77Q mutations in Vpr, were generated by introducing punctual mutations in HIV NL4-3 WT with the QuikChange XL Site-Directed Mutagenesis kit (Stratagene, Agilent Technologies, La Jolla, CA), using the following primers:

Mutation	Forward primer	Reverse Primer
Q65R	5'-agtggaagccataataagaattctgagacaactgctgtttattcattttcaga-3'	5'-tctgaaatgaataaacagcagttgtctcagaattcttattatggcttccact-3'
R77Q	5'-gaattatgcctattctgctatgttgacacccaattctgaaatgaata-3'	5'-tattcatttcagaattgggtgtcaacatagcagaataggcataattc-3'

The LZRS-Vpr-IRES-NGFR retroviral vector used for complementation of HIV viruses with a wild-type Vpr protein during production, was constructed by PCR amplification of the *vpr* gene from the NL4-3-IRES-HSA vector and introduction into LZRS-IRES-NGFR (Stove et al., 2005), expressing Nerve Growth Factor Receptor as a marker gene.

Production of HIV

HIV production was done by transfection of 293T cells with Calcium Phosphate Transfection Kit (Life Technologies) or JetPei® (Polyplus, Sélestat, France), according to manufacturer's instructions using 5 µg of HIV plasmid per 750,000 cells. For VSV-pseudotyped viruses, cells were transfected with 1 µg pMD.G plasmid (Stove et al., 2005) and 4 µg of HIV plasmid instead. Medium was refreshed 24 h after transfection. Viral supernatant was harvested after

48 h and centrifuged at 900 g for 10 min, to clarify the supernatant from remaining cells. Viral titer was determined by measuring reverse transcriptase activity in the supernatant with the SG-PERT assay (as described in Vermeire et al., 2012). Production of VSV-pseudotyped HIV NL4-3 WT and dVpr viruses used in context of MDDC experiments was performed as previously described (Puigdomenech et al., 2013).

For production of wild-type and *gag*-mutated VSV-pseudotyped HIV-1 LAI dEnv dNef viruses complemented with a wild-type capsid protein, 293T cells were transfected with 2.7 µg HIV plasmid, 1.7 µg pCMV-ΔR8.91 (kindly provided by Dr. N. Manel (Manel et al. 2010)) and 0.7 µg pMD.G plasmid using JetPei®. Viral supernatant was harvested as indicated above.

For complementation of wild-type and mutated HIV NL4-3-HSA-I virions with a wild-type Vpr protein, 293T cells were transfected with 1.7 µg HIV plasmid and 3.3 µg LZRS-Vpr-IRES-NGFR (Vpr complemented) or LZRS-IRES-NGFR (control vector) using JetPei®. Viral supernatant was harvested as indicated above and transfection efficiency was determined at moment of harvesting by surface staining with anti-CD24-APC and anti-NGFR-PE (clone ME20.4, Chromaprobe, Maryland Heights, MO). Percentage of $CD24^+NGFR^+$ cells was determined by flow cytometry (FACSCalibur, BD Biosciences) and always > 40 %. Efficacy of Vpr complementation using this protocol was previously validated by measurement of Vpr protein levels by Western Blot. In some experiments, supernatants of complemented viruses with low titer were concentrated using PEG-it precipitation of viral particles following manufacturer's instructions (PEG-it^{TM} Viral Precipitation Solution, System Biosciences, Mountain View, CA) and subsequent resuspension in IMDMc in 1/5 of the original volume.

HIV-1 group M A8 isolate (92UG029) (Bachmann et al., 1994) was obtained from the NIH AIDS Research and Reference Reagent Program. The HIV-1 group M D2 (VI203) (Louwagie et al., 1993) and HIV-2 CI85 (Arien et al., 2005) were previously isolated from patients attending the AIDS clinic at the Institute of Tropical Medicine in Antwerp, Belgium, with the approval of the ethical committee after written informed consent. Virus stocks were initially obtained after propagation in short-term cultures of PBMCs as described before (Arien et al., 2005) and subsequently expanded by infection and culture of Jurkat CD4 CCR5 cells. To this end, cells were plated at 5×10^5 cells per well of a 12-well flat-bottom plate (BD Biosciences) in 1 mL IMDMc and 1 mL of PBMC-derived viral supernatant was added to the cells (viral titer ranging from 350-1150 mU RT/ mL, equivalent to 35-210 ng p24/mL). Supernatant of the Jurkat CD4 CCR5 cells was collected at peak of infection and centrifuged at 900 g for 10 min, to clarify the supernatant from remaining cells.

<u>Infection with HIV</u>

For HIV infection of primary $CD4^+$ T cells, 250,000 cells were seeded per well in a 96-well flat-bottom plate (BD Biosciences) in RPMIc supplemented with 20 ng/mL IL-2 (final

concentration). Depending on the experiment 6-1700 mU RT (equivalent to 1 – 300 ng p24 of HIV virus was added per well in a final volume of 200 μL, with low concentrations (1-40 ng p24) used for long-term experiments (read-out until day 5-7 after infection) and high concentrations (60-300 ng p24) used for short-term experiments (last read-out at day 1-3 after infection)). Cells were subsequently spinoculated at 950 g for 90 min at 32 °C. Medium was refreshed with RPMIc (+ IL-2) immediately after spinoculation and for half of the volume every 2 days during subsequent culture. At the indicated time points after infection, cells were harvested to determine HIV infection levels and remaining cells were lysed in 700 μL QIAZOL (miRNeasy mini kit, QIAGEN, Venlo, The Netherlands) according to manufacturer's instruction. Supernatant of the cells was collected after spinoculation of the cells at 350 g for 10 min at 4 °C. Lysates and supernatants were stored at -80 °C until further use.

For HIV-1 infection in the presence of IFN-α and/or IFN-β neutralizing antibodies, primary CD4$^+$ T cells were resuspended immediately after spinoculation in medium containing 1/100,000x diluted goat anti-human IFN-β serum (925, kindly provided by Dr. Van Damme, Rega Institute, KUL, Belgium (Van Damme et al., 1987)) and/or 6,000 neutralizing units (NU)/ mL rabbit polyclonal anti-human IFN-α antibody (PBL Assay Science, Piscataway, NJ). Every 2 days, new medium with antibodies was added to the cells. For HIV-1 infection in the presence of antiretrovirals, primary CD4$^+$ T cells were plated in medium with 5 μM nevirapine, 0.5 μM raltegravir or 1 μM ritonavir (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Germantown, MD) 1 h before infection and antiretrovirals were maintained at these concentrations during infection

MT4 cells were infected with HIV NL4-3-GFP-I viruses by plating 1.5 x 10^6 cells per well of a 6-well flat-bottom plate (BD Biosciences) and addition of 165-350 mU RT (equivalent to 30-60 ng p24) of virus in a total volume of 6 mL IMDMc. Cells were subsequently spinoculated at 950 g for 90 min at 32 °C. 48 h-72 h after infection, cells were collected and used for co-culture with primary CD4$^+$ T cells.

For infection of MDDCs, 8 x 10^4 MDDCs were seeded in flat-bottomed 96-well plates. MT4C5 cells were infected with VSV-pseudotyped HIV NL4-3 WT or dVpr, 24-48 h before the start of the co-culture. Donor T cells were stained with Far Red DDAO-SE (Life Technologies). MT4C5 cells were then co-cultured with MDDC at a 1:2 ratio (T:MDDC). Non-infected MT4C5 cells were used as negative controls. Co-cultures were performed for 72 h. VLPs carrying Vpx were produced as previously described (Puigdomenech et al., 2013) and added to MDDCs 2 h before HIV-1 infection and maintained during the experiment.

Origin and production of lentiviral vectors

Lentiviral TRC1 pLKO.1-puro vectors (Sigma-Aldrich, St. Louis, MO) encoding shRNA are listed in Table S3 and were mostly obtained from the BCCM/LMBP Plasmid collection,

Department of Biomedical Molecular Biology, Ghent University, Belgium (http://bccm.belspo.be/about/lmbp.php). For knock-down of IRF3, a pLKO.1-puro vector targeting IRF3 was kindly provided by Dr. N. Manel (Institut Curie, Paris, France) (Manel et al. 2010). As a control, a pLKO.1-puro vector encoding a non-targeting scrambled shRNA (SHC-002) was purchased from Sigma-Aldrich. Lentiviruses were produced in 293T cells by transfection with the pLKO.1 vectors and the MISSION® Lentiviral Packaging Mix using FuGENE® HD Transfection Reagent (Promega, Leiden, The Netherlands) as described before (Landi et al., 2014). Medium was refreshed 24 h after transfection. Viral supernatant was harvested after 48 h and centrifuged at 900 g for 10 min, to clarify the supernatant from remaining cells.

<u>Knock-down of host genes by lentiviral vector transduction and co-culture with HIV-1 infected cells</u>

pDC depleted primary CD4$^+$ T cells were transduced immediately after isolation from peripheral blood with pLKO.1 lentiviral vectors. Cells were plated at 300,000 cells in 55 µL RPMIc per well of a 96-well flat bottom plate and 45 µL of lentiviral vector supernatant was added to each well in the presence of 20 ng/mL IL-2, 1 µg/mL PHA and 8 µg/mL polybrene. Cells were then spinoculated for 30 min, 950 g at 32 °C. After 24 h, fresh RPMIc containing IL-2 and PHA was added to the cells. 48 h after transduction puromycin (1.2 µg/mL final concentration, Sigma Aldrich) was added to the cells. After 72 h, PHA/IL-2 stimulation was ended by removing all medium and adding fresh RPMIc containing IL-2 and puromycin. 5 days after transduction cells were counted using MACSquant® Analyzer or using Flow-count Fluorospheres (Beckman Coulter, Suarlée, Belgium) and FACSCalibur flow cytometer, according to manufacturer's instructions. Cells were subsequently plated at 1.2×10^5–1.5×10^5 cells per well of a 96-well flat bottom plate and an equal amount of HIV-infected or non-infected MT4 cells were added in a total volume of 200 µL fresh RPMIc (+ IL-2). Remaining primary CD4$^+$ T cells were lysed in QIAZOL and used for target gene expression analysis. 24 h after the start of the co-culture, cells were lysed in QIAZOL and used for IFN-β expression analysis. IFN-β mRNA induction was not observed after co-culture of HIV-infected MT4 cells and primary CD4+ T cells in the presence of the HIV-1 reverse transcriptase inhibitor nevirapine (data not shown).

<u>Quantitative real-time PCR (qPCR)</u>

RNA was isolated from QIAZOL lysates using the miRNeasy mini kit, either manually or with QIAcube (Qiagen), according to manufacturer's instructions. RNA (max. 1 µg) was subsequently treated with amplification-grade DNAse I (Life Technologies) and used for synthesis of cDNA with Superscript® III reverse transcriptase and random primers (Life

Technologies), all according to manufacturer's instructions. Depending on the gene to measure, cDNA was subsequently 3x (for target genes)-15x (for reference genes) diluted by addition of Nuclease-Free Water (NFW) (Ambion, Life Technologies) and 5 μL of diluted cDNA was used as input for qPCR. For qPCR measurement of *YWHAZ, UBC, IFIT1*, IFN-α and *IRF3*, forward (FWD) and reverse (REV) primers were used at a final concentration of 300 nM in combination with 2x concentrated LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics, Vilvoorde, Belgium) in a final reaction of 15 μL. For measurement of IFN-β (*IFNB1*), FWD and REV primers were used at a final concentration of 500 nM and a double-quencher probe (56-FAM/ZEN/3′ Iowa Black FQ) was used at a final concentration of 250 nM in combination with 2x concentrated LightCycler® 480 Probes Master mix (Roche Diagnostics) in a final reaction of 15 μL. Primers and probe were manufactured by IDT (Leuven, Belgium). For measurement of *MB21D1* (cGAS), *TMEM173* (STING) and *TBK1*, 20x concentrated commercial Prime PCR Sybr® Green assays (Bio-Rad Laboratories, Temse, Belgium) were used in combination with 2x concentrated LightCycler® 480 SYBR Green I Master mix in a final reaction of 15 μL. Primer/probe sequences or assay IDs are show in Table S1. qPCR reactions were performed in 384-well plates (LightCycler® 480 Multiwell Plates 384, white, Roche Diagnostics) on the on the LightCycler® 480 II instrument (Roche Diagnostics) using following program: 10 min at 95 °C; 45 cycles of amplification: 10 s at 95 °C, 1 min at 60 °C.

For each sample, qPCR reactions were performed in duplo. A non-template control (NFW instead of cDNA) and a serial 10-fold dilution series of cDNA derived from poly I:C stimulated PBMCs (standard curve) was included for measurement of each gene on each plate. Cycles of quantification (Cq) values were generated by the LightCycler® 480 software 1.5.0 according to the second-derivative maximum method. Amplification efficiency of each qPCR assay was tested during primer/assay validation and varied from 85-105 %. Melting curve analysis was performed for the not previously validated SYBR Green assays (*IFIT1*, IFN-α and *IRF3*) and showed a single peak. Calibrated normalized relative quantities (CNRQs) were calculated for each target gene in each sample based on obtained Cq values, with the qBasePlus Software (Biogazelle, Zwijnaarde, Belgium), using *YWHAZ* and *UBC* as reference genes and using target- and run- specific amplification efficiencies. GeNorm analysis (implemented in qBase Plus) was used to select *YWHAZ* and *UBC* as stable reference genes in prior experiments.

<u>Measurement of secreted type 1 IFN bioactivity</u>

HL-116 cells were plated 16 h prior to the assay at 40,000 cells per well of a 96-well-flat bottom plate in 200 μL DMEMc. Medium was removed and 100 μL culture supernatant or recombinant IFNα, together with 100 μL DMEMc was added. Cells were incubated for 7-8 h,

washed with PBS and lysed (Cell Culture Lysis 5X Reagent, Promega). Lysates were either processed immediately or stored at -80°C until further use. For measurement of luciferase activity, 20 µL cell lysate was transferred to 96-well white solid plates (Costar/Corning, Amsterdam, The Netherlands) and luciferase assay was conducted using 50 µL Luciferase reagent (Luciferase Assay System, Promega) in a TriStar LB 941 automated plate reader (Berthold Technologies, Bad Wildbad, Germany). IFN levels are expressed in IU/mL as equivalent of IFNα1b (Immunotools, Friesoythe, Germany) bioactivity for primary CD4$^+$ T cells and as equivalent of IFNα2a (PBL Assay Science) bioactivity for MDDC experiments, both determined by inclusion of a dilution series of the recombinant IFNα in each experiment.

Measurement of MxA and HIV infection levels.

Intracellular staining for p24 or MxA in primary CD4$^+$ T cells was performed with the Fix and Perm Cell kit (AN DER GRUB Bio Research, Susteren, The Netherlands) according to manufacturer's instructions using following antibodies: HIV-1 core antigen PE (phycoerythrin)-conjugated mAb (Clone KC57, Beckman Coulter, Suarlée, Belgium) for p24 or anti-MxA (anti-MX1) (rabbit polyclonal, Abcam, Cambridge, UK) as primary antibody and Alexa Fluor® 660 goat anti-rabbit IgG (Life Technologies) as secondary antibody for MxA. For HIV NL4-3-HSA-I infected cells, surface staining was performed with an anti-CD24-APC (allophycocyanin) antibody (HSA; mouse clone M1/69, BioLegend, San Diego, CA). Cells were analyzed by flow cytometry (FACSCalibur using CellQuest Pro software (BD Biosciences) or FlowJo 887 (Tree Star, Ashland, OR) or Flowing Software (Turku Centre for Biotechnology, University of Turku) for analysis). For HIV-2 CI85 infected cells, surface staining was performed with an anti-CD4-APC antibody (clone M-T466, Miltenyi Biotec). Percentage of HIV-1 infected cells was determined as percentage of HSA, GFP or p24 expressing cells and for HIV-2 CI85 as percentage of CD4^{low} cells.

To evaluate HIV-1 infection levels in MDDCs, cells were intracellularly stained with anti-HIV-Gag (KC57-PE, Beckman-Coulter) and Gag-PE and Far Red DDAO-SE levels were analyzed by flow cytometry.

Statistical analysis and software

Figures were created with Microsoft PowerPoint and GraphPad Prism 5.0 software. Statistical tests were performed with GraphPad Prism 5.0 as indicated in the figure legends

Table S1. Properties of the qPCR assays used.

Related to Experimental Procedures.

Official gene symbol	Detected Refseq transcripts	Primer/probe sequence source	Primer/ probe identity	Primer/probe sequence	Ampli-con length
YWHAZ	NM_001135702.1, NM_001135701.1, NM_001135700.1, NM_001135699.1, NM_145690.2, NM_003406.3	RTprimerDB ID: 9 (Pattyn et al., 2003)	FWD	5'-ACTTTTGGTACATTGTGGCTTCAA-3'	94
			REV	5'-CCGCCAGGACAAACCAGTAT-3'	
UBC	NM_021009.6	RtprimerDB ID: 8 (Pattyn et al., 2003)	FWD	5'-ATTTGGGTCGCGGTTCTTG-3'	133
			REV	5'-TGCCTTGACATTCTCGATGGT-3'	
IFIT1	NM_001548.4	Designed with Primer-BLAST (Ye et al., 2012)	FWD	5'-GATCTCAGAGGAGCCTGGCTAA-3'	84
			REV	5'-TGATCATCACCATTTGTACTCATGG-3'	
IFNA cluster (IFNA10, IFNA17, IFNA4, IFNA2, IFNA8, IFNA7, IFNA14, IFNA21, IFNA16, IFNA6, IFNA1, IFNA13, IFNA5)	NM_002171.2, NM_021268.2, NM_021068.2, NM_000605.3, NM_002170.3, NM_021057.2, NM_002172.2, NM_002175.2, NM_002173.2, NM_021002.2, NM_024013.2, NM_006900.3, NM_002169.2	RTprimerDB ID: 3541 (Pattyn et al., 2003)	FWD	5'-GTGAGGAAATACTTCCAAAGAATCAC-3'	93
			REV	5'-TCTCATGATTTCTGCTCTGACAA-3'	
IFNB1	NM_002176.2	Designed with PrimerQuest Assay design IDT	FWD	5'-GCTTCTCCACTACAGCTCTTTC-3'	115
			REV	'5-CAGTATTCAAGCCTCCCATTCA-3'	
			PROBE	56-FAMTTCAGTGTC/ZEN/AGAAGCTCCTGTGGCAA/3IABkFQ	
IRF3	NM_001197125.1, NM_001197124.1, NM_001197123.1, NM_001197122.1, NM_001571.5	Viemann et al., 2011	FWD	5'-AGGCCACTGGTGCATATGTTC-3'	108
			REV	5'-CCTCTGCTAAACGCAACCCTT-3'	
MB21D1	n.a.	qHsaCID0009796 Bio-rad Laboratories	/	n.a.	110
TMEM173	n.a.	qHsaCID0010565 Bio-rad Laboratories	/	n.a.	93
TBK1	n.a.	qHsaCID0018552 Bio-rad Laboratories	/	n.a.	141

Table shows properties of primers used for qPCR: official gene symbol according to the HUGO Gene Nomenclature Committee database (www.genenames.org); detected Refseq transcripts are those obtained by Primer-BLAST (Ye et al. 2012) using the indicated primer sequences with default parameters; FWD: forward primerr, REV: reverse primer, n.a. not available

Table S2. HIV-1 encoding vectors used in this study.

Related to Experimental Procedures.

Parental vector	Variant	Virus referred to as:	Source	Reference
NLENG1-IRES	WT	HIV NL4-3-GFP-I	Dr. D.N. Levy, New York University college of Dentistry, New York, NY	Kutsch et al., 2002; Levy et al., 2004
NL4-3-IRES-HSA	WT	HIV NL4-3-HSA-I	Dr. M.J. Tremblay; Faculté de Médecine, Université Laval, Québec, Canada	Imbeault et al., 2009
	Tat-mutated	HIV NL4-3-HSA-I TatSTOP	Constructed in house	See Materials and methods
	Vpr-mutated	HIV NL4-3-HSA-I dVpr	Constructed in house	See Materials and methods
	WT	HIV NL4-3	NIH AIDS Research and Reference Reagent Program	Adachi et al., 1986
pNL4-3	Integrase-mutated	HIV NL4-3 D116N	Dr. Z. Debyser, Laboratory for Molecular Virology and Gene Therapy, KU Leuven, Belgium	Mutation introduced in HIV NL4-3 (according to Engelman et al., 1995)
	Vpr-deleted	HIV NL4-3 dVpr	Dr. Margottin-Goguet, Institute Cochin, Paris, France	Le Rouzic et al., 2008
	Vpr Q65R mutant	HIV NL4-3 Q65R	Constructed in house	See Materials and methods
	Vpr R77Q mutant	HIV NL4-3 R77Q	Constructed in house	See Materials and methods
pLaiΔEnv-GFP3	WT	HIV LAI dEnv dNef	Dr. N. Manel, Institut Curie, Paris, France	Manel et al., 2010
	Capsid G89V mutant	HIV LAI dEnv dNef G89V	Dr. N. Manel, Institut Curie, Paris, France Constructed in house	Manel et al., 2010
	Capsid T54A/N57A mutant	HIV LAI dEnv dNef T54A/N57A		Manel et al., 2010
	Capsid Q63A/Q67A mutant	HIV LAI dEnv dNef Q63A/Q67A		Manel et al., 2010
pBR-NL43-I-EGFP	WT	HIV NL4-3-I-EGFP		Schindler et al., 2003
	Vpu-mutated	HIV NL4-3-I-EGFP dVpu	Constructed in house	Rucker et al., 2004; *nef*-IRES-eGFP introduced as described in Sauter et al., 2009
	Vpr-mutated	HIV NL4-3-I-EGFP dVpr		Rucker et al., 2004; *nef*-IRES-eGFP introduced as described in Sauter et al., 2009
	Vpu-mutated and Vpr-mutated	HIV NL4-3-I-EGFP dVpu dVpr		Rucker et al., 2004; *nef*-IRES-eGFP introduced as described in Sauter et al., 2009
pNL4-3 UIE	WT	HIV NL4-3-Vpu-IRES-Env NL4-3 WT (M)	Constructed in house	Sauter et al., 2009
	Vpu NL4-3 S52 mutant	HIV NL4-3-Vpu-IRES-Env NL4-3 S52		Sauter et al., 2009
	Vpu NL4-3 STOP	HIV NL4-3-Vpu-IRES-Env NL4-3 STOP		Sauter et al., 2009
	Vpu NL4-3 deleted	HIV NL4-3-Vpu-IRES-Env NL4-3 deleted		Sauter et al., 2009
	Vpu HIV-1 M JRCSF	HIV NL4-3-Vpu-IRES-Env JRCSF (M)		Sauter et al., 2009
	Vpu HIV-1 M Yu2 (adapted)	HIV NL4-3-Vpu-IRES-Env Yu2 (M)		Sauter et al., 2009
	Vpu HIV-1 O 13127	HIV NL4-3-Vpu-IRES-Env 13127 (O)		Sauter et al., 2009
	Vpu SIV Cpz Pts Tan1	HIV NL4-3-Vpu-IRES-Env Cpz Pts Tan1		Sauter et al., 2009
	Vpu SIV Cpz Ptt MP7	HIV NL4-3-Vpu-IRES-Env Cpz Ptt MP7		Sauter et al., 2009

Table shows different HIV-1 constructs used in this study, indicating the parental wild-type (WT) vector and variants used; the name by which this virus is referred to in the study, the source of the vector and the reference describing construction of the vector if published.

Table S3. List of pLKO.1-puro shRNA-encoding lentiviral vectors used for knock-down of host proteins. Related to Experimental Procedures.

Origin lentiviral vector	Target gene (official symbol)	Refseq	TRC number / reference	Target sequence	Targeted gene region
BCCM/LMBP[a]	MB21D1	NM_138441.2	TRCN0000148694	CTGCCTTCTTTCACGTATGTA	CDS
BCCM/LMBP[a]	MB21D1 (#2)	NM_138441.2	TRCN0000149984	CAACTACGACTAAAGCCATTT	CDS
BCCM/LMBP[a]	MB21D1 (#3)	NM_138441.2	TRCN0000146282	CTTTGATAACTGCGTGACATA	CDS
BCCM/LMBP[a]	TMEM173	NM_001301738.1, NM_198282.3	TRCN0000161052	GCTGGCATGGTCATATTACAT	CDS
BCCM/LMBP[a]	TBK1	NM_013254.3	TRCN0000003182	GCAGAACGTAGATTAGCTTAT	CDS
Dr. N. Manel, Institut Curie, Paris, France	IRF3	NM_001197125.1, NM_001197126.1, NM_001197123.1, NM_001197122.1, NM_001571.5	Manel et al. 2010	CTGCCTGGATGGCCAGTCACAC	CDS
Sigma-Aldrich	Non-targeting scrambled shRNA control (SHC-002)	Not applicable	Not applicable	Not applicable	Not applicable

Table shows official gene symbol of the shRNA targeted gene according to the HUGO Gene Nomenclature Committee database (www.genenames.org); Refseq code of the targeted transcripts (Reference sequence database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)); TRC number of the clone from the Sigma Mission® TRC1 lentiviral library, sequence in the gene transcript targeted by the shRNA and corresponding region in the transcripts targeted (CDS: coding sequence).
[a]Sigma Mission® TRC1 vector obtained through the BCCM/LMBP Plasmid collection, Department of Biomedical Molecular Biology, Ghent University, Belgium (http://bccm.belspo.be/about/lmbp.php)

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Chapter IV :

Discussion and future perspectives

The pathogenesis of HIV-1 infection is characterized by a long-lasting battle between the virus and the human immune system. Despite development of a very active immune response, the virus is never eliminated and rarely controlled during natural infection. Eventually, the immune system becomes exhausted and is no longer able to keep HIV replication (and other infections) at bay, leading to AIDS. A considerable part of HIV-1 research has focused on defining immune responses against the virus as well as understanding the mechanisms that drive immune exhaustion and dysfunction. The final aim here is to artificially boost these responses or prevent/repair their deterioration in a way to functionally cure the infection [1]. Type 1 interferon (IFN-I) responses are an interesting target in this regard for several reasons. First, they are able to suppress HIV-1 replication *in vitro* and early *in vivo* responses are thought to play a crucial role in limiting the initial viral spread. Second, despite a beneficial effect of early responses, long-term exposure to IFN-I likely contributes to hyper-immune activation and dysfunction and may therefore drive immune exhaustion in the chronic phase of HIV-1 infection. Third, the virus has adopted strategies to counteract IFN-I responses, which further adds to immunological failure and may present alternative therapeutic targets [2-4]. A prerequisite for targeted modulation of these responses is an in depth understanding of the origin and mechanisms that regulate IFN-I secretion during HIV-1 infection. With this in mind, we aimed at evaluating the potential of the main HIV-1 targets, activated CD4$^+$ T cells, to produce IFN-I in response to HIV and to subsequently asses host and viral factors that mediate IFN-I induction in these cells.

SG-PERT assay as a valid alternative for retroviral quantification

The mechanistic evaluation of IFN-I responses required the use of many HIV-1 variants and lentiviral vectors and introduced early-on the need for a fast retroviral quantification method. In **Chapter III-1** we report on the optimization and evaluation of an SG-PERT assay that allows quantification by measurement of reverse transcriptase (RT) activity. We show that viral titer determination by SG-PERT correlates well with other frequently applied methods for retroviral quantification. Furthermore, it outperforms the "gold standard" method p24 ELISA by a lower inter-run variation, higher linear range and (much) lower cost. It is also considerably faster and more amenable to standardization than functional quantification methods, which are dependent on infection or transduction of cells. An overview of advantages and disadvantages of the SG-PERT assay is provided in Table 1.

A primary field of application of this assay would be in virology and molecular biology research environments. Given the increased use of lentiviral vectors as a tool for high-throughput screening, faster and cheaper methods for quantification are highly appealing. Measurement of RT activity further offers the advantage that theoretically any type of retrovirus can be detected with a single method, while other assays are usually limited to

detection of virus-specific proteins or nucleic acid sequences. For HIV research an additional advantage may be the shorter half-life of RT activity compared to p24 proteins as reported by others [5, 6], since this could provide a better estimation of active viral particle production during long-term culture. Lenti- and retroviral vectors are also increasingly used in gene therapy protocols, reaching over 20% of vector-based clinical trials in 2014 [7]. Quality control for clinical grade vectors often involves assessment of total/functional particle ratio's and also requires screening for possible replication competent retroviruses/lentiviruses (RCR/RCL) [8]. The SG-PERT assay may also find applications in this regard, as alternative for p24 ELISA [9, 10] or more labor-intensive PERT assays [11, 12]. Although our assay has a comparable sensitivity to standard p24 ELISA, this would be largely insufficient for use in a clinical setting as viral load assay. As discussed in Chapter III-1, we estimate that 20-50 particles per reaction can be accurately quantified by SG-PERT and fewer particles may even be detectable. However, the input of the reaction is currently limited to 0,5 µL. This is mainly due to an extensive dilution step of the viral lysates, that was added to avoid possible PCR inhibitory effects of culture medium [13]. When higher sensitivity is required, ultracentrifugation of the sample could be performed to allow prior removal of inhibitory substances and further concentration of viral particles. This strategy was successfully applied on patient plasma previously, for viral load assessment with a more labor-intensive PERT variant [14, 15]. In Switzerland, such PERT assays are standardly requested for HIV confirmation testing in patients with low viral load upon diagnosis [16]. In this context, the by nature sequence independent PERT is applied to avoid possible underestimation of viral load, which may arise from sub-optimal detection of certain HIV-1 variants by the sequence-based standard viral load test. If combined with ultracentrifugation or RT purification methods [17], the SG-PERT assay may also be able to quantify low viral load levels in patients under cART. However, given the requirement of 20-50 particles per reaction for accurate quantification, the assay is unlikely to exceed sensitivity of current RNA-based viral load assays, which can detect as few as 20-40 RNA copies/mL in plasma without prior need for ultracentrifugation [18].

Table 1. Strengths (+) and weaknesses (-) of the SG-PERT assay as a retroviral quantification method

+	-
High linear range	Detection of non-functional viral particles
Applicable for all types of retroviruses	Lower sensitivity compared to eg. viral RNA quantification methods
Relative low cost	
Fast procedure	
Low inter-run variation	

HIV-infected CD4^{+} T cells as potential source of IFN-I

In a first part of **Chapter III-2** we employed several assays to evaluate the occurrence of IFN-I responses during HIV replication in primary CD4^{+} T cells. We found that both HIV-1 laboratory adapted strains as well as different primary HIV-1 or HIV-2 isolates trigger a bioactive response in these cells, characterized by induction of IFN-I and several ISGs. This finding has some interesting implications.

As discussed before, both correlative evidence [19-21] and *in vivo* manipulation of IFN-I responses [22-26] implicate IFN-I produced in the chronic phase of HIV/SIV infection as a potential driver of hyper-immune activation and dysfunction (and thus pathogenesis). Our data now indicate that part of this IFN-I may originate from HIV-infected CD4^{+} T cells. Indeed, although an IFN-I signature is evident in chronically infected patients or pathogenic models of SIV infection, the cellular sources of chronic IFN-I have remained elusive [27]. A recent study in humanized mice showed that targeted depletion of plasmacytoid dendritic cells (pDCs) led to a significant (although not complete) reduction of IFN-I levels during chronic HIV-1 infection [28]. In contrast, inhibition of pDC responses during pathogenic SIV infection indicated a rather transient contribution of these cells to the IFN-I response [29] and IFN-α producing pDCs were barely detected in spleens of chronically infected HIV-1 patients. In the later study, IFN-α did co-localize with other cell types, including T cells [30]. Given the correlation between levels of HIV replication and IFN-I we observed, CD4^{+} T cells are most likely to contribute to IFN-I secretion *in vivo* mainly at sites where high levels of infection are reached, such as the lymph nodes or the GALT. The latter constitutes the largest reservoir of activated CD4^{+} T cells in the body and a marked IFN-I signature is indeed observed in gut biopsy samples from chronically infected HIV-1 patients [31, 32]. Future *in situ* or flow-cytometric evaluations of patient-derived lymphoid tissue will be needed to identify potential i*n vivo* niches of IFN-I producing CD4^{+} T cells. In this regard, it will also be interesting to asses CD4^{+} T cells derived from different compartments and different CD4^{+} T cell subsets for their *in vitro* capacity to produce IFN-I during infection. Differences between peripheral CD4^{+} T cells and those derived from lymphoid tissue have been observed in terms of inflammasome activation by HIV-1 after innate signaling [33, 34]. Previous exposure of T cells to cytokines in the lymphoid tissue and upon T cell differentiation may affect their IFN-I production capacity by eg. modulating expression levels of the innate sensor or the NF-κB activation status [35].

A second direct, but perhaps surprising implication is that HIV does not prevent its own innate sensing in its main target cells. Given the very adaptive nature of the virus and its wide ability to divert the host cellular pathways, induction of the primarily antiviral IFN-I cytokines by the virus might be unexpected. Our results indeed indicate an antiviral effect of HIV-induced IFN-I, since addition of IFN-I neutralizing antibodies enhanced viral replication. Certain ISGs with anti-HIV activity that are not counteracted by HIV-1 accessory proteins,

such as MX2 or other yet to be identified ISGs [36-38], are likely responsible for this effect. The extent of the antiviral effect we observed, was however rather modest. Previous studies have indicated a larger resistance of HIV-1 to IFN-I when added after initial establishment of infection in the culture (eg. after integration) and the remaining effect is highly dependent on the amount of IFN-I [39, 40]. We could therefore speculate that the virus allows a certain level of IFN-I induction -and its antiviral effect- as a trade-off for other effects of IFN-I or the innate immune response, that can be exploited to its advantage. This may be e.g. enhanced recruitment and activation of potential target cells, impeding antiviral T-cell responses or even direct stimulatory effects on viral replication [19, 41-44]. In this regard, it will be interesting to also assess the panel of chemokines and cytokines, besides IFN-I, that is induced by innate sensing of the virus in CD4$^+$ T cells. Furthermore, it will be important to evaluate IFN-I induction with a larger panel of primary HIV-1 isolates. The use of HIV-1 founder viruses might learn us if and how innate sensing in CD4$^+$ T cells also affects early phases and establishment of infection, as this could reflect in a different induction ability of these viruses.

IFN-I induction by post-integration cGAS-mediated sensing and regulation by Vpr and Vpu

In the second part of **Chapter III-2** we investigated the underlying mechanism of the HIV-induced IFN-I response, both from a host and viral perspective. By using shRNA-mediated knock-down of cellular IFN pathway proteins and a panel of mutated HIV variants, we show that IFN-I is induced through a post-integration mechanisms that requires the cytosolic DNA sensor cGAS and its downstream signaling molecules. In addition, we demonstrate that this response is regulated by two newly expressed HIV-1 accessory proteins: Vpr potentiates induction, while Vpu suppresses the response. This indicates a mechanistic model in which HIV DNA is sensed by cGAS upon productive infection, through the assistance of newly expressed viral replication products. Especially the newly produced Vpr protein seems to be an important driver of sensing after integration, since a functional Vpr was required for full IFN-I induction and virion-incorporated Vpr seemed insufficient to mediate this effect (Figure 4.1).

The ability of cGAS to sense HIV infection was demonstrated by several groups [45-48] soon after its initial identification as a DNA pattern recognition receptor [49]. Importantly, until now, activation of cGAS has never been observed under "natural" HIV-1 infection conditions. Indeed, while HIV-1 efficiently triggers cGAS in monocytic THP-1 cell lines [45, 48], its activation in primary DCs and macrophages only occurs upon addition of Vpx [45, 46] or when using specific HIV-1 capsid mutants [46, 47]. The loss of Vpx by HIV-1 and cloaking of the HIV-1 capsid with host proteins were consequently suggested as strategies specifically acquired by the virus to evade cGAS sensing [47, 50-52]. Our data now indicate that cGAS

activation can occur in a natural HIV-1 infection context. Consequently, this implicates the receptor for the first time as a potential mediator of IFN-I response in HIV-1 patients. A small-scale study by Nissen et al. did not detect an association between cGAS mRNA levels and markers of immune activation in HAART-naïve HIV-1 infected individuals [53]. However, variation in cGAS expression levels was very limited among the included subject, which may hamper efficient correlation assessment. It would be interesting to further evaluate cGAS activity in CD4^{+} T cells from different patient groups (eg. patients with different virological or immunological response to treatment, long-term non-progressors or elite controllers) or alternatively asses the occurrence of genetic variability in cGAS or STING (eg. single nucleotide polymorphisms) [54] among HIV-1 patients.
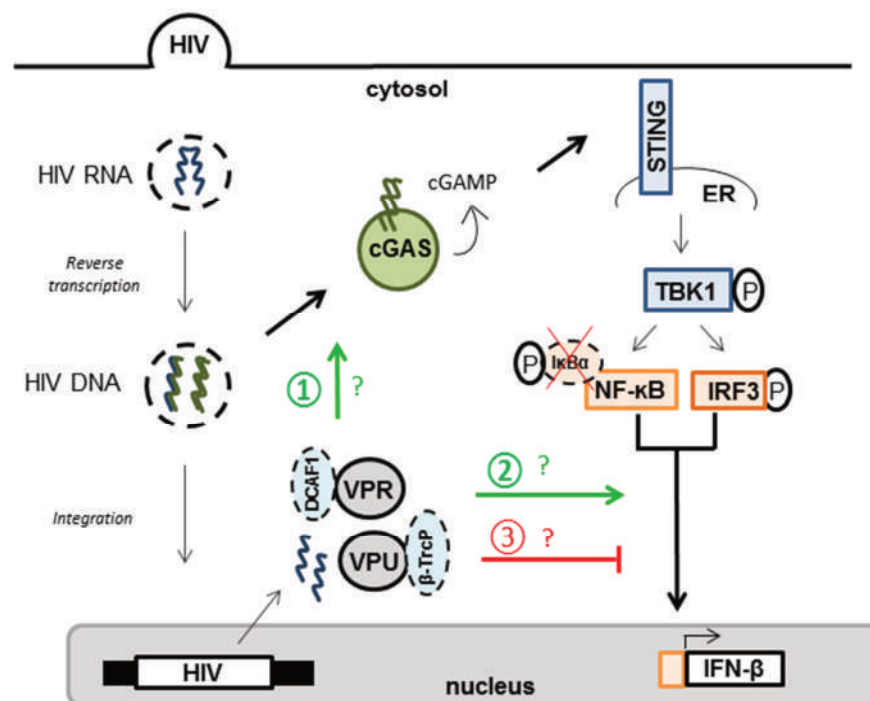

Figure 4.1 Mechanistic model of HIV innate sensing in activated CD4^{+} T cells. Productive HIV infection triggers the cytosolic sensor cGAS, which results in activation of the STING-TBK1-IRF3 signaling pathway and NF-κB. Both IRF3 and NF-κB subsequently bind to the IFN-β promoter to induce its expression. Given the known DNA sensing properties of cGAS, activation is most likely induced by HIV DNA products, created upon reverse transcription. However, IFN-I induction only occurs after integration and expression of the provirus, indicating that additional newly expressed viral products mediate the sensing . Newly expressed Vpr potentiates IFN-I induction by an unknown mechanism that is most likely dependent on its interaction with the host factor DCAF1. Vpr might enhance HIV DNA binding to cGAS by unmasking it from its capsid-host factor shield (1) or might potentiate downstream signaling by eg. activating NF-κB (2). Conversely, Vpu counteracts IFN-I induction possibly by recruiting the E3 ubiquitin ligase component β-TcrP and interfering with NF-κB activation (3).

An important point to consider in our model is the origin of the HIV DNA that is recognized by cGAS. Post-integration sensing of HIV DNA implicates that both an integrated viral copy and cytosolic HIV DNA need to be present in the cell. A successfully integrated virus is however not expected to leave cytosolic DNA fragments behind. As such, the DNA sensed by cGAS most likely originates from a separate infection event. In this regards, re-infection of cells containing an expressing virus might be rather rare *in vivo* since HIV-1 has developed several mechanism to prevent viral entry of productively infected cells [55, 56] and most infected CD4+ T cells have a relative short half-life [57]. HIV DNA can however stay behind in the cytosol due to the frequent failure of HIV-1 reverse transcription and/or nuclear import [58-61] and such cells could be targeted for subsequent productive infection. In HIV-1 patients, the proportion of cells containing unintegrated DNA largely exceeds those with integrated DNA for both resting and activated CD4+ T cells. Most of this DNA is thought to consist of linear unintegrated DNA [62, 63], which may exist both in the cytoplasm and the nucleus. At sites where virus concentrations are high or cells are tightly packed, multiple simultaneous co-infections of a cell may also occur. Different studies employing *in situ* analysis of splenic tissue of HIV-1 patients have described the presence of multiple proviruses per cell [64-66] and co-infection of cells is a prerequisite for generation of recombinant HIV-1 variants [67]. Furthermore, formation of viral synapses between infected and uninfected cells is known to result in directed delivery of multiple virions at once [68-71]. Even though most of these particles will fail to establish productive infection, their reverse transcription products could be targeted for cGAS detection by a successfully integrated virus. The simultaneous transmission of multiple "DNA-providing" virions during cell-to-cell transfer might contribute to the higher IFN-I production that is observed during infection of Vpx treated dendritic cells [72] and primary CD4+ T cells (see chapter III-2) by co-culture compared to infection with free virions, as IFN-I induction in both cell types is mediated by post-integration cGAS-dependent sensing.

Although post-integration sensing of HIV DNA by cGAS has indeed been suggested before in Vpx treated dendritic cells [46, 50], we cannot rule out that other viral components (eg. RNA, proteins) are sensed after integration. A strong argument for a DNA-sensing driven process is the complete abrogation of IFN-I induction upon cGAS knock-down we observed, combined with observations that cGAS is a nucleic-acid binding protein but seems unable to recognize RNA or respond to RNA viruses without DNA replication intermediates [49, 73, 74]. Two studies have suggested that cGAS would be able to co-operate with other sensors, although so far only other DNA sensors were found to collaborate with cGAS [48, 75]. To further define the sensing mechanism it will be important to determine which separate and combined viral products are able to induce IFN-I in primary CD4+ T cells and which of them are able to "rescue" IFN-I induction by an integrase deficient virus.

Another point worth discussing is the opposing effect of two viral proteins on the same process. HIV accessory proteins mediate their effects by hijacking specific host proteins and pathways. However, targeting of these factors could have unintended side-effects and might only be beneficial to the virus in certain target cells or at certain stages of the viral replication cycle. Induction of G2 arrest by Vpr and apoptotic effects of certain accessory proteins were suggested to be such by-products of accessory protein actions [76-79]. Furthermore, opposing effects of different accessory proteins on eg. apoptosis [80, 81], NF-κB activation [82-84] and cell cycle progression [85-87] have been reported and could represent ways of the virus to deal with non-beneficial effects when they present. Given the antiviral nature of IFN-I, it is possible that potentiation of post-integration sensing by Vpr is such a side-effect of a required Vpr-host interaction. As discussed before, the innate immune responses resulting from this post-integration Vpr interaction may have certain benefits for viral spread, such as recruitment and activation of surrounding HIV target cells. However, high levels of IFN-I are likely to be avoided by the virus. As such, the virus may have employed another late HIV-1 protein, Vpu, to partially counteract the Vpr effect and keep IFN-I levels in check. Indeed, an IFN-I suppressing effect of Vpu was observed before [88-90], but the evolutionary drive of this effect remains unclear. Since Vpu is only present in the infected cell after viral integration, it is likely to counteract a mechanism of post-integration IFN-I induction, which had not been reported during natural HIV-1 infection until now. In this regard it will be interesting to evaluate *vpu* and *vpr* alleles of the HIV-1 ancestral lentiviruses and non-M HIV-1 viruses, to see if Vpr and Vpu effects have co-evolved together. Furthermore, alleles of multiple HIV-1 M isolates should be tested to asses preservation of the Vpu and Vpr effect. While all primate lentiviruses encode a Vpr protein, some of them (eg. HIV-2) lack Vpu [91]. The one HIV-2 isolate tested in our study had a similar capacity to induce IFN-I as HIV-1 viruses. It will be interesting to see if Vpr proteins of these and other viruses without Vpu have with a weaker IFN-I potentiating ability or if these viruses might employ other viral proteins to counteract the Vpr effect.

Future research will be required to fully understand the mechanism behind the HIV-1 Vpu and Vpr effect. Our model does provide some interesting clues for research. First, it will be important to asses if newly expressed Vpr can act as a sole factor to enable post-integration sensing or cooperates with other newly synthesized HIV products. As discussed above, we can evaluate if expression of Vpr in trans can rescue IFN-I induction by an integrase-deficient virus. To avoid extensive cytopathic effects of long-term Vpr expression, the use of an inducible retro- or lentiviral construct to introduce Vpr in primary CD4+ T cells will likely be required. In order to envision how Vpr can enhance post-integration sensing, it is necessary to consider how sensing is prevented before integration. As discussed in Chapter III-2, it has been suggested that viral DNA is shielded by complexes of HIV-1 capsid and host factors cyclophilin A (CypA) [46, 50] and/or CPSF6 [47]. As such, newly expressed Vpr might play a role in unmasking DNA for cGAS binding. Interestingly, Vpr is also known to

interact with CypA [92, 93]. High levels of Vpr accumulating after integration might eventually compete with capsid for interaction. Alternatively, Vpr may expose HIV-1 DNA through direct interaction with DNA [94] or through HIV DNA binding proteins that are induced or relocated by Vpr, such as the DNA damage response proteins Ku70, Ku80 and PARP-1 [95-97]. In an alternative model, Vpr may influence factors of the cGAS pathway. A low efficiency of signaling transduction following DNA sensing has been suggested in CD4^{+} T cells by one group [98], although active signaling was implied by others [61, 95]. It is possible that Vpr enhances the threshold for successful signaling by eg. activation of a signaling protein such as NF-κB [83, 99] or by inducing proteasomal degradation of an inhibiting factor. To discriminate between these two models it will be interesting to evaluate if Vpr can potentiate IFN-I induction by alternative stimulators of the cGAS pathway (eg. dsDNA or DNA viruses [49, 100]) in primary CD4^{+} T cells. Evaluation of the intracellular redistribution and phosphorylation of different cGAS signaling proteins (eg. TBK1, IRF3 and members of the NF-κB pathway) can subsequently be used to pinpoint the stage of the Vpr effect in cGAS stimulated or HIV-infected cells. Alternatively, it may be useful to evaluate levels of 2′3′-cGAMP in HIV wild-type and ΔVpr infected cells, which is produced by cGAS upon DNA binding [101, 102]. An HIV-specific effect of Vpr at this stage, would favor the first model in which Vpr enhances binding of HIV DNA to cGAS. Finally, future correlation studies of Vpr's ability to potentiate IFN-I induction and other known Vpr functions, through use of Vpr mutants, knock-down of Vpr interacting proteins or treatment with inhibitors, can lead to further mechanistic insights. We already showed that Vpr-DCAF1 interaction is important for its IFN-I potentiating effect. Many of the above suggested mechanisms might however depend on formation of this complex, given its broad and incompletely characterized role during HIV-1 replication [76, 103-105]. Since DCAF1 is used by Vpr to target cellular proteins for proteasomal degradation, it could be interesting to search for known cellular regulators of cGAS or NF-κB signaling among Vpr interacting proteins. Large scale *in cellulo* [106] and *in silico* [107-109] interactome studies aimed at identifying new viral-host protein interactions are actively being performed and multiple databases have been developed to explore identified HIV protein interaction partners [110, 111]. Such tools and datasets may provide useful clues for future research.

For Vpu, we show that suppression of IFN-I induction is independent of Vpu's ability to counteract the host factor tetherin. This protein was previously reported to act as an innate sensor of budding viral particles, thereby inducing activation of NF-κB but not IRF3 [90, 112]. Although tetherin-mediated sensing could therefore further enhance the innate response, on its own it is likely insufficient to induce IFN-I production [90]. The presence of a viral innate suppressive mechanism in addition to tetherin counteraction, indicates that other mechanisms of sensing need to be counteracted by the virus. In our study, IFN-I induction by a wild-type virus was completely blocked upon cGAS knockdown. It will however be interesting to also evaluate *vpu*-deleted viruses in cGAS knockdown cells, to see

if other types of sensing occur and are suppressed by Vpu in primary CD4^{+} T cells. Knock-down of tetherin alone and in combination with cGAS could furthermore learn us if tetherin-mediated NF-κB activation enhances cGAS-mediated IFN-I induction. To narrow down the stage of the Vpu effect in the IFN-I signaling pathway, similar strategies as proposed for Vpr can be employed. As discussed in Chapter III-2, previous Vpu studies may already provide some clues about the molecular mechanism in this regard: although sometimes controversial, Vpu has been described to deplete [113] or cleave IRF3 [114], as well as to directly suppress activation of NF-κB [82, 89, 115, 116]. Interestingly, the latter has been linked to recruitment of β-TcrP by Vpu [82, 115] and our results indicate a similar requirement for β-TcrP-Vpu interaction to counteract HIV-mediated IFN-I induction in primary CD4^{+} T cells. In these previous studies, Vpu was suggested to sequester β-TcrP and thereby prevent β-TrCP dependent degradation of the NF-κB inhibitor IκBα, which in turn prevents translocation of NF-κB to the nucleus [82, 115]. It would therefore be interesting to evaluate if differences in IκBα levels and subcellular localization of NF-κB upon HIV-1 wild-type and HIV-1 ΔVpu infection can be detected in primary CD4^{+} T cells.

General conclusion and implications

In this work we have extensively characterized the IFN-I response against the HIV-1 virus in its main target cells. We demonstrate that activated CD4^{+} T cells are able to sense HIV through cGAS and accordingly produce IFN-I, which indicates them as a potential source of elevated IFN-I in HIV-1 patients. Although this will require *in vivo* confirmation, they may represent new targets for strategies aimed at suppressing hyper-immune activation during HIV-1 infection. Inhibition of IFN-I production or signaling as a therapy is still at early stages of evaluation, but in different small trials a decrease in immune activation or disease progression was observed [22, 23, 25, 26]. One of the strategies used in this regard is treatment with chloroquine, which inhibits endosomal acidification and thereby TLR7-dependent IFN-I production by pDC's. However, cGAS-mediated sensing is unlikely to be affected and IFN-I production may therefore be incompletely counteracted with this strategy. Options for sensor- (and cell-) specific blocking of IFN-I production instead of IFN-I signaling could however be useful, since they are less likely to disturb global anti-viral and anti-tumor functions of IFN-I and would also prevent the pro-inflammatory effects of NF-κB activation upon sensing. Furthermore, the cellular origins of IFN-I may differ at different stages of the infection and cell-types may therefore differently contribute to beneficial (early) versus disease aggravating (late) effects of IFN-I. In one study, chloroquine administration to patients at early stages of infection actually led to an increase in viral replication [117], indicating blockage of antiviral IFN-I effects. Although this needs further *in vivo* confirmation, pDC's are considered to be the main source of early IFN-I [27], while CD4^{+} T cells are more likely to contribute at chronic stages of infection. As such, targeting of cGAS may be worth considering in patients with insufficient immunological response to cART or

remaining signs of chronic immune activation under cART. Given the recent identification of cGAS as a DNA sensor, a specific inhibitor of the protein is not yet available. However, structural studies indicate that both the catalytic pocket of the cGAS enzyme as well as the cGAMP binding pocket of STING may be amendable to small molecule inhibition [101, 118].

We further show that the innate immune response to HIV-1 in CD4$^+$ T cells is subjected to a high level of viral regulation: IFN-I induction is efficiently prevented before integration and controlled by two viral accessory proteins after integration. The inability of HIV-1 to evade innate sensing, the potentiating effect of Vpr and the incomplete suppression by Vpu may indicate that the virus strives to reach a delicate balance in sensing. Following further mechanistic elucidation, development of strategies aimed at disturbing this balance could lead to new therapeutic options. Such strategies can serve two different goals: targeting the IFN-I suppressing effect of Vpu or disturbing the mechanisms that prevent viral sensing prior to integration (and Vpu expression) may elevate IFN-I induction to levels that are sufficient to hamper viral spread, while targeting the Vpr potentiating effect could alternatively be used limit production of IFN-I. Similarly to targeting cGAS, the latter may find an application in patients with insufficiently controlled hyper-immune activation under cART. For the former strategies, it is difficult to predict if they will confer any additional advantage in suppressing ongoing viral replication compared to current antiretroviral agents. However, they may be useful as part of an immunological booster in "shock and kill" strategies, in which enhancing antiviral immunity will most likely be required to clear the "awakened" virus [119]. Alternatively, they could find an application in prophylactic strategies. Allowing an adequate innate immune response upon initial exposure may be sufficient to prevent the struggling virus from establishing infection.

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Vermeire J, Iannucci V, Naessens E, Van Landeghem K, Vanderstraeten H, Van Damme J , Verhasselt B. Primary CD4$^+$ T cells produce type I IFN in response to HIV-1 infection. BSM Annual Symposium, Microbial Diversity for Science and Industry, Brussels, November 2013 – oral presentation

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